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Stable Isotopic and Molecular Biological Tools to Validate Biodegradation of 1,4-Dioxane

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

by

Peerapong Pornwongthong

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ABSTRACT OF THE DISSERTATION

Stable Isotopic and Molecular Biological Tools
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Doctor of Philosophy in Civil Engineering
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Professor Shaily Mahendra, Chair

1,4-Dioxane, a probable human carcinogen, is a heterocyclic ether increasingly found as a contaminant in water supplies. Recent studies have reported that 1,4-dioxane can be biodegraded by a variety of microorganisms, and bioremediation may be an effective strategy for 1,4-dioxane contaminated sites. However, reliable monitoring tools to validate biodegradation of 1,4-dioxane are still lacking. Molecular biological tools and stable isotope-based tools have been previously applied as diagnostic tools for monitored natural attenuation and engineered bioremediation of various organic and inorganic compounds. In this study, molecular biological tools were used for determining bacterial populations, and for associating 1,4-dioxane biodegradation with relative copy numbers of phylogenetic and functional genes. These biomarkers were amplified using primers designed from the genome sequence data of 1,4-dioxane-degrading bacterium *Pseudonocardia dioxanivorans* CB1190, and were correlated with measured biodegradation rates. The results revealed that abundance of DXMO and 16S rRNA were in agreement with 1,4-dioxane biodegradation rates, and could be used to illustrate the inhibitory effect of co-contaminant transition metals Cu(II), Cd(II), Ni(II), and organic ligands such as tannic acid and L-cysteine. It should be recognized that biomarkers provide an indirect

association between genes and enzyme activity. Factors regulating protein synthesis and catalytic activities of enzymes are not captured by nucleic acid-based biomarkers. This complicates the interpretation of biomarkers for predicting biodegradation rates. Compound specific isotope analysis (CSIA) could be used as another diagnostic tool to assess 1,4-dioxane biodegradation. In this study, hydrogen and carbon isotope analyses of 1,4-dioxane were successfully developed to determine isotope signatures of commercial 1,4-dioxane, and applied to determine kinetic isotope fractionation associated with biodegradation in both pure and mixed cultures, as well as abiotic degradation of 1,4-dioxane. During biodegradation, both ^{2}H and ^{13}C were enriched, while abiotic processes could enrich only ^{2}H in residual 1,4-dioxane. This indicated that combined carbon and hydrogen isotope analyses of 1,4-dioxane allow differentiation of biological processes from abiotic mechanisms. Availability of stable isotopic and molecular biological tools will allow environmental engineering professionals to include bioremediation as an effective strategy in the cleanup of specific environmental contaminants.

The dissertation of Peerapong Pornwongthong is approved.

Michael Stenstrom

Irwin Suffet

Jennifer Jay

Shaily Mahendra, Committee Chair

University of California, Los Angeles

2014

To All of My Loves

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SELECTED PUBLICATIONS

1. Pornwongthong, P., M. Bill, M. E. Conrad, and S. Mahendra (2014). Carbon and Hydrogen Stable Isotope Fractionation during Biotic and Abiotic Degradation of 1,4-Dioxane (in review).
2. Pornwongthong, P., G. Paradis, and S. Mahendra (2014). Development and Application of Compound-Specific Carbon Isotope Analysis Method for Validating 1,4-Dioxane Biodegradation (in review).
3. Pornwongthong, P., Mulchandani, A., Gedalanga, P., and Mahendra, S. (2014) Transition Metals and Organic Ligands Influence Biodegradation of 1,4-Dioxane, *Applied Biochemistry and Biotechnology 173*, 291-306.
4. Gedalanga, P. B., P. Pornwongthong, R. Mora, S.-Y. D.Chiang, B. Baldwin, D. Ogles, and S. Mahendra (2014). Identification of Biomarker Genes to Predict Biodegradation of 1,4-Dioxane. *Applied and Environmental Microbiology* 80 (10). 3209-3218.
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CHAPTER 1

BACKGROUND AND OBJECTIVES

1.1. Introduction. 1,4-Dioxane, a probable human carcinogen and a confirmed animal carcinogen (*1, 2*), is an emerging contaminant of concern in surface water and groundwater resources. It has been mainly used as a stabilizer of 1,1,1-trichloroethane, but it is known as a by-product of polyester textile production through the esterification of terephthalic acid with ethylene glycol, and an unwanted ethoxylated by-product in various personal care products (*3, 4*). Contamination of surface water and groundwater often results from improper storage and accidental spill of industrial wastes containing 1,4-dioxane (*3, 4*). While conventional water and wastewater treatment processes, including air stripping and carbon adsorption, were ineffective in treating 1,4-dioxane in contaminated water (*5*), many reports describe that advanced oxidation technologies, i.e., UV photoxidation (*6, 7*), chemical oxidation (*8, 9*), anodic oxidation (*10*) and sonolysis (*11-14*) are effective for removal of 1,4-dioxane from contaminated water. However, operation of these processes for groundwater remediation requires long-term pumping, and relies on expensive and energy-consuming technologies (*3, 4*). Numerous studies in the past decades demonstrated that 1,4-dioxane is biodegradable in natural as well as controlled environments (*4, 15-29*) suggesting the possibility of using bioremediation as an alternative treatment strategy. Bioremediation is an economically attractive, ecological friendly and effective technology for 1,4-dioxane remediation, and can be used in conjunction with or as an alternative to physical-chemical treatment technologies already installed at the contaminated sites. However, reliable instruments for assessing the effectiveness of 1,4-dioxane biodegradation are still limited.

Recently, a variety of advanced tools have been developed to assess, monitor and validate bioremediation of various organic compounds. The tools, including molecular biology tools (MBTs) and compound specific isotope analysis (CSIA), have been effectively applied as diagnostic tools for monitored natural attenuation (MNA) and engineered bioremediation. MBTs

provide biomolecular characteristics of microbial communities capable of biodegrading environmental contaminants. Quantification of the phylogenetic and functional genes by quantitative Polymerase Chain Reaction (qPCR) can determine the abundance of a specific bacterial population capable of carrying out biodegradation. In addition, quantification of functional genes also demonstrates physiological characteristics of bacterial communities. CSIA is a technology for isotopic ratio analysis which is applicable for examining the alteration of isotope ratios during chemical and biological degradation of organic compounds. CSIA provides a strong evidence for characterizing biodegradation, and can be applied to predict the extent of biodegradation or identify biodegradation mechanisms (*30, 31*). Recently, CSIA was successfully applied to assess biodegradation of chlorinated solvents (*32-35*), methyl *tert*-butyl ether (MTBE) (*31, 36, 37*), BTX (*38-42*) and polycyclic aromatic hydrocarbon (*43-45*) under natural and engineered environments.

1.2. Research Goals and Objectives. The goals of this research are to develop isotopic and molecular biological tools for evaluating biodegradation of 1,4-dioxane. The specific objectives of this study are to:

- apply molecular biological tools to indicate biodegradation of 1,4-dioxane in pure cultures
- demonstrate the applications of molecular biological tools for assessing potential biodegradation of 1,4-dioxane in environmental samples
- develop analytical methods for measuring stable isotopic fractionation of carbon and hydrogen

- quantify ^{13}C and ^{2}H isotope fractionation of 1,4-dioxane during abiotic degradation and biodegradation of 1,4-dioxane

1.3. Dissertation Overview. This dissertation is organized into 7 chapters. The background, problem statement, goal and objectives of this research are outlined in *Chapter 1*. Previously available literature relevant to this research is reviewed in *Chapter 2*. *Chapter 3* describes the individual effects and interactive effects of heavy metals and organic ligands on biodegradation of 1,4-dioxane, and illustrates applications of qPCR to quantify population of laboratory pure cultures. *Chapter 4* reveals applications and limitations of molecular biological tools to assess biodegradation of 1,4-dioxane in environmental samples. *Chapter 5* describes the principles of CSIA and development of carbon-CSIA as a tool for assessing biodegradation of 1,4-dioxane. Similarly, *Chapter 6* describes principles and development of 2D-CSIA as a monitoring tool and its applications for validating 1,4-dioxane degradation. Lastly, *Chapter 7* summarizes the key findings and prospects future directions building upon this research.

CHAPTER 2

LITERATURE REVIEW

2.1. 1,4-Dioxane

2.1.1. Characteristics. 1,4-Dioxane (*p*-dioxane, diethylene oxide, 1,4-diethylene dioxide, glycol ethylene ether; CAS Number 123-91-1; Figure 2.1) was first synthesized by A. V. Lourenço in 1863 as product of a reaction between ethylene glycol and 1,2-dibromoethane (*3*). 1,4-Dioxane is a heterocyclic ether that contains four carbon, eight hydrogen and two oxygen atoms in a ring. Oxygen atoms in 1,4-dioxane can form hydrogen bonds with water making the compound hydrophilic and fully miscible in water and most organic solvents. 1,4-Dioxane also has excellent solvency (*46*). The structure of 1,4-dioxane is symmetrical indicating the high stability of the compound. As a result, it is inert and relatively immune to hydrolysis, hydrogenation, sulfonation, and oxidation at natural conditions (*46, 47*). At room temperature, 1,4-dioxane is a liquid and has a butanol-like odor (*3*). Some selected properties of 1,4-dioxane are outlined in Table 2.1.

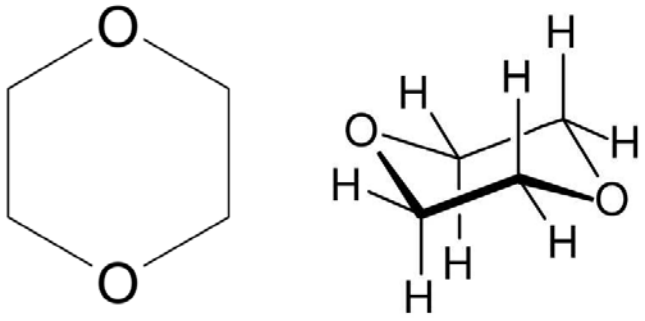

Figure 2.1. Chemical Structure of 1,4-Dioxane

Table 2.1. Selected properties of 1,4-dioxane (adapted from (*3*))

Parameters	**1,4-Dioxane**
Chemical formula	$C_4H_8O_2$
Polarity of the compound	Polar
Molecular weight (g/mole)	88.11
Solubility in water (ppm)	Miscible
Vapor pressure (mmHg)	29 at 20 ˚C; 38.09 at 25 ˚C
Melting point (˚C)	11.85
Boiling point (˚C)	101.2

2.1.2. Production and Uses.

2.1.2.1. Production. 1,4-Dioxane production commonly involves dehydration and ring closure of ethylene glycol with a strong acid catalyst, usually sulfuric acid (Figure 2.2) (*3*). Alternatively, 1,4-dioxane can be produced by dimerization of ethylene oxide or by heating bis(2-chloroethyl)ether with 20% sodium hydroxide reactant (*48, 49*).

1,4-Dioxane was first commercialized in 1929 (*48*). The production of 1,4-dioxane was directly related to the production of the chlorinated solvent 1,1,1-trichloroethane (methyl chloroform, TCA) as 1,4-dioxane was mainly used as a stabilizer for that solvent. The largest demand of 1,4-dioxane was recorded in the late 1950s and early 1960s. In 1985, the world production of 1,4-dioxane was estimated at 30.8 million pounds (25 million pounds in United States) which accounts for 3% of the methylene chloride production (*49*). In 1995, production of 1,4-dioxane

dramatically decreased to 22 million pounds after the use of methyl chloroforms, considered to be ozone-depleting substances, had declined (*50*). At present, manufacturing levels of 1,4-dioxane are likely to be significantly less due to changing use patterns (*48*).

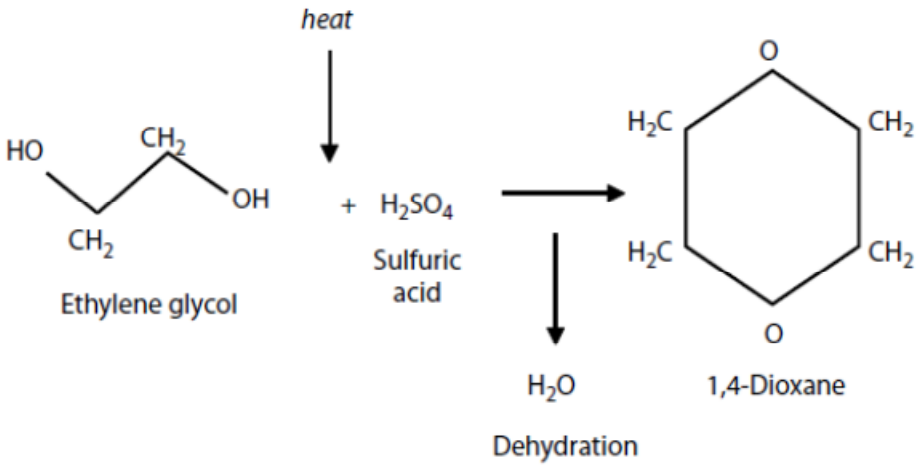

Figure 2.2. A Common Method for 1,4-Dioxane Production. The process involves dehydration of ethylene glycol with strong acid catalyst (sulfuric acid) (*3*)

2.1.2.2. Uses

2.1.2.2.1. Solvent Stabilizer. Chlorinated compounds, which have been used for degreasing, textile cleaning, wool processing, preparation of inks, paints and varnishes, and production of pharmaceuticals, can be decomposed when exposed to environmental stresses. The primary causes of solvent decomposition include oxidation, hydrolysis, pyrolysis, photooxidation and reaction with alkaline metals (*3*). The decomposition of the solvents produces hydrochloric acid that can corrode the metal parts in systems where the solvents are used. Therefore, a stabilizer, a chemical that inhibits decomposition of the solvents, has to be spiked into the chlorinated solvents.

The primary use of 1,4-dioxane is as a stabilizer for chlorinated solvents such as methyl chloroform and dichloromethane. It functions as a metal inhibitor which can deactivate the reaction between the solvents and aluminum which is a major cause of solvent decomposition. 1,4-Dioxane can be found up to 8% by weight of the solvents and 90% of 1,4-dioxane produced annually was used for stabilizing methyl chloroform (*3*).

2.1.2.2.2. Other Uses. 1,4-Dioxane was reported to be a solvent directly used in many industries and activities including textile fabrication, cellulose membrane production, pharmaceutical production, liquid scintillation, flame retardant production, rubber and plastic manufacturing, syntheses of various organic compounds, natural and synthetic resin production, polishing agent manufacturing, paper industry, etc (*3, 4*). 1,4-Dioxane is also an unwanted by-product in ethoxylated surfactants, personal care products (detergents, shampoos, sundries, and cosmetics), pesticides, herbicides, terephthalate esters (polyester), gasoline antiknock agent, adhesives, paints and printer ink (*3, 4*).

2.2. 1,4-Dioxane in the Environment. 1,4-Dioxane is a water contaminant in many locations throughout the United States and many areas over the world. Widespread contamination of 1,4-dioxane is usually due to the releases and accidental spills of industrial and commercial products containing 1,4-dioxane into water resources. It was believed that contamination of 1,4-dioxane in the environment predominantly originates from TCA-contaminated groundwater, and chemical and municipal treatment plant effluents (*4*). In addition, 1,4-dioxane is highly soluble in water and generally resistant to degradation. Thus, it tends to migrate to a great extent in groundwater and surface water, and remains persistent in the environment. As a result, 1,4-dioxane has been detected in all types of water bodies including surface water sources,

groundwater aquifers, landfill leachates, wastewater streams, and even in drinking water supplies.

2.2.1. Surface Water. 1,4-Dioxane is frequently detected as a contaminant in the lower Mississippi River (*5*). In Westville, Indiana, creeks near a former waste oil refinery were impacted by 1.4-dioxane. It was found that 29 samples from the creeks contained 1,4-dioxane at concentrations ranging from 54 to 140 μg L^{-1} (*51*). In Japan, 1,4-dioxane at concentrations ranging from 1.9 to 94.8 μg L^{-1} were detected in 83 of 95 samples collected from river, ocean, and groundwater at various locations, and only seven rivers contained low levels of 1,4-dioxane (0.1-1.6 μg L^{-1}) (*52*).

2.2.2. Groundwater. 1,4-Dioxane has impacted groundwater in many locations throughout the United States. At various US Air Force installations, 1,4-dioxane was concurrently found with trichloroethylene in chlorinated solvent groundwater plumes (*53*). For example, 1,4-dioxane plume travelled farther than the chlorinated contaminants plume (3,500 ft. vs. 1,500 ft.) (*3*) and moved farthest among five major contaminants at the Seymour Recycling Corporation Hazardous Waste Site in Indiana (*54*). Similar trends were also observed at the Air Force Plant 44 in Tucson, Arizona and the San Jose solvent recycling facilities in San Jose, California (*3*).

Adamson et al. (*55*) studied behavior and occurrence of 1,4-dioxane in California groundwater. Based on a survey of more than 2000 impacted sites where groundwater had been contaminated by chlorinated solvents and/or 1,4-dioxane, 194 of these sited were polluted with 1,4-dioxane, with 95% containing one or more chlorinated solvents. It was shown that 1,4-dioxane frequently co-occur with 1,1,1-trichloroethane. The analyses also revealed that at the sites contaminated with both 1,4-dioxane and chlorinated solvents, 1,4-dioxane plumes were found to be shorter

than chlorinated solvent plumes at 63% of these sites. These results suggested that 1,4-dioxane plume had not migrated beyond chlorinated solvent plumes.

A shallow and a deep aquifer at the Pall/Gelman Sciences, Inc. site in Ann Arbor, Michigan has been heavily impacted by 1,4-dioxane as the sole contaminant (*56*). In 1970s, approximately 850,000 pounds of the compound was used to produce triacetate filters (*56*). Wastewater from the factory containing as high as 25 mg L^{-1} 1,4-dioxane was directly discharged into filtration lagoons resulting in contamination of surface water and groundwater at this location (*56, 57*).

2.2.3. Landfill Leachates. 1,4-Dioxane is likely to be detected in leachates released from landfills receiving industry wastes and products (*3*). As early as 1981, 1,4-dioxane (0.5-2.4 μg L^{-1}) was detected in 37% of groundwater samples collected near Army Creek Landfill located in Wilmington, DE (*58*). 1,4-Dioxane was found in landfill leachate of Operating Industries, Inc. (OII) located in Monterey Park, CA at concentrations as high as 19,000 μg L^{-1} (*59*). In Japan, 87.5% of leachate samples from landfills contained 1,4-dioxane at concentrations between 1.1 and 190 μg L^{-1} in 1994 (*60*). In 2000, a landfill survey by USEPA estimated the mean concentration of 1,4-dioxane in leachate from all municipal landfills as 118 μg L^{-1} (*61*).

2.2.4. Wastewater. 1,4-Dioxane has been detected in many industrial and municipal wastewater treatment plants (*62*). For example, wastewater effluent discharged from an anaerobic treatment unit at H Co. in the Gumi Industrial Complex, South Korea contained 1,4-dioxane, which is a by-product from polyester manufacturing processes at the facility, at an average concentration of 31 mg L^{-1} (*63*). In Spain, 50% of effluent samples from a polyethylene terephthalate (PET) resin plant contained 1,4-dioxane at concentrations higher than 1,000 μg L^{-1} and the remaining samples had 1,4-dioxane in the range of 100 to 1000 μg L^{-1} (*50*).

1,4-Dioxane has been found in effluents of municipal wastewater treatment plants in Michigan and Japan. In Japan, concentrations of 1,4-dioxane in sewage up to 90 mg L^{-1} were recorded (*52*). Households discharging liquid soaps and detergents are likely the sources of 1,4-dioxane contamination in these municipal wastewaters. In the city of Ann Arbor, Michigan, raw and treated wastewater samples were analyzed for 1,4-dioxane. According to three sampling events, the average 1,4-dioxane concentration in the influent and effluent was determined as 3 and 2 μg L^{-1}, respectively. The sources of the contaminations in that study were not specified (*64*).

2.2.5. Drinking Water. Contamination of 1,4-dioxane in drinking water is an important public health concern. 1,4-Dioxane has contaminated many water supplies and systems. In California, 1,4-Dioxane at or greater than 1 μg L^{-1} has been found in 79 drinking water sources and 35 systems since 2000 (*65*). Interestingly, more than 60% of these contaminations were found in Los Angeles County (52 drinking water sources and 20 systems) (*65*). In 1995-1996, 1,4-dioxane at concentrations between 0.2–1.5 μg L^{-1} was detected in tap water samples in six cities of Kanagawa Prefecture in Japan (*66*).

In many cases, contamination of 1,4-dioxane in drinking water supplies led to the closure of water supply wells. In Bedford, MA, four municipal supply wells were closed because of contamination with TCE and 1,4-dioxane (2,100 μg L^{-1}) (*67*). In Ann Arbor, MI, operation of two municipal supply wells was suspended in April 2001 due to the contamination of 1,4-dioxane (1-24 μg L^{-1}). 1,4-Dioxane contaminated in these water supply wells was likely discharged from the Pall/Gelman Sciences Inc. site where 1,4-dioxane was used as a solvent for microporous cellulose filter production (*56*). Contamination of 1,4-dioxane also caused municipal well closings in California (*68*).

2.3. Fate and Transport of 1,4-dioxane in Surface and Subsurface Water. Fate and transport of 1,4-dioxane in surface and subsurface water involves many chemical, physical and biological processes. In this section, the principles as well as the effects of each mechanism on fate and transport of 1,4-dioxane will be described.

2.3.1. Photolysis. 1,4-dioxane in surface water can be transformed by photolysis via either direct or indirect mechanism.

2.3.1.1. Direct Photolysis. Ultraviolet light with wavelengths less than 290 nm (shorter than the wavelengths of light penetrating the troposphere), can directly photolyze 1,4-dioxane; hence, it mainly relates to the atmospheric fate of 1,4-dioxane (*3*). UV photoreaction was described in many studies under various UV wavelengths (*69-72*). Hentz and Parrish (*71*) proposed photolysis pathway of vaporized 1,4-dioxane at the wavelength of 147 nm (Eq 2.1 – 2.3). It was shown that photolysis of 1,4-dioxane produced various volatile solid by-products, such as p-formaldehyde and trioxane. In addition, it is likely that 1,4-dioxane is transformed to 1,4-dioxane dimers and alcohol isomers when exposed to the UV light (Figure 2.3) (*69*).

$$C_4H_8O_2 \xrightarrow{h\nu} C_2H_4 + 2CH_2O \qquad \text{(Eq 2.1)}$$

$$C_4H_8O_2 \xrightarrow{h\nu} C_2H_4 + CH_2O + H_2 + CO \qquad \text{(Eq 2.2)}$$

$$C_4H_8O_2 \xrightarrow{h\nu} H_2 + \text{Unidentified product} \qquad \text{(Eq 2.3)}$$

2.3.1.2. Indirect photolysis. Indirect photolysis involves the reaction of radicals produced from the reactions of sunlight with water vapor in the atmosphere or in surface water with 1,4-dioxane. Figure 2.4 demonstrates how 1,4-dioxane reacts with hydroxyl radicals (from (*73*)). Briefly, 1,4-dioxane (a) reacts with a hydroxyl radical followed by addition of oxygen forming a

1,4-dioxyl radical (b). The 1,4-dioxyl radical converts NO to NO_2 and produces an alkoxyl radical (c) which is rapidly be transformed to the $HC(O)O(CH_2)_2OCH_2$ radical (d) via a ring-opening reaction. The alkoxyl radical is further transformed by addition of oxygen into the molecule producing peroxyl radical $HC(O)O(CH_2)_2OCH_2O_2$ (e). The peroxyl radical converts NO to NO_2 forming $HC(O)O(CH_2)_2OCH_2O$ (f), which is able to react with oxygen to produce ethylene glycol diformate or EDF (g). As described in Maurer et al. (*74*), EDF can degrade from successive reactions with hydroxyl radicals and produce many products such as formic acid anhydride (FAA), formic acid (FA), carbon monoxide (CO), and alkyl diformates, etc. The formic acid-related products can result in an increase in the atmospheric acid burden (*3*).

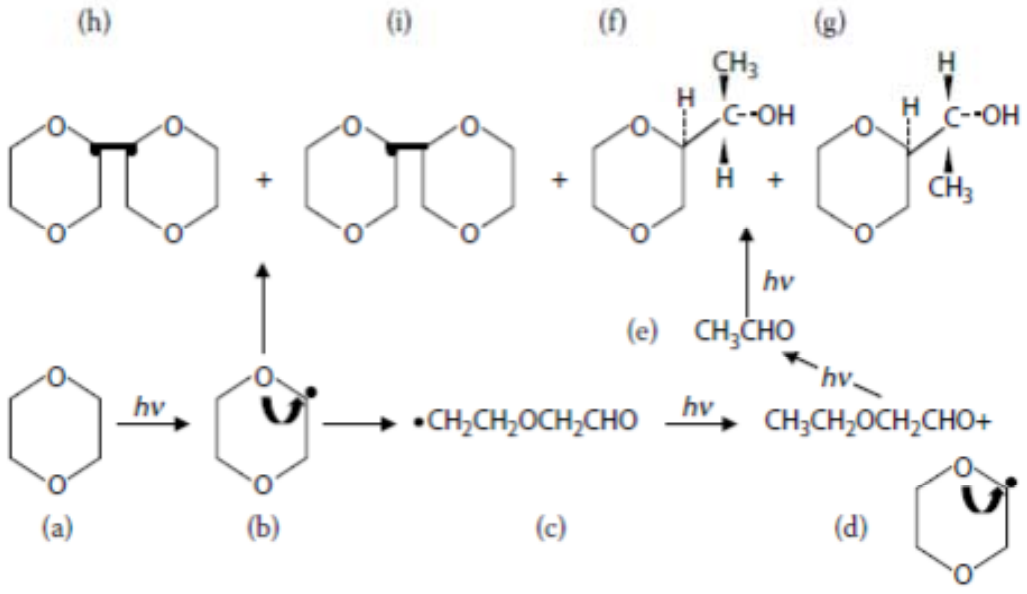

Figure 2.3. Laboratory Photolysis of 1,4-Dioxane. Dioxyl radicals (b) are formed by abstracting hydrogen from 1,4-dioxane (a). The dioxyl radicals can undergo either dimers formation (h and i) or bond cleavage producing a radical (c). The radical (c) can be further transformed to ethoxyacetaldehyde (d) and acetaldehyde (e), respectively when exposed to the UV light. Lastly, acetaldehyde (e) can be photo-reduced to the alcohol isomers (f and g) (*69*).

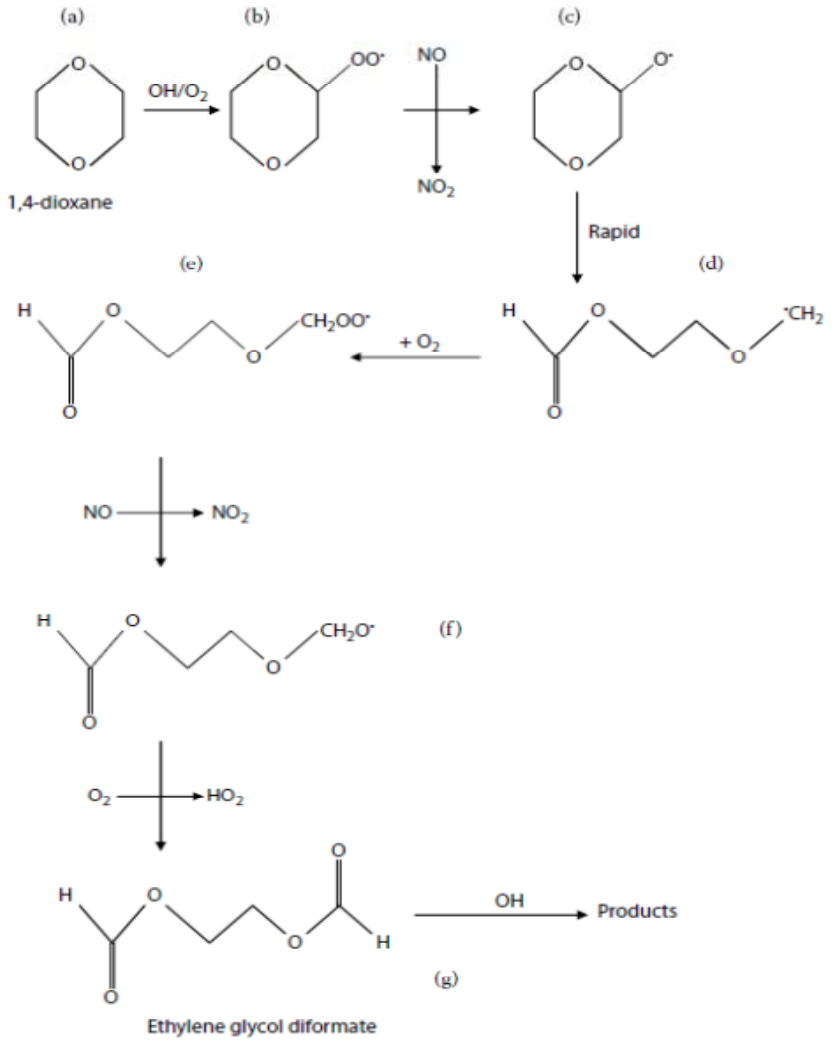

Figure 2.4. Photo-oxidation Pathway for 1,4-Dioxane in the Presence of NO_x (from (*73*)).

2.3.2. Advection and Diffusion. Advection and diffusion are two transport processes controlling the fate of 1,4-dioxane in surface and subsurface water. Advection is important in streams and rivers where flows are governed by gravity and water velocity, while diffusion, thermal stratification and wind-induced currents are important in lakes. Contaminant transport by advection in groundwater is very slow. Therefore, diffusion is the primary mechanism that controls the migration of contaminants in subsurface (*3*). Note that diffusion of a contaminant is controlled by concentration gradients, solubility, and temperature (*75*).

The diffusion of 1,4-dioxane and other contaminant in subsurface water can be described by Fick's first law:

$$J = \Theta_{total} \cdot D_{eff} \frac{dC}{dz} \quad \text{(eq 2.4)}$$

where J is the flux density (in μg s cm^{-2}), Θ_{total} is total porosity (dimensionless), D_{eff} is effective diffusity (in m^2 d^{-1}) and dC/dz is the solute concentration gradient. Barone et al. (*76*) calculated a diffusion coefficient for 1,4-dioxane in pure water at 22 ˚C as 10.6 x 10^{-6} cm^2 s^{-1} and reported a measured diffusion coefficient (D_{eff} = 4.0 x 10^{-6} cm^2 s^{-1}) and retardation factor of 2.19 in the soil plug diffusion of a clay.

2.3.3. Sorption. Barone et al. (*76*) studied plume behavior of 1,4-dioxane that passed through saturated and unsaturated soils. It was demonstrated that the 1,4-dioxane plume migrated quickly due to its high solubility and low affinity for sorption to soil organic matter. An adsorption coefficient (K_d) for 1,4-dioxane was determined based upon laboratory diffusion tests in a saturated and undisturbed clayey soil (*76*). A clay soil with grain sizes of clay (45%), silt (43%), sand (10%), and gravel (2%), and mineralogy of the sub-gravel grains as calcite/dolomite (34%), quartz and feldspars (15%), illite (25%), chlorite (24%), and smectite, (2%) possessed a K_d value for 1,4-dioxane of 0.17 mL g^{-1} based upon a measured diffusion coefficient of 4 x10^{-6} cm^2 s^{-1}, soil organic carbon content of 0.58% and the soil cation exchange capacity of 10 milliequivalents per 100 grams dry weight (*76*). These results are similar to those of Lyman et al. (*77*) who reported small K_d (0.1) and K_{oc} (log K_{oc} = 1.23) for 1,4-dioxane in clay. This evidence indicates that 1,4-dioxane would not preferentially sorb to soil particles.

2.3.4. Biodegradation. At least eight genera of monooxygenase-expressing bacteria have been reported to biodegrade 1,4-dioxane under aerobic conditions in the laboratory (*18, 78, 79*).

These bacteria can biodegrade 1,4-dioxane via metabolic (growth-supporting) or cometabolic mechanisms, and their mineralization does not produce nor accumulate any toxic intermediates and terminal products (*16, 18*). Among these bacteria, an aerobic Actinomycetes bacterium known as *Pseudonocardia dioxanivorans* CB1190 (Figure 2.5) is capable of using 1,4-dioxane as its sole carbon and energy source (*17, 20*). Recently, the genome of *P. dioxanivorans* CB1190 was successfully sequenced and annotated (*80*). The average G+C content of the genome was determined as 73.1%. The genome comprises of 4 replicons, including the chromosome (7.1 Mb), plasmid 1 (circular, 192 kb), plasmid 2 (circular, 137 kb with gaps), and plasmid 3 (linear, 15 kb), 46 tRNA genes and three copies of the rRNA genes. Annotation of coding sequences which were predicted with Prodigal (http://prodigal.ornl.gov) was performed using Pfam, KEGG, and COG databases. A total of 6,799 candidate protein-encoding genes were annotated. In addition, the analysis of *P. dioxanivorans* CB1190's genome revealed eight putative gene clusters encoding at least 17 monooxygenases (*80*).

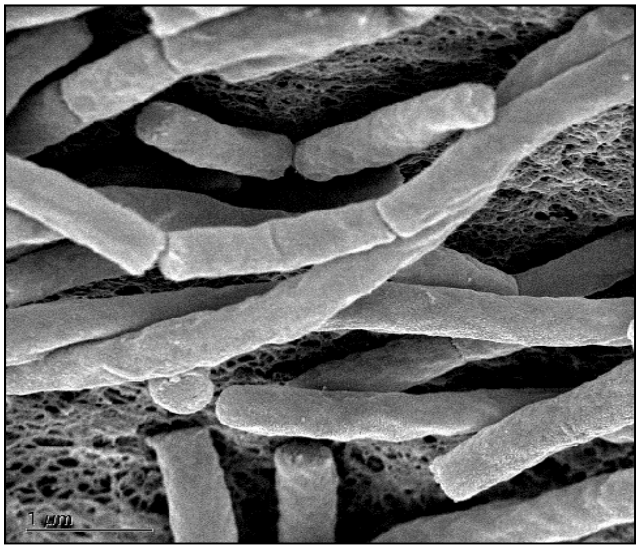

Figure 2.5. A scanning Electron Micrograph of *Pseudonocardia dioxanivorans* CB1190 (*17*)

Many studies have been conducted to identify intermediate and terminal by-products and key functional genomics related to biodegradation pathway of 1,4-dioxane (Figure 2.6) (*16, 81-84*). The studies reveal that monooxygenase enzymes, particularly tetrahydrofuran/dioxane monooxygenase, play an essential role in 1,4-dioxane degradation since they catalyze the initial step of the biodegradation pathway (*16, 18, 83*). Monooxygenases hydroxylate 1,4-dioxane by adding a single oxygen atom from molecular oxygen into the organic chemical and reduce the second oxygen atom to water (*16*). The activity of monooxygenases was previously proposed to be related to the two reactions in the 1,4-dioxane degrading pathway, the transformation of 1,4-dioxane to 2-hydroxy-1,4-dioxane and the hydroxylation of 2-hydroxyethoxyacetic acid to 1,2-dihydroxyethoxyacetic acid (*16*). However, the recent transcriptomic evidences suggested that protein product of *thmADBC* in *P. dioxanivorans* CB1190 oxidizes 1,4-dioxane, but it is unable to catabolize 2-hydroxyethoxyacetic acid in the biodegradation pathway (*82*). It is possible that by-product 2-hydroxyethoxyacetic acid is catalyzed by non-monooxygenase enzymes. Grostern et al. (*81*) used microarray to observe expression of genes in *P. dioxanivorans* CB1190 grown in three different substrates in the 1,4-dioxane biodegradation pathway. It was proven in this study that monooxygenase gene cluster located on plasmid pPSED02, plasmid-borne genes associated with expression of putative aldehyde dehydrogenases, an aldehyde reductase and an alcohol oxidoreductase, and chromosomal genes related to glyoxylate metabolisms (through glyoxylate carboligase pathway) were up-regulated. The results from microarray analyses and ^{13}C-labeled dioxane amino acid isotopomer analysis demonstrated that monooxygenases as well as non-monooxygenase enzymes play an essential role in 1,4-dioxane biodegradation, and suggested that metabolites of 1,4-dioxane are routed through the glyoxylate carboligase pathway via three

carbon compound, likely to be phosphoglyceric acid (*81*). Recently, biomarkers for 1,4-dioxane biodegradation were identified based upon these previous studies (*84*).

Besides monooxygenase-expressing bacteria, fungi can also biodegrade 1,4-dioxane. *Cordyceps sinensi*, a fungus isolated from soil, could mineralize 1992 mg L^{-1} 1,4-dioxane within three days of incubation (*85*). Biodegradation of 1,4-dioxane by the fungus could generate ethylene glycol, glycolic acid, and oxalic acid as intermediate by-products. Skinner et al. (*86*) evaluated the capability of a filamentous fungus *Graphium* sp. to metabolize or co-metabolize heterocyclic ethers including 1,4-dioxane and tetrahydrofuran. It was found that both propane- and THF-grown mycelia were able to cometabolically biodegrade 1,4-dioxane.

Biodegradation of 1,4-dioxane in environmental samples (mixed consortia) has also been observed. Sei et al. (*21*) evaluated the biodegradation potential of 1,4-dioxane in the natural environment. The experiment revealed that soil samples from a drainage area of the chemical factory could reduce 100 mg L^{-1} of 1,4-dioxane to below detection limit (0.8 mg L^{-1}) within 33 days. It was also shown that 1,4-dioxane could be co-metabolized in the presence of 100 mg L^{-1} tetrahydrofuran in an activated sludge sample and 69% of 1,4-dioxane could be removed within 14 days with this condition. Hence, it can be concluded that the potential for 1,4-dioxane degradation is not ubiquitously distributed in the natural environment, and the differences in ability of 1,4-dioxane degradation depend upon the different types of 1,4-dioxane degradation bacteria inhabiting soil samples (*21*). Han et al. (*87*) demonstrated that mixed consortia in activated sludge samples collected from biological wastewater treatment systems installed in three polyester manufacturing corporations in Republic of Korea could remove 52% to 100% 1,4-dioxane. It was proven in that study by using 16S rRNA cloning method that the difference

in biodegradation capability of those activated sludges was due to the different microbial diversity in the samples.

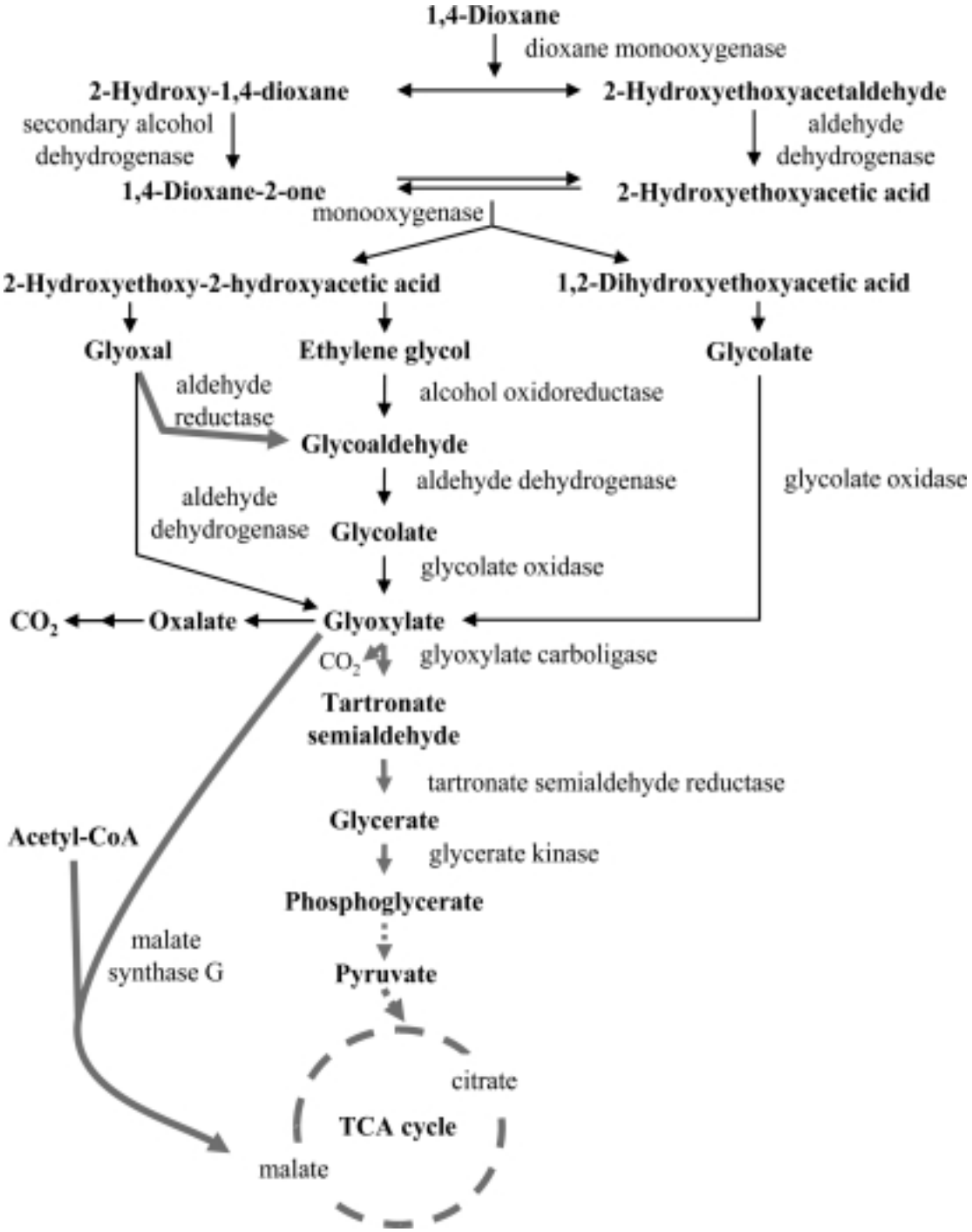

Figure 2.6. 1,4-Dioxane Mineralization by Monooxygenase-Expressing Bacteria ((*81*) after (*16*))

Mahendra et al. (*88*) investigated the effects of 1,1,1-trichloroethane and 1,1-dichloroethene, which are common co-contaminants of 1,4-dioxane in polluted groundwater, on biodegradation of 1,4-dioxane by various bacteria. The results suggested that biodegradation of 1,4-dioxane could be inhibited by these two co-contaminants. For 1,4-dioxane-metabolizing strain *P. dioxanivorans* CB1190, the inhibition was not competitive and was reversible. In contrast, the inhibition was competitive and reversible in 1,4-dioxane-cometabolizing strain *Pseudonomas mendocina* KR1 and in toluene monooxygenase-expressing *Escherichia coli*. These results suggested that biodegradation of 1,4-dioxane could be inhibited by various environmental factors, and it is important to consider the presence of these chlorinated solvents during natural monitoring attenuation and engineered bioremediation.

2.3.5. Other Processes. Other processes tend to have minor effects on the fate of 1,4-dioxane. For example, 1,4-dioxane cannot undergo transformation by dissociation when dissolved in water because its molecule does not contain a functional group capable of dissociation (which is a characteristic of stabilizers) (*3*). In addition, 1,4-dioxane is resistant to hydrolysis because there is no hydrolyzable functional group on the molecule (*89*).

2.4. Heath Effects. Toxicity of 1,4-dioxane in humans was evaluated based upon evidence from laboratory animals and limited data from human exposure. The major exposure routes of 1,4-dioxane are inhalation, ingestion, and dermal contact (*90*). Inhalation exposure and dermal contact of 1,4-dioxane is likely to occur in the occupational setting while oral exposure in humans results from ingestion of contaminated foods and drinking water. 1,4-Dioxane exposures may result in toxicities to the respiratory tract, central nervous system, liver, and kidney (*2, 48*). In addition, 1,4-dioxane is a probable carcinogen in human (*1*).

2.4.1. Acute and Short-Term Effects. 1,4-Dioxane has low acute toxicity. Inhalation and ingestion of 1,4-dioxane at toxic doses can cause serious systematic consequences, particularly to the liver and kidneys (*91*). Dermal exposure of 1,4-dioxane can cause pain and irritation to the skin upon prolonged or repeated contact (*3, 92*).

In human volunteer studies, inhalation exposure to high concentration of 1,4-dioxane (>200 mg L^{-1}) for several minutes produced irritations of the eyes, nose, and throat (*93-96*). In contrast, no significant change in clinical symptoms, e.g. blink frequency, nasal swelling, pulmonary function (*96*) or inflammatory markers in plasma (i.e., C-reactive protein and interleukin-6), was observed following exposure to 20 mg L^{-1} 1,4-dioxane for two hours (*97*). Deaths following both inhalation and dermal exposure of 1,4-dioxane have been reported (*98, 99*). Barber (*98*) revealed four deaths after exposure to high (unspecified) concentrations of 1,4-dioxane. The deaths were reported within 5-8 days of the onset of illness and the cause of death was determined as renal failure (hemorrhagic nephritis) in conjunction with liver injury (centrilobular necrosis). Johnstone (*99*) reported death due to both inhalation and dermal exposure (approximate 500 mg L^{-1}) in air, which is roughly equivalent to 257 mg kg^{-1} over an eight-hour work day) of 1,4-dioxane for a week. At autopsy, hemorrhagic nephritis and liver necrosis were recorded with perivascular widening indicating systematical and neurological effects (anoxia and cerebral edema) (*99*).

Acute toxicity of 1,4-dioxane has also been investigated in laboratory animals. The deaths of laboratory animals following inhalation of 1,4-dioxane vapor were reported in many studies (*95, 100, 101*). Inhalation exposure of 1,4-dioxane at concentrations of 1,000-30,000 mg L^{-1} to guinea pigs for 8 hours exhibited the irritation of mucous membranes, altered respiration, narcosis, pulmonary edema and congestion, and hyperemia of the brain at the higher doses (*93*).

Inhalation exposure prominently involved liver and kidney injuries, respiratory failure due to lung edema and brain edema indicating neurological effects of 1,4-dioxane (*2, 48*). The 4-hour inhalation LC for rats is 14,260 mg L^{-1} (*91*).

Oral exposure of 1,4-dioxane at high doses can cause death in mice, rats and guinea pigs (*101-103*). LD_{50} values for oral exposure of 1,4-dioxane in various laboratory animals are listed in Table 2.2. Oral exposure of 1,4-dioxane manifested clinical signs of depression including staggered gait, narcosis, paralysis, coma, and death (*100, 102, 104, 105*). Similar to inhalation exposure, oral exposure often resulted in severe liver and kidney degeneration in laboratory animals (*102, 104-108*). Moreover, histopathological lesions in the nasal cavity and the brain could be observed in rats exposed to 1,4-dioxane in drinking water for two weeks (*107*).

Deaths following dermal exposure of 1,4-dioxane were observed in rabbits, and LD_{50} was estimated as 7.6 g kg^{-1} (*91*).

Table 2.2. LD_{50} values for 1,4-dioxane in oral studies (single-dose gavage)

Animal	LD_{50} values (mg kg^{-1})	Reference
Female Nelson Rat	6400	(*101*)
Male Wistar Rat	7120	(*103*)
Rat	5400	(*102*)
Mouse	5900	(*102*)
Guinea Pig	3150	(*103*)
	4030	(*102*)

2.4.2. Chronic Effects. The epidemiology studies in occupational workers exposed to 1,4-dioxane did not provide evidence of significant effects in humans (*109, 110*). However, these data are inconclusive since the sample size of studied population and number of cases available to identify health risks to low-level exposure was very small.

Long term effects of 1,4-dioxane have been evaluated in animal studies. Chronic and subchronic exposures of 1,4-dioxane vapor were assessed in many studies (*96, 111-113*). Fairley et al. (*96*) studied the effects of inhalation exposure of 1,4-dioxane. Rabbits, guinea pigs, rats and mice (3-6 species per group) were exposed to various concentrations of 1,4-dioxane vapor for 16.5 hours per week. At 10000 mg L^{-1} concentration, only one rat survived after a 7-day exposure. Deaths of other animals (six guinea pigs, three mice and two rats) were recorded within the first five days. Acute vascular congestion and severe damage to the liver (from cloudy swelling of hepatocyte to large areas of necrosis) and kidney (degeneration of the cortical tubules with vascular congestion and hemorrhage) were observed in these animals. Kidney and liver damages were also observed in dead and surviving animals exposed to 1,4-dioxane at concentrations of 1000, 2000, or 5000 mg L^{-1}. Using data of this study, EPA has adopted 1000 mg L^{-1} 1,4-dioxane as LOAEL for liver and kidney degeneration in rats, mice, rabbits, and guinea pigs.

Chronic oral exposure of 1,4-dioxane was conducted in many studies. Kociba et al. (*114*) conducted 1,4-dioxane toxicity tests with Sherman rats of both sexes. The rats were fed with 100, 1000, or 10000 mg 1,4-dioxane L^{-1} in drinking water for 716 days. Rats receiving 10000 mg L^{-1} exhibited lower body weight gain, survival rate, and water consumption. According to a histopathological study, the animals drinking water containing 1,4-dioxane at the concentration of 1000 or 10000 mg 1,4-dioxane L^{-1} had developed renal tubular epithelial and hepatocellular

degeneration and necrosis. A NOAEL of 9.6 and 19 mg kg^{-1} body weight day^{-1} was determined for male and female rats, respectively. Liver and kidney injuries due to chronic oral exposure of 1,4-dioxane were also observed by Argus et al. (*115*), JBRC (*107*), Kano et al. (*116*), NCI (*117*) and Yamazaki et al. (*118*).

2.4.3. Developmental and Reproductive Toxicity. Giavini et al. (*119*) revealed the developmental toxicity of 1,4-dioxane in Sprague-Dawley rats. Oral exposure of 1033 mg 1,4-dioxane kg^{-1} bodyweight day^{-1} during days 6-15 of gestation could develop maternal toxicity evidenced by reduced food consumption. Oral exposure of 1,4-dioxane at 258, 516, or 1033 mg 1,4-dioxane kg^{-1} bodyweight day^{-1} did not exhibit any adverse effects on numbers of implantations, live fetuses, postimplantation loss or major malformations. At high dose exposure, reduced fetal weight and delayed ossification of the sternebrae were observed as embryotoxicities. Based on the decreases in maternal food consumption and fetal weight and delayed ossification, the NOAEL for developmental toxicity was determined at 516 mg 1,4-dioxane kg^{-1} bodyweight day^{-1} (*119*) .

Reproductive effects of 1,4-dioxane have not been reported yet.

2.4.4. Carcinogenicity. Chronic oral exposure of 1,4-dioxane in drinking water could develop liver tumors through cytotoxicity followed by regenerative hyperplasia in rats and mice, and nasal cavity, mammary, and peritoneal gland tumors in rats only (*107, 114, 116-118, 120*). According to these studies, International Agency for Research on Cancer (IARC) classified 1,4-dioxane as a member in group 2B: possibly carcinogenic to humans due to *inadequate evidence* in humans and *sufficient evidence* in animal experiments for the carcinogenicity of 1,4-dioxane (*1*). U.S. Environmental Protection Agency (USEPA) classified 1,4-dioxane as

group B: likely carcinogenic to human by all routes of exposure based on the evident from animal studies (*2*). Similarly, National Toxicology Program (NTP) of the Department of Health and Human Services classified 1,4-dioxane as reasonably anticipated to be a human carcinogen (*121*). Dourson et al. (*120*) recently proposed a reference dose (RfD) of 0.05 1,4-dioxane kg^{-1} bodyweight day^{-1} to protect against regenerative liver hyperplasia in rat and mice.

2.5. Standards and Guidelines. To date, 1,4-dioxane in water is not regulated by USEPA. However, 1,4-dioxane was added into the third contaminant candidate list (CCL), a list of contaminants that are not regulated by national primary drinking water regulations, in 2008 (*122*). Recently, 1,4-dioxane has been included in the federal Unregulated Contaminant Monitoring Requirement Rule 3 (UCMR 3) list, a list of unregulated contaminants that are anticipated or known to occur in public water systems (*123, 124*). UCMR 3 requires the analyses of 1,4-dioxane using EPA method 522 in the water systems as a regulation under the Safe Drinking Water Act (*124*).

Currently, USEPA has not yet established federal maximum contaminant level (MCL) or MCL goal for this constituent. However, several states apply an advisory guidance level for limiting 1,4-dioxane concentrations in drinking water (*3*). In March 2005, Colorado was the first state to regulate 1,4-dioxane (*3*). Recently, Colorado has established an interim groundwater quality cleanup standard of 0.35 $\mu g\ L^{-1}$ which is based on one-in-a million increased chance of developing cancer (*125*). New Hampshire has established reporting limit of 0.25 $\mu g\ L^{-1}$ for all public water supplies (*126*). While California has set a notification level for 1,4-dioxane in drinking water as 1 $\mu g\ L^{-1}$ (*65*), Massachusetts has established the guideline level of 0.3 $\mu g\ L^{-1}$ (*127*).

2.6. Current Treatment Technologies for 1,4-Dioxane

2.6.1. Physical/Chemical Processes. Bowman et al. (*128*) demonstrated that an air stripper designed to remove chlorinated solvents was able to reduce 1,4-dioxane from 610 μg L^{-1} to only 430 μg L^{-1}. Air stripping has been used to treat contaminants at US Air Force Plant 44 in Tucson, Arizona (*3*). Two-stage air-stripping towers with a loading capacity of ~5,000 gallons per minute at this site are effective to remove trichloroethylene and 1,1-dichloroethene. However, the maximum removal rate for 1,4-dioxane was only 10% even when a high air:water ratio was applied to the system. These results indicate that the air-stripping system as a primary means to remove 1,4-dioxane would not achieve the clean-up goal of 6.1 μg 1,4-dioxane L^{-1}.

Chemical treatment can be effectively applied to remove 1,4-dioxane from groundwater. For example, chlorine oxidation at 75 ˚C was proven to efficiently treat 1,4-dioxane in wastewater (*129*). The optimal pH for chlorine oxidation of 1,4-dioxane was determined as 5.2 indicating that hypochlorous acid was the preferred oxidant to treat 1,4-dioxane (*129*). Unfortunately, chlorine oxidation for 1,4-dioxane produces disinfection by-products which are 1000 times more toxic than 1,4-dioxane (*4*). This indicates that disinfection by-products of 1,4-dioxane can be generated from water and wastewater treatment plants using chlorination and can be a public health concern.

Many studies revealed that activated carbon was not very effective in removing 1,4-dioxane. Removal of 1,4-dioxane by activated carbon derived from pecan and walnut shells was demonstrated but only approximately 50% removal efficiency could be achieved (*5*). McGuire et al. (*130*) studied the removal of 1,4-dioxane during passage through granular activated carbon (GAC) column. It was found that 67% of 1,4-dioxane could be adsorbed by GAC in the column.

2.6.2. Advanced Oxidation Processes. 1,4-Dioxane can be substantially degraded by advanced water treatment technologies such as UV photoxidation (*6, 7*), chemical oxidation (*8, 9*), anodic oxidation (*10, 131*) and sonolysis (*11-14*). Degradation of 1,4-dioxane is usually improved by using two or more of these processes in conjunction. Fenton's reagent (ferrous iron) can be used with hydrogen peroxide. It was reported that 97% reduction in 1,4-dioxane can be achieved in 10 hours by exposure to Fenton's reagent with a 12:1 ratio of hydrogen peroxide to 1,4-dioxane (*129*). Degradation pathway associated with Fenton treatment of 1,4-dioxane was proposed based on radical reaction mechanism and by analysis of Fenton reaction-treated samples by Fourier transform infrared (FTIR) spectrometry (*132*). The organic chemicals including ethylene glycol, glycolic acid, oxalate anion and formic acid were proven as by-products of Fenton treatment of 1,4-dioxane (*132*). Recent study demonstrated that heat-activated persulfate oxidation completely degraded 1,4-dioxane within 3-80 hours and higher persulfate concentration could enhance the degradation (*133*). It was found that 96% of carbon from 1,4-dioxane degradation by persulfate oxidation was converted to various by-products including acetaldehyde, acetic acid, glycolaldehyde, glycolic acid, carbon dioxide, and hydrogen ions.

Stefan and Bolton (*7*) showed that UV in combination with hydrogen peroxide could be used to attain 90% reduction of 1,4-dioxane in five minutes. Moreover, employing a catalyst with UV light was shown to improve degradation of 1,4-dioxane. For example, 99% reduction of 1,4-dioxane could be obtained by applying UV (300 nm wavelength) with a titanium dioxide catalyst (*6*). Recently, Choi et al. (*10*) and De Clercq et al. (*131*) demonstrated the application of anodic oxidation by boron-doped diamond electrodes to effectively and economically remove 1,4-dioxane. Hydroxyl radicals and ultrasonic waves produced from sonication enhance 1,4-dioxane degradation (*11-13*). It is likely that hydroxyl radicals and other free radicals play an essential

role in the mineralization (*12*). In addition, it has been shown that zero-valent iron, ferrous iron, persulfate or hydrogen peroxide boosts sonolytic degradation of 1,4-dioxane (*14*). Evaluation of synergistic effects between sonolysis and photocatalysis by titanium dioxide was also reported (*134*). The results from that study suggest that addition of titanium dioxide and hydrofluoric acid-treated titanium dioxide enhance 1,4-dioxane degradation by activation of the photocatalyst surface, improving mass transport of organic molecules and preventing aggregation in samples.

Advanced oxidation processes have been successfully applied to treat 1,4-dioxane in aquifers. A technology called HiPOx system which integrates hydrogen peroxide treatment with ozonation was effective in remediating 1,4-dioxane and chlorinated solvents at groundwater treatment facilities in the San Gabriel Basin, CA (*135*). Similar approach has been successfully applied to remove 1,4-dioxane at the Pall-Gelman Sciences site in Michigan. More than 100,000 gallons of 1,4-dioxane-contaminated water was removed from the aquifer at the site leading to the reduction in plume concentrations (*56*). These applications demonstrate the usefulness of advanced chemical oxidation for the treatment of 1,4-dioxane.

2.6.3. Bioremediation. Monooxygenase-expressing bacteria have been proven to degrade 1,4-dioxane via growth-supporting or cometabolic mechanism (*18*). An aerobic Actinomycetes bacterium, namely *Pseudonocardia dioxanivorans* CB1190, is capable of using 1,4-dioxane as its sole carbon and energy source (*17, 20*). Discovery of strain CB1190 and other monooxygenase-expressing bacteria that can catabolize 1,4-dioxane confirms that bioremediation of 1,4-dioxane is possible. Based upon this knowledge, many authors built and tested bioreactors to treat 1,4-dioxane. Zenker et al. (*136*) investigated the laboratory-scale trickling filter to remove 1,4-dioxane in the presence of tetrahydrofuran as the growth substrate for microorganism. The trickling filter which was operated for approximately one year could

remove 93 – 97% of 1,4-dioxane at various loading conditions. The authors suggested that biodegradation of 1,4-dioxane requires the presence of tetrahydrofuran since 1,4-dioxane was degraded only after tetrahydrofuran was depleted. Han et al. (*63*) demonstrated the application of an up-flow biological aeration filter (UBAF) packed with tire chips to remove 1,4-dioxane from wastewater discharged from a polyester manufacturing company in South Korea. In batch experiments, the UBAF could remove 99.5% of 1,4-dioxane from the influent with 25.6 mg L^{-1} 1,4-dioxane. In continuous flow mode, the average removal efficiency was estimated at 71.4%. The optimal empty bed contact time (EBCT) and air to liquid flow rate (A:L) in continuous mode were determined at 8.5 hours and 30:1, respectively. Interestingly, microbial analysis revealed six groups of microorganisms in biofilms, and members of these groups are known to produce soluble methane monooxygenase.

Several authors suggested that monitored natural attenuation and engineered bioremediation are possible treatment strategies for 1,4-dioxane. Kelley et al. (*137*) demonstrated that 100 mg L^{-1} 1,4-dioxane could be completely removed from soil bioaugmented with *Amycolata* sp. CB1190 (now classified as *P. dioxanivorans* CB1190) and stimulated with poplar root extract within 45 days of experiment. The results suggested that CB1190 could be used as a bioremediator for 1,4-dioxane in soil. Li et al. (*19*) examined the ability of environmental samples to degrade 1,4-dioxane at low temperatures, and tested the possibility of using biostimulation and bioaugmentation treatment to enhance the degradation of 1,4-dioxane. Microcosms were prepared with groundwater and soil from an impacted site in Alaska at Arctic ground water temperature (4 ˚C). The results showed that 1,4-dioxane (50 mg L^{-1}) significantly disappeared in biologically active microcosms relative to an autoclaved control. Moreover, microcosms amended with 1-butanol (100 mg L^{-1}) and inorganic nutrients (70 mg-N L^{-1} and 310 mg-P L^{-1})

removed a significant amount of 1,4-dioxane. Arulazhagan et al. (*138*) revealed that addition of tetrahydrofuran, glucose, and yeast extract could enhance biodegradation of 1,4-dioxane by consortia enriched from industrial sludge, suggesting the possibility of using biostimulation strategy to enhance 1,4-dioxane bioremediation in industrial wastewater treatment systems. Chiang et al. (*139*) presented many lines of evidence to illustrate that biodegradation of 1,4-dioxane was naturally occurring in an aerobic aquifer contaminated with 1,4-dioxane and trichloroethene. The study provided evidence that monitored natural attenuation may be a promising strategy for 1,4-dioxane remediation at certain contaminated sites.

2.6.4. Phytoremediation and Constructed Wetlands. Aitchison et al. (*140*) demonstrated that phytoremediation is a possible technology for 1,4-dioxane remediation. In their study, hybrid poplar trees (*Populus deltoids* × *nigra*, DN34, Imperial Carolina) were grown in half-strength Hoagland's solution with the initial concentration of 22.7 mg 1,4-dioxane L^{-1} and in soil spiked with 10 mg 1,4-dioxane kg^{-1} soil. The results showed that 54% of 1,4-dioxane could be removed from the hydroponic solution by the plants within 9 days. The plants were also able to remove 53% of 1,4-dioxane in soil within 15 days. Since 1,4-dioxane is hydrophilic, the compound is readily translocated to the leaves. It was shown that approximate 80% of the uptake 1,4-dioxane was transpired from leaf surfaces to the atmosphere where photodegradation of 1,4-dioxane likely took place (*140*). Kelley et al. (*137*) showed that biodegradation of 1,4-dioxane in reactors planted with hybrid poplar trees could remove more 1,4-dioxane within 26 days than unplanted soil regardless whether strain CB1190 was bioaugmented. It is possible that 1,4-dioxane removal in the reactors resulted from evapotranspiration and biodegradation in root zone. This study suggested that bioaugmented phytoremediation is probably a promising strategy to treat 1,4-dioxane in shallow contaminated site. Ward (*141*) conducted a two-year study to investigate

the capability of a constructed wetland with and without planted cottonwood trees on 1,4-dioxane removal. It was found that 18-48 % of 1,4-dioxane was uptake by the cottonwood trees. Based on mass balance assessment, 1,4-dioxane uptake efficiency positively correlated to plant transpiration rates. Experiments in open ponds where the tank discharges were retained demonstrated that 1,4-dioxane could be also removed by volatilization and UV photodegradation. Recently, phytoremediation system by a stand of coniferous trees was also applied to treat 1,4-dioxane in recovered groundwater (*142*).

2.7. Environmental Diagnostic Tools for Validating Biodegradation. Environmental diagnostic tools are group of advanced and emerging tools used to determine chemical and biological characteristics of environmental samples including soils, sediments, surface water, and groundwater. Although the traditional analytical methods (i.e., analyses of chemical concentrations in environmental samples) can be used to demonstrate the removal or change in concentrations of organic chemicals, it is not always sufficient to determine fate of the compounds in the environments. Environmental diagnostic tools; in contrast, can provide insightful and important information for determining fates of organic compounds, and can be used to complement those traditional methods. These advanced tools are; thus, valuable in the environmental management decision-making processes typically involving site characterization, monitoring, remediation, and closure.

Environmental diagnostic tools can be categorized into three distinct groups: (i) isotopic, (ii) molecular biological and (iii) sampling tools. Key features and applications of each environmental diagnostic tool are summarized in Table 2.3.

Table 2.3. Features and Applications of Environmental Diagnostic Tools (modified from (*143*))

Category	Environmental Diagnostic Tools	Principle	Purpose	Availability for 1,4-Dioxane [Ref]
Isotopic Tools	Compound Specific Isotope Analysis	Analyzes the relative abundance of various stable isotopes (e.g., $^{13}C/^{12}C$, $^{2}H/^{1}H$) of the component elements of contaminants. Degradation processes can cause measureable shifts in the isotopic ratios.	Determine whether contaminant degradation is occurring. Investigate the degradation mechanism. Identify contaminant source.	No (first developed in this study)
	Isotope Probing	Detects the presence of an added synthesized form of the contaminant containing a stable isotope (e.g., ^{13}C) or a radioisotope (e.g. ^{14}C). If contaminant biodegradation is occurring, the isotope will be detected in biomolecules (e.g., phospholipids, DNA), intermediate by-products and metabolites (e.g., CO_2).	Determine whether biodegradation of a specific contaminant is occurring. Identify the microorganisms responsible for this activity. Identify biodegradation pathway of contaminants.	Yes (*139*)
Molecular Biological Tools (MBTs)	Polymerase Chain Reaction (PCR)	Amplifies (makes copies of) the genetic material of microorganisms to levels that can be further analyzed using other techniques.	Detect microorganisms or target genes responsible for contaminant biodegradation. Process genetic material for use in other environmental diagnostic tools.	Yes (applied in this study) (*84, 144, 145*)
	Quantitative polymerase chain reaction (qPCR)	Quantifies a target gene based on DNA or RNA.	Quantify the abundance and expression of specific functional genes, microorganisms, or groups of microorganisms responsible for contaminant biodegradation.	
	Microbial fingerprinting methods	Differentiates, and in some cases identifies, microorganisms by unique characteristics of universal biomolecules, including phospholipid fatty acids (PLFA) and nucleic acids (DNA and RNA).	Provide a profile of the microbial community. Identify a subset of the microorganisms present. Quantify living biomass.	Yes for DNA (DGGE method) (*144*)
	Microarrays/ Geochip	Detects and estimates the relative abundances of hundreds to tens of thousands of genes simultaneously.	Provide a comprehensive evaluation of the microbial diversity and community composition.	Yes (*81, 144*)
	Enzyme activity Probes (EAPs)	Detect the transformation of surrogate compounds that resemble specific contaminants.	Quantify the activity of microorganisms with specific biodegradation capabilities.	Yes (monooxygenase assay) (*18*)
	Fluorescence *in situ* hybridization (FISH)	Detects the presence of targeted genetic material in an environmental sample.	Estimate the number of and/or relative activity of specific microorganisms or groups of microorganisms.	No
Sampling Tools	Sampling methods	Active sampling methods (e.g., low-flow groundwater sampling) and passive microbial sampling devices in which subsurface microorganisms colonize a solid matrix.	Methods for collection of biomass from environmental media to be used in conjunction with environmental diagnostic tools.	Yes (Bio-Trap® samplers) (*139*)

2.7.1. Isotopic Tools

2.7.1.1. Compound Specific Isotope Analysis (CSIA). CSIA has been applied as a monitoring tool for assessing abiotic degradation and biodegradation of various organic contaminants (*32, 33, 139, 146-149*). During chemical and biological degradation processes of organic contaminants, the molecules with light isotopes tend to be degraded faster than those containing heavy isotope. As a result, the ratios of heavy (e.g., ^{13}C and ^{2}H) to light stable isotope (e.g., ^{12}C and ^{1}H) often increase in residue compound. The change in the ratios, termed as isotopic fractionation, can be used to predict the extent of degradation of the contaminants. CSIA provides a measurement of the isotopic ratios, thus it is valuable as a monitoring tool for characterizing degradation mechanisms as well as quantifying the contaminant degradation based on stable isotope data from field sites (*31, 150*). CSIA can be also used as a forensic tool for identifying the true source of contamination based on the stable isotopic signatures of the compound. CSIA was proven as an effective tool to validate biodegradation of various organic contaminants (*31-33, 147-149, 151-157*). As a tool for monitoring biodegradation, CSIA aids in judging whether *in situ* or engineered bioremediation might be an appropriate strategy to remedy a contaminated site (*158*). Recently, this technology has been adopted to verify the biodegradation of various dissolved organic contaminants such as chlorinated solvents, fuel oxygenates, and petroleum hydrocarbons (*30, 31*).

CSIA involves a three-step process accomplished by using gas chromatograph-combustion-isotope ratio mass spectrometry (GC-C-IRMS). The principles behind the instrument include: (1) separation of carbon-containing compounds using gas chromatography, (2) conversion of the separated compounds to carbon dioxide in a combustion oven, (3) removal of water produced in combustion and introduction of carbon dioxide derived from each compound into mass

spectrometer for isotope analysis (*30*). Following ionization of carbon dioxide in a mass spectrometer, the fixed Faraday cups separate ions with different mass-to-charge-ratios. Figure 2.7 is schematic diagram showing the components of a GC-C-IRMS.

CSIA involves with the measurement of the relative abundance ratio of two stable isotopes, i.e., ^{12}C and ^{13}C for carbon, and ^{2}H and ^{1}H for hydrogen, etc. These ratios are calculated relative to the international standard (generally V-PDB for carbon and V-SMOW for hydrogen) to ensure interlaboratory compatibility and accuracy. The measured values are reported as $\delta^{13}C$ or $\delta^{2}H$ for carbon and hydrogen, respectively. Equations 2.1 and 2.2 show how these terms are computed with the unit of per mille (‰).

$$\delta^{13}C\ (‰) = [(({}^{13}C/{}^{12}C)_{sample} - ({}^{13}C/{}^{12}C)_{standard})/({}^{13}C/{}^{12}C)_{standard})] \times 1000 \quad \text{(Eq. 2.1)}$$

$$\delta^{2}H\ (‰) = [(({}^{2}H/{}^{1}H)_{sample} - ({}^{2}H/{}^{1}H)_{standard})/({}^{2}H/{}^{1}H)_{standard})] \times 1000 \quad \text{(Eq. 2.2)}$$

In biochemical and abiotic reactions, molecules containing the lighter isotopes (i.e., ^{12}C) tend to react more rapidly than molecules containing heavier isotopes (i.e., ^{13}C). Thus, the ratio of ^{13}C to ^{12}C would increase as the biodegradations or abiotic reactions proceed. The shift of stable isotope ratio is known as stable isotope fractionation. Carbon isotope fractionation can be described by a fractionation factor alpha (α) as described in equation 2.3.

$$\alpha = (R_a/R_b) = [(1000 + \delta^{13}C_a)/(1000 + \delta^{13}C_b)] \quad \text{(Eq. 2.3)}$$

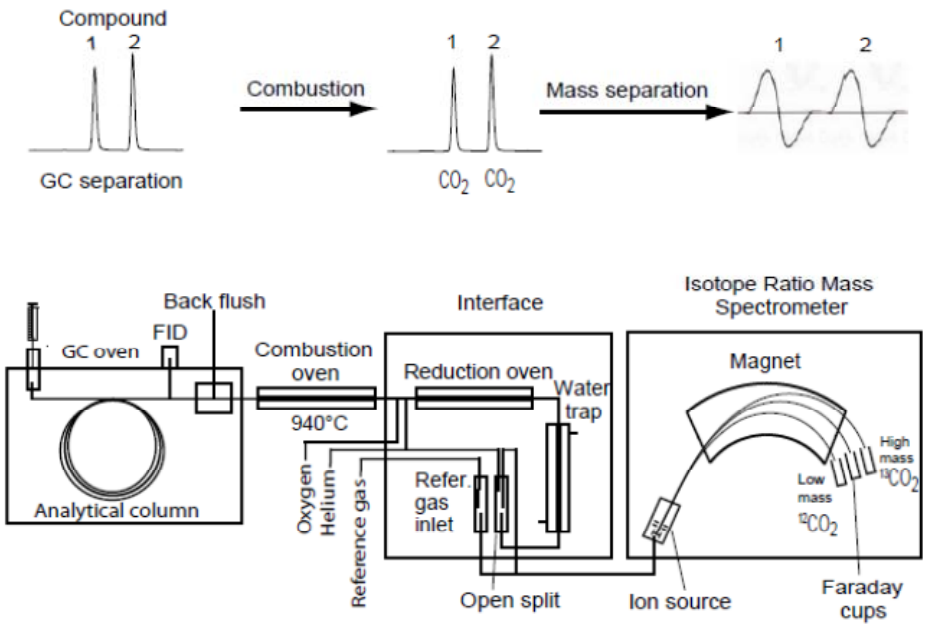

Figure 2.7. Schematic Diagram Showing Components of a GC-C-IRMS (from (*30*))

Note that R is the stable isotope ratio of the compound and subscripts a and b refer to either the compound at time zero (t=0) and a later time point (t=t) or a compound in the source zone versus a down gradient zone. Stable isotope fractionation is typically described by the Rayleigh equation (equation 2.4).

$$R_t = R_0 f^{(\alpha-1)} \qquad \text{(Eq. 2.4)}$$

According to equation 3.4, R_t is the stable isotope ratio of the compound at time t, R_0 is the initial stable isotope ratio of the compound and f is the remaining concentration ratio (C/C_0) of compound compared at time t and zero. The concentrations (C and C_0) of the studied compound (i.e., chlorinated solvents) are mostly determined by gas chromatograph (GC) coupled with a detector, either flame ionized detector (FID) or mass spectrometer (MS). Rayleigh equation (equation 3.4) can be rearranged to produce simple linear correlation equations that are more convenient for practical applications (equation 2.5-2.7) (*30*).

$$\ln(R_t/R_0) = (\alpha - 1)\ln f \quad \text{(Eq. 2.5)}$$

$$1000 \times \ln(R_t/R_0) = \varepsilon \ln f \quad \text{(Eq. 2.6)}$$

$$1000 \times \ln [(1000 + \delta_t) / (1000 + \delta_0)] = \varepsilon \ln f \quad \text{(Eq. 2.7)}$$

A new term nominated as epsilon (ε) is introduced in equation 2.6 and 2.7. This term is defined as the stable isotope enrichment factor which is equal to (α-1) x 1000 (*159*). The enrichment factor is useful for comparing the levels of fractionation among degradation mechanisms. Larger fractionation results in more negative epsilon values. Epsilon values can be determined as a slope of the graph 1000 x ln $[(1000 + \delta_t) / (1000 + \delta_0)]$ versus lnf.

2.7.1.2. Isotope Probing. Isotope probing techniques are based on the incorporation of ^{13}C- or radioisotope-labeled substrate to biological building blocks such as DNA, RNA, protein and lipid of environmental microorganisms capable of biodegrading the compound. Due to safety reasons, stable isotope probing (SIP) is more common in field applications. The separation of labeled-building block by isopycnic centrifugation and microbial fingerprint analysis of these biomolecules can provide phelogenetic and functional information of active microorganisms involving metabolism of a particular compound. Because of high sensitivity with the use of small samples, high specificity and cost-effectiveness of the SIP methods, these techniques are increasingly applied as diagnostic and monitoring tools in many environmental microbiology applications (*160*).

2.7.2. Molecular Biological Tools. Molecular biological diagnostic tools are used to evaluate the biodegradation capacities of environmental microorganisms by analyzing biomolecules usually functional genomics including DNA, RNA, proteins (enzymes) and phospholipid-derived

fatty acids (PLFAs). Specifically, the tools can be applied to (i) determine the presence of specific microorganisms capable of biodegrading specific compound in the environment, (ii) identify unknown microorganisms involved in biodegradation of contaminants, and (iii) evaluate whether microorganisms present in the environment are actively degrading the compounds. Examples of molecular biological tools are polymerase chain reaction (PCR), quantitative polymerase chain reaction (qPCR), microbial fingerprint methods (i.e., denaturing gradient gel electrophoresis (DGGE), 16S rRNA sequencing, PLFA analysis and terminal restriction fragment length polymorphism (T-RFLP)), functional gene arrays (i.e., microarray and GeoChip), enzymatic activity probes (EAPs) and fluorescent *in situ* hybridization (FISH). The description and applications of these molecular biological tools are described in Table 2.3.

2.7.3. Sampling tools. Various sampling methods have been developed to collect microorganisms from an environment (i.e., groundwater) for further microbial analyses using isotopic and/or molecular biological tools. Sampling methods can be divided into two categories: active and passive microbial sampling methods. Active microbial sampling methods involves with collection of samples from single point of time by using traditional sampling tools used for collecting soil and groundwater samples for analyses of contaminants. Examples of active sampling tools are ranging from commonly peristaltic pumps for groundwater collection to direct split spoon and push coring sampling for soil collection. Analyses of these samples by environmental diagnostic tools provide a snapshot of microbial community. Thus, to investigate how microbial community changes over time, multiple sampling collections are required.

In contrast, passive microbial samplings methods involve the use of passive sampler tools for collecting microorganisms over a period of time for the ultimate goals of better understanding the potential of biodegradation in an environment. Passive microbial sampling devices typically

a solid matrix material contained within a permeable housing. While a variety of solid matrix materials (i.e., sterile sand, granular activated carbons, glass or ceramic beads and glass wool) have been used, Bio-Trap® sampler, a commercially available passive microbial device, is commonly used. In general, environmental microorganisms tend to attach to a surface rather than freely floating in suspension. Bio-Trap® sampler, containing BioSep® beads which are a composite of 25% Nomex® fiber and 75% power activated carbon, provides a large surface area for these microorganisms to attach, colonize and form biofilm. Bio-Trap® can be used to quantify microorganisms capable of biodegrading contaminants, evaluate natural and engineered bioremediation, investigate diversity and shift of bacterial community during engineered bioremediation, and compare microbial populations among the contaminated sites. In addition, Bio-Trap® sampler can be baited with various amendments or compounds to determine site characteristics, and assist selection of bioremediation strategy for groundwater treatment.

2.8. Environmental Diagnostic Tools for Validating Biodegradation of 1,4-Dioxane. This section summarizes the development and applications of the available environmental diagnostic tools to study and validate biodegradation of 1,4-dioxane.

2.8.1. Enzymatic Activity Probes. Monooxygenase assay was applied to detect enzymatic activities related to biodegradation of 1,4-dioxane. Mahendra et al. (*18*) applied a colorimetric assay which was based on the conversion of naphthalene to 1-napthol by the enzyme, and color-generating reaction between 1-napthol and o-dianisidine, a chemical probe, to determine oxygenase activities. While the assay had limited range of quantification and relied upon 1-naphthol which is a very unstable product, it was successful to qualitatively determine monooxygenase activities in 1,4-dioxane-degrading bacteria.

2.8.2. Radioisotope Probing. Radioisotope probing can provide evidence of 1,4-dioxane biodegradation which can be used to confirm metabolic biodegradation and incorporation of the compound in laboratory cultures and environmental samples. Mahendra et al. (*16*) successfully used radioisotope-labeled 1,4-dioxane to determine intermediate and terminal products during 1,4-dioxane biodegradation by *P. dioxanivorans* CB1190. The results demonstrated that ^{14}C was incorporated into CO_2 suggesting that the bacterium can mineralize 1,4-dioxane. Furthermore, ^{14}C was detected in cell pellets which suggested incorporation of 1,4-dioxane into biomass. Li et al. (*144*) conducted a microcosm study using ^{14}C-1,4-dioxane to assess biodegradation potential of 1,4-dioxane in groundwater. The results revealed mineralization capacity of microcosm constructed with groundwater from the source zones (6.4% $^{14}CO_2$ recovery during 15 days, representing over 60% of the amount degraded), suggesting metabolic biodegradation of 1,4-dioxane by indigenous micororganisms in the groundwater.

2.8.3. PLFA-SIP and Bio-Trap® Samplers. Chiang et al. (*139*) used isotopic and sampling tools to validate *in situ* biodegradation of 1,4-dioxane. The authors applied stable isotope probing to validate the ability of indigenous microorganisms to biodegrade 1,4-dioxane and trichloroethene. The results from phospholipid fatty acid analysis with stable isotope probes (PLFA-SIP) of the microbial community revealed incorporation of ^{13}C into microbial biomass and dissolved inorganic carbon in Bio-Trap® samplers baited with ^{13}C-labeled 1,4-dioxane indicating metabolic biodegradation of 1,4-dioxane in the monitoring wells. This study provided a direct evidence to support monitored natural attenuation as a remedial strategy at specific contaminated sites.

2.8.4. Functional Gene Arrays. Functional gene arrays including microarrays and GeoChip have been used to explore biodegradation mechanism of 1,4-dioxane and determine relative

abundance of functional genes in environmental samples. Beside the study using microarrays to investigate biodegradation pathway by Grostern and coworkers (*81*) (described in section 2.3.4), Li et al. (*144*) applied functional gene array (GeoChip) to investigate relative abundance of functional genes in groundwater samples contaminated with 1,4-dioxane. The analyses suggested that genes encoding for soluble diiron monooxygenases (SDIMOs), especially group-5 SDIMOs (i.e., tetrahydrofuran monooxygenase and propane monooxygenase), were abundant in every tested samples including background samples with no exposure of 1,4-dioxane. However, in the source zone, group-5 *thmA*-like gene was enriched (2.4-fold over the background). The authors suggested the selective pressure by 1,4-dioxane at the source zone with high 1,4-dioxane concentration in majority of samples, demonstrating presence but not high sensitivity of SDIMOs to 1,4-dioxane.

2.8.5. PCR-DGGE. PCR-DGGE have been applied in many studies to illustrate diversity and presence of microorganisms and genes associated with 1,4-dioxane biodegradation. For instance, Li and coworkers (*144*) demonstrated the application of PCR-DGGE to investigate diversity of SDIMOs in various groundwater microcosms. The results revealed the presence of *ThmA*-like gene in all groundwater samples collected from four different sites suggesting that THF/dioxane monooxygenase-expressing microorganisms (likely Actinomycytes) may be widespread in groundwater which is not surprising because monooxygenase-expressing microbes are ubiquitous in the subsurface containing organic matter. Arulazhagan et al. (*138*) applied PCR-DGGE to identify bacterial strains present in an enriched consortium from an activated sludge capable of biodegrading 1,4-dioxane. The results revealed the presence of these bacteria in the consortium: bacterium enrichment culture clone strain AYS1 (JQ419749), *Runella* sp.

AYS2 (JQ419750), *Achromobacter* sp. AYS3 (JQ419751), *Marinobacter* sp. AYS4 (JQ419752) and *Rhodanobacter* sp. AYS5 (JQ419753).

2.8.5. qPCR and RT-qPCR. Quantitative polymerase chain reaction (qPCR) and reverse transcriptase qPCR have been used to determine abundance and activity of bacterial multicomponent genes involving biodegradation of 1,4-dioxane. Chiang et al. (*139*) applied CENSUS qPCR analysis (developed by Microbial Insights, Inc.) to evaluate the abundance of gene targets representing microorganisms or enzymes capable of catabolizing 1,4-dioxane and trichloroethene. The gene targets included a gene specific to methane oxidizing bacteria (mathanotrophs) and genes expressing soluble methane oxygenase (sMMO), phenol hydroxylase/toluene 2-,3-,4-monooxygenase (PHE) and toluene-3,4-monooxygenase (RMO). The analyses of DNA extracted from Bio-Trap® samplers and groundwater samples collected from monitoring wells at a site polluted with 1,4-dioxane and trichloroethene revealed the abundance of methanotrophs and genes expressing the enzymes, suggesting the possibility of natural 1,4-dioxane biodegradation at the investigated sites. Gedalanga and coworkers (*84*) developed primers, based on *in silico* analysis of *P. dioxanivorans* CB1190, for qPCR and RT-qPCR analyses to investigate abundance and expression of select monooxygenases and dehydrogenases in *P. dioxanivorans* CB1190 and in mixed bacterial cultures in industrial activated sludge samples actively biodegrading 1,4-dioxane. The results suggested that the presence of genes including dioxane monooxygenase (DXMO), propane monooxygenase (PrMO), alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) are promising indicators of 1,4-dioxane biodegradation. However, it was proven that analysis of gene abundance alone was insufficient to predict actual biodegradation. Time-course gene expression analyses during biodegradation of 1,4-dioxane by *P. dioxanivorans* CB1190 and the bacterial

consortium were also conducted in the same study. Direct association between expression of DXMO and PrMO and 1,4-dioxane removal rates was illustrated. In addition, the expression of DXMO, PrMO, and ALDH demonstrated a time-dependent relationship with 1,4-dioxane biodegradation. These results also revealed that abundance and expression of functional gene biomarkers were better predictors of 1,4-dioxane biodegradation than the taxonomic 16S rRNA genes. Based on these evidence, it was concluded that bacterial monooxygenase and dehydrogenase gene targets together serve as an effective biomarkers for assessing, monitoring and validating biodegradation of 1,4-dioxane in various environments.

CHAPTER 3

TRANSITION METALS AND ORGANIC LIGANDS INFLUENCE BIODEGRADATION OF 1,4-DIOXANE

3.1. Introduction. Monooxygenase enzymes catalyze oxidation of a broad range of organic compounds with oxygen serving as terminal electron acceptor. Methane monooxygenase (MMO) is known to oxidize over 300 compounds (*161*) and the ammonia monooxygenase (AMO) is also reported to cometabolically degrade trichloroethene (TCE) (*162*). These enzymes contain transition metals as cofactors in the catalytic site to facilitate transferring electrons from one redox couple to another (*163, 164*). While transition metals are essential elements for biological activity and enzyme expression, they may also be toxic at elevated concentrations, attacking nucleic acids and enzymatic pathways (*165*). *In vivo* reduction of transition metals can lead to the formation of reactive oxygen species, which produce oxidative stress in microorganisms (*166*). A study by Jahng and Wood (*167*) demonstrated that copper, nickel, and zinc inhibit soluble MMO (sMMO) activity in purified enzyme assays. This enzyme is responsible for the cometabolic degradation of 1,4-dioxane by methanotrophic bacteria. According to Grosse and coworkers (*168*), Cu(II), Ni(II), and Zn(II) ions inhibited the reductase, and Cu(II) may also influence other components of the sMMO. In the presence of 1 mM concentration of Cu(II), Ni(II), and Zn(II) ions, the loss of *in vitro* sMMO activity was 95, 40 and 80% respectively (*168*). Previous studies have determined that sMMO activity is most influenced by copper, followed by zinc and nickel (*167-169*) in accordance with the Irving-Williams series (*170*). Bacterial populations are also very sensitive to cadmium concentrations. In ammonia oxidizing bacteria (AOB), ammonia oxidation and a shift in dominant AOB with increasing cadmium concentrations, followed by resistance to inhibition was reported (*171*). Although transition metals at high concentrations may negatively influence enzyme activity and are toxic to microorganisms, several microorganisms have developed a variety of mechanisms to protect against metal toxicity, such as decreased uptake, binding of the metal by proteins or

extracellular polymers, formation of insoluble metal sulfides, volatilization, and enhanced export from the cells (*172*).

It is important to understand which forms of trace metals are available to bacteria for uptake. Metal speciation (the distribution of metals in free, chelated, and precipitated forms) is governed by total metal concentrations, pH, and complex interactions between the metals, ligands, major ions, and particles (*173*). Trace metals tend to be complexed by organic and inorganic ligands in natural waters and soils (*174*). For example, copper, nickel, and zinc can be complexed by biologically produced organic ligands (*175-177*). Copper forms stronger organic complexes than most other divalent metals (*178*). There have also been reports of competition of copper and zinc for strong ligands in the environment (*173*). It was also found that transition metals, especially soft metals (i.e., cadmium), can readily bind to sulfhydryl groups of proteins. This binding has an effect on the structure and function of the enzymes. This illustrates the importance of binding capability of a metal to proteins in determining its toxicity (*179*).

The objective of this study was to investigate the effects of transition metals on biodegradation of 1,4-dioxane by monooxygenase-expressing bacteria. Such data will lead to an improved understanding of 1,4-dioxane biodegradation rates and overall viability of 1,4-dioxane degrading microorganisms in waste treatment systems and at contaminated sites.

3.2. Materials and Methods.

3.2.1. Chemicals. ACS grade 1,4-dioxane ($C_4H_8O_2$), copper (II) chloride dihydrate ($CuCl_2{\cdot}2H_2O$), nickel(II) chloride hexahydrate ($NiCl_2{\cdot}6H_2O$), zinc(II) sulfate heptahydrate ($ZnSO_4{\cdot}7H_2O$), tannic acid, alginic acid, and L-cysteine were procured from Sigma Aldrich (St. Louis, MO). ACS grade cadmium sulfate 8/3 hydrate ($3CdSO_4{\cdot}8H_2O$) was purchased from

Fisher Scientific (Fair Lawn, NJ). Bovine serum albumin (BSA) protein and Suwannee River natural organic matter (SRNOM) were obtained from Thermo Scientific (Fair Lawn, NJ) and the International Humic Substances Society (Saint Paul, MN), respectively. Transition metal and organic ligand stock solutions were prepared by dissolving required masses of metal salts or organic ligands in deionized water. The stock solutions were filter-sterilized and stored at 4 ˚C for a short period prior to use.

3.2.2. Bacterial Strain and Culture Conditions. *P. dioxanivorans* CB1190 was grown in ammonium mineral salt medium (AMS) with 100 mg L^{-1} 1,4-dioxane as the sole carbon and energy source (*20*). The culture was maintained for at least five transfers to induce robust monooxygenase activity. Culture purity was examined morphologically by plating cultures onto AMS agar plate with 440 mg L^{-1} 1,4-dioxane or R2A agar plates, as well as sequencing of the 16S rRNA gene.

3.2.3. 1,4-Dioxane Biodegradation in the Presence of Transition Metals and/or Organic Ligands. Biodegradation of 1,4-dioxane by *P. dioxanivorans* CB1190 was carried out in 100 mL glass media bottles with screw caps (Fisher Scientific, Fair Lawn, NJ). The culture medium was kept at 20% of total volume of the container (i.e., 20 mL in 100 mL media bottle) to prevent oxygen mass transfer limitations. Cultures were incubated at 30 °C with 150 rpm agitation, and aseptic conditions were maintained to prevent contamination. In biodegradation studies, initial concentration of 1,4-dioxane was 120 mg L^{-1} in the bacterial cultures, which were inoculated with 1% v/v *P. dioxanivorans* CB1190 and exposed to transition metal salts individually. Metal salts were added from the stock solutions to yield Cd(II), Cu(II), Ni(II), and Zn(II) final concentrations of 0, 1, 10, or 20 mg L^{-1}. To confirm the role of free ions, organic ligands were added in the presence of each metal in the bacterial cultures. To estimate independent effects of

ligands on 1,4-dioxane biodegradation, they were also added separately in metal-free cultures. Alginic acid, tannic acid, and L-cysteine were added to obtain final concentrations of 0, 0.005, 0.05, and 0.5 mM, whereas BSA protein and SRNOM were amended to a final concentration of 0, 0.01, 0.1, and 1 mg L^{-1}. Concentrations of 1,4-dioxane were monitored by gas chromatography, and biodegradation rates were estimated by calculating the change of 1,4-dioxane concentration in each sample over a period of time. Aliquots of 0.5 mL culture samples were also collected and stored at -20 ˚C for DNA extraction and analyses of gene abundance. All experiments were conducted in duplicate bottles. Duplicate abiotic controls in each condition were maintained throughout this study.

3.2.4. Modeling of Transition Metal Speciation. Aqueous metal speciation in AMS medium was predicted using the United States Geological Survey geochemical modeling program PHREEQC version 3 (*180*). The input data to the modeling programs were concentrations of AMS medium components, pH fixed at 7, pe 4, temperature 25 ˚C and molar amounts of total metal added into the medium. Oxygen and carbon dioxide equilibria were included in the model considering that the system was open to air.

3.2.5. Gas Chromatography. Analysis of 1,4-dioxane was accomplished using a Hewlett-Packard 6890 Chromatograph (GC) equipped with a Flame Ionization Detector (FID) (Hewlett-Packard, Atlanta, GA) and a Restek® Stabilwax-DB capillary column (30 m length x 0.53 mm id x 1 μm film thickness; Restek, Bellefonte, PA). 1,4-Dioxane was measured by manually injecting 2 μL of culture samples, which were previously filtered by 0.45 μm syringe filters, into the injector set at 220 °C in splitless mode. The oven temperature was initially set at 80 °C for 3 min then raised at 20 °C min^{-1} to 140 °C, where it was held for 1 min. Detector temperature was set at 250 °C. Resolution of the 1,4-dioxane peak occurred at a retention time of 3.7 minutes.

1,4-Dioxane standards covering the range of the experimental concentrations were prepared by diluting pure 1,4-dioxane in deionized water and used for building calibration curves. The accuracy of the method was better than ± 4% and the method detection limit was estimated at 0.80 mg L^{-1}.

3.2.6. DNA Extraction. A phenol/chloroform extraction method was used to extract total nucleic acids from 0.5 mL of cells cultured in the presence of metals and/or organic ligands as previously described (*84*). Briefly, bacterial cells were lysed by heating to 65°C for 2 min, bead beating for 2 min with a Mini Bead Beater (Biospec Products, Bartlesville, OK), incubating at 65°C for 8 min, and bead beating again for 2 min followed by phenol/chloroform/isoamyl alcohol purification. Nucleic acids were precipitated from solution by addition of 0.1 volume 3 M sodium acetate, 1 volume isopropanol, and incubated overnight at -20°C. The precipitate was collected by centrifugation at 20,000 x g for 30 min at 4°C and washed with 70% ethanol. Purified extracts were resuspended in 100 μL DNase and RNase-free water and quantified using a Nanodrop 2000C Spectrophotometer (Thermo Scientific, Wilmington, DE). All extracts were stored at -80°C until ready for qPCR analysis.

3.2.7. Quantitative Polymerase Chain Reaction (qPCR). Cell abundance was determined by quantification of both functional and phylogenetic genes in *P. dioxanivorans* CB1190 using qPCR. The 16S rRNA gene and dioxane monooxygenase gene (DXMO) were chosen as the phylogenetic and functional gene targets, respectively. DXMO catalyzes the first reaction of 1,4-dioxane biodegradation while the 16S rRNA gene is commonly used in phylogenetic analysis of bacteria. The complete genomic sequence of *P. dioxanivorans* CB1190 reveals that three copies of the 16S rRNA gene and one copy of DXMO are found per cell (*181*). These gene targets were amplified using primers and thermal cycling parameters as previously described (*84*) with slight

modification. Briefly, qPCR reactions were performed in a total volume of 20 µL containing 1X Luminaris Color HiGreen High ROX qPCR Master Mix (Thermo Scientific, Waltham, MA), 0.3 µM of each primer (IDTDNA, Coralville, IA), and 2 µL of template DNA. Standard curves for the DXMO and 16S rRNA gene targets were generated using genomic DNA extracted from pure cultures of *P. dioxanivorans* CB1190, and the minimum detection limit for the primer sets used in this study was 1.3×10^3 copies mL^{-1}.

3.2.8. Statistical Analyses. One-way ANOVA (ANOVA: single factor) followed by Bonferroni's post-hoc comparisons tests with significance levels of 5% were conducted to test for significant differences of biodegradation rate and gene abundance between treatments. All statistical analyses were executed using Microsoft® Office Excel 2007 with Analysis Toolpak add-in software (Microsoft Corporation, Redmond WA).

3.3. Results

3.3.1. Influence of Transition Metals on 1,4-Dioxane Biodegradation. Biodegradation of 1,4-dioxane was influenced to varying degrees by independent exposures to the four transition metals (Figure 3.1). In this study, percent biodegradation rates[1] were computed and compared (Figure 3.2). In the absence of any added transition metal beyond AMS recipe (referred to as metal-free or positive control), *P. dioxanivorans* CB1190 biodegraded 120 mg L^{-1} 1,4-dioxane to below detection limit within four days with an average biodegradation rate of 30.23 ± 2.86 mg L^{-1} d^{-1}. Biodegradation of 1,4-dioxane by cultures containing 1 mg L^{-1} Cd(II) or Ni(II), or 1, 10, or 20 mg L^{-1} Zn(II) was indistinguishable from the metal-free controls. Cultures with addition of 1 mg L^{-1} Cu(II), 10 or 20 mg L^{-1} Cd(II), or 10 or 20 mg L^{-1} Ni(II) experienced longer

[1] % Biodegradation rate = (Biodegradation rate of the tested condition/Biodegradation rate in metal-free control) x 100

initial lag, but ultimately degraded 1,4-dioxane to below detection limit within six days. Addition of 10 and 20 mg L^{-1} Cu(II) decreased 1,4-dioxane biodegradation rates by nearly 90% to 2.84 ± 1.42 and 2.79 ± 0.07 mg L^{-1} d^{-1}, respectively.

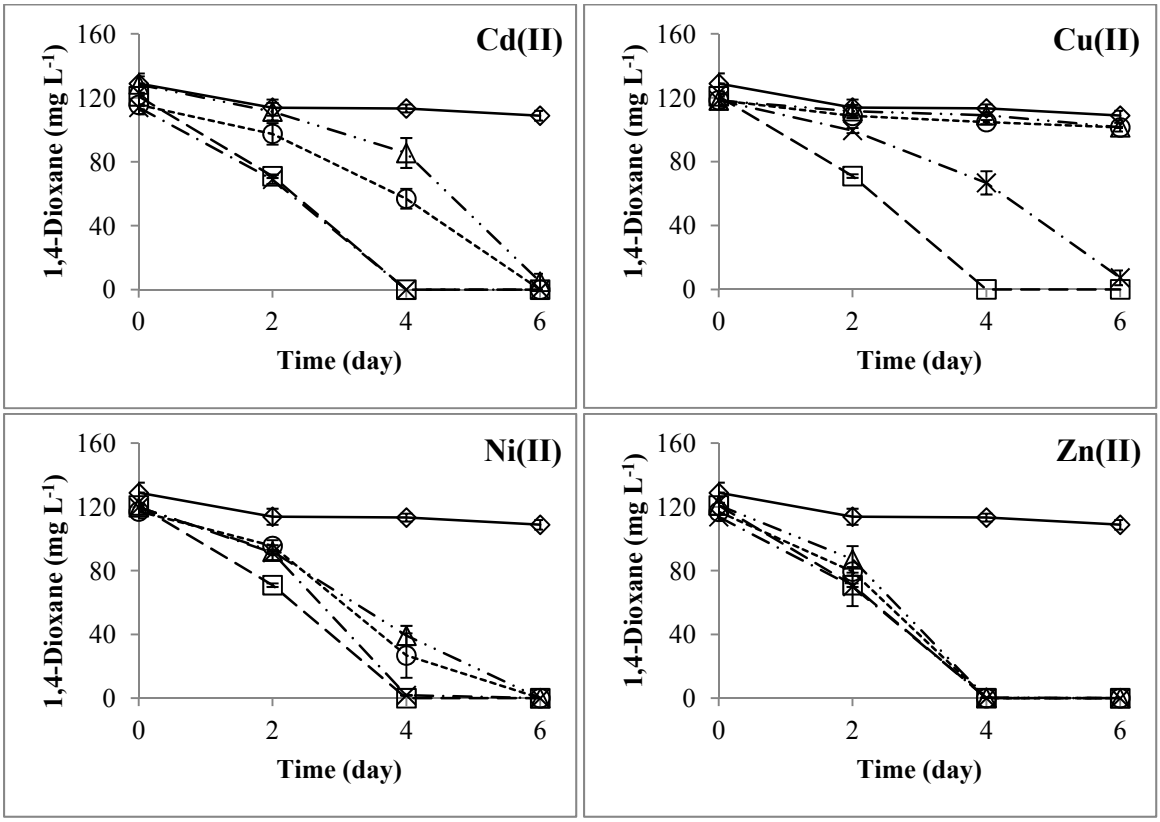

Figure 3.1. 1,4-Dioxane biodegradation by *Pseudonocardia dioxanivorans* CB1190 in presence of four transition metals at three different concentrations of 1 mg L^{-1} (crosses), 10 mg L^{-1} (circles) and 20 mg L^{-1} (triangles). Diamonds represent abiotic control as negative control and squares represent metal-free control as positive control. Inhibitory effect of transition metals on 1,4-dioxane biodegradation was measured to be in the following order: Cu(II) > Cd(II) ≥ Ni(II) > Zn(II). The error bars correspond to the range of duplicate samples.

3.3.2. Interactive Effects of Cu(II) and Organic Ligands on 1,4-Dioxane Biodegradation. Bioavailability and toxicity of Cu(II) could be alleviated by some organic ligands (Figure 3.3) resulting in improved 1,4-dioxane biodegradation rates (Figure 3.4). While metal-free controls degraded 1,4-dioxane to below detection within 8 days, at biodegradation rates ranging from

12.57 ± 0.15 to 17.09 ± 0.24 mg L^{-1} d^{-1}, cultures containing 2 mg L^{-1} Cu(II) produced biodegradation rates between 0.35 ± 0.51 and 1.15 ± 0.80 mg L^{-1} d^{-1}, but could not completely biodegrade 1,4-dioxane. 1,4-Dioxane biodegradation rates were higher when 0.05 or 0.5 mM L-cysteine, or 0.005 mM tannic acid were added to the cultures in the presence of 2 mg L^{-1} Cu(II). Under these conditions, nearly 100%, 100%, and 44% of the initial 1,4-dioxane was degraded at rates of 15.19 ± 2.60, 16.14 ± 0.18 and 7.54 ± 1.03 mg L^{-1} d^{-1}, respectively. These biodegradation rates were significantly different from those determined for cultures exposed to 2 mg L^{-1} Cu(II) ($p < 0.05$) without ligands. Biodegradation rates with addition of 2 mg L^{-1} Cu(II) were higher at all concentrations of alginic acid, but they were not significantly different from Cu(II) controls ($p = 0.18 – 0.25$). Addition of BSA or SRNOM in cultures with 2 mg L^{-1} Cu(II) did not improve biodegradation rates (Figure 3.4).

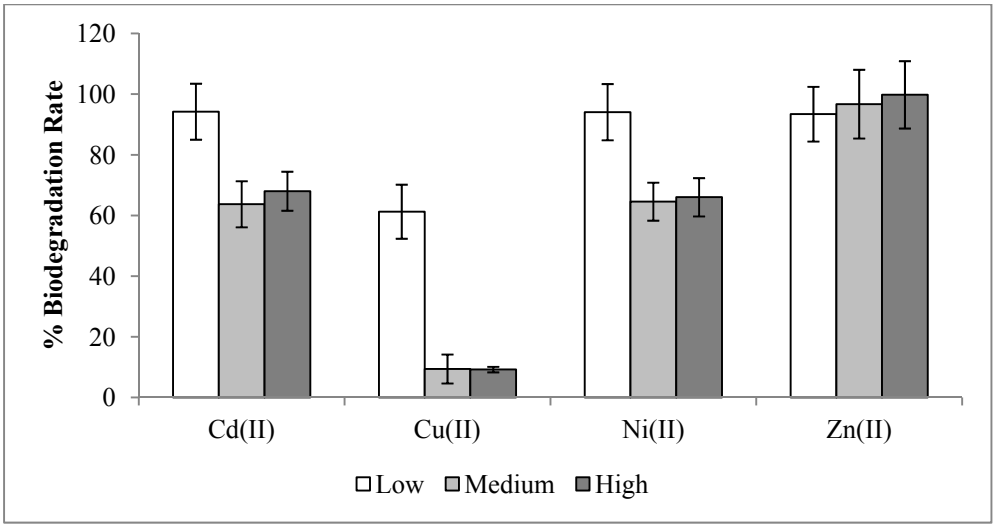

Figure 3.2. Effects of four different transition metals at three different concentrations on the rate of 1,4-dioxane biodegradation by *Pseudonocardia dioxanivorans* CB1190. % Biodegradation rate plotted on y-axis is biodegradation rate measured in the presence of metals normalized with that established for metal-free positive controls. White columns, 1 mg L^{-1} transition metal; light grey columns, 10 mg L^{-1} transition metal; dark grey column, 20 mg L^{-1} transition metal. Addition of Cd(II) to 10 and 20 mg L^{-1}, Cu(II) at all tested concentrations, and Ni(II) at 10 and 20 mg L^{-1} significantly affected 1,4-dioxane biodegradation rates. The error bars correspond to the range of duplicate samples.

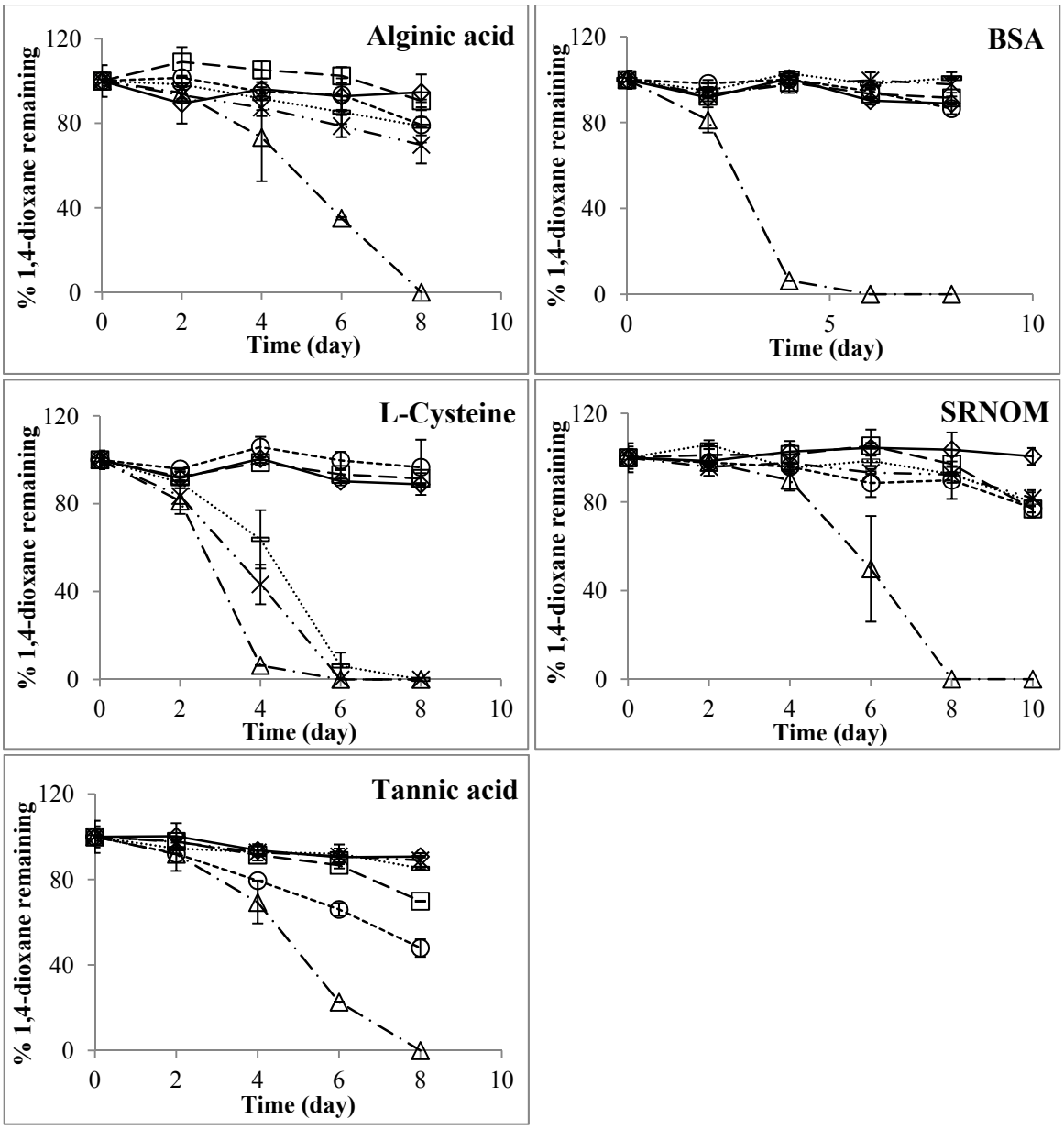

Figure 3.3. 1,4-Dioxane biodegradation by *Pseudonocardia dioxanivorans* CB1190 exposed to 2 mg L^{-1} Cu(II) concurrently with each of the five organic ligands. Low, medium and high concentrations of the ligands are represented by circles, dash signs and X signs, respectively. Diamonds represent abiotic control as negative control, squares represent cultures with addition of 2 mg L^{-1} Cu(II) alone, and triangles represent metal-free control as positive control. Alginic acid, L-cysteine and tannic acid were added at 0.005, 0.05 and 0.5 mM, whereas BSA and SRNOM were added at 0.01, 0.1 and 1 mg L^{-1}. Only low concentrations of tannic acid, and medium and high concentrations of L-cysteine were able to partially mitigate the inhibitory effects of 2 mg L^{-1} Cu(II) on biodegradation activity. The error bars correspond to the range of duplicate samples.

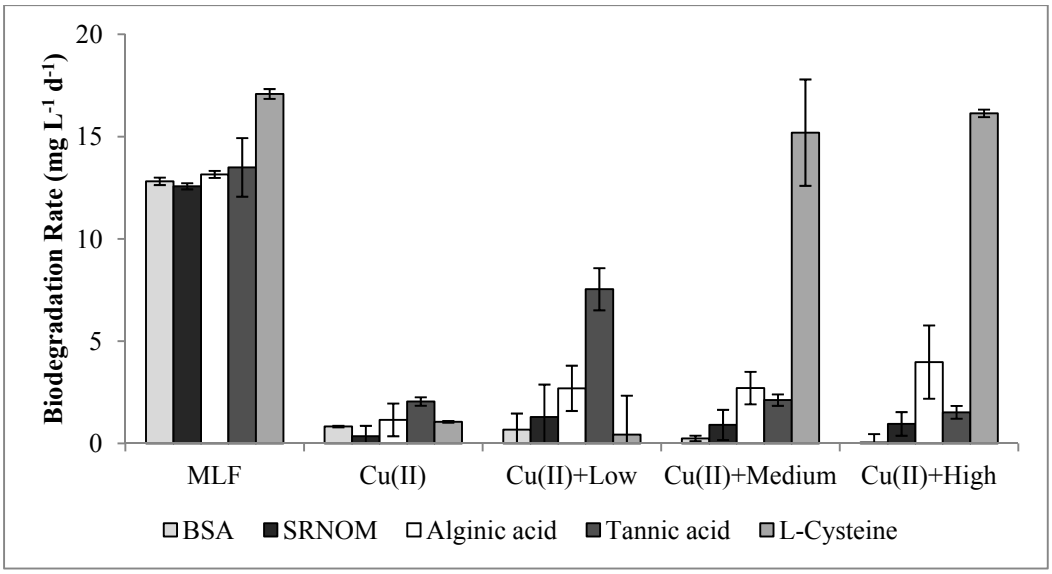

Figure 3.4. 1,4-Dioxane biodegradation by *Pseudonocardia dioxanivorans* CB1190 exposed to 2 mg L^{-1} Cu(II) simultaneously with each of the five organic ligands added at three different concentrations. Inhibition of 1,4-dioxane biodegradation due to the presence of 2 mg L^{-1} Cu(II) was mitigated by L-cysteine at 0.05 and 0.5 mM, and tannic acid at 0.005 mM. This supports the hypothesis that organic ligands decreased bioavailability and toxicity of Cu(II) resulting in improved 1,4-dioxane biodegradation rates. The error bars correspond to the range of duplicate samples.

3.3.3. Influence of Organic Ligands on 1,4-Dioxane Biodegradation. Exposure of *P. dioxanivorans* CB1190 to alginic acid, tannic acid, L-cysteine, BSA protein, or SRNOM also resulted in altered biodegradation rates (Figure 3.5). Although ligand-free controls could degrade 1,4-dioxane within 8 days, with biodegradation rates between 12.57 ± 0.15 and 17.09 ± 0.24 mg $L^{-1} d^{-1}$, 1,4-dioxane concentrations in abiotic and ligand-amended abiotic controls did not change over time. Removal of 1,4-dioxane to below detection limit was observed in most cultures with addition of organic ligands except with the amendments of tannic acid (0.05 and 0.5 mM) and L-cysteine (0.5 mM). Surprisingly, cultures with addition of 0.05 and 0.5 mM tannic acid and 0.5 mM L-cysteine could remove only 29%, 11%, and 23% of initial 1,4-dioxane within 8 days at rates of 4.25 ± 0.09, 1.54 ± 0.27, and, 2.83 ± 1.50 mg $L^{-1} d^{-1}$, respectively.

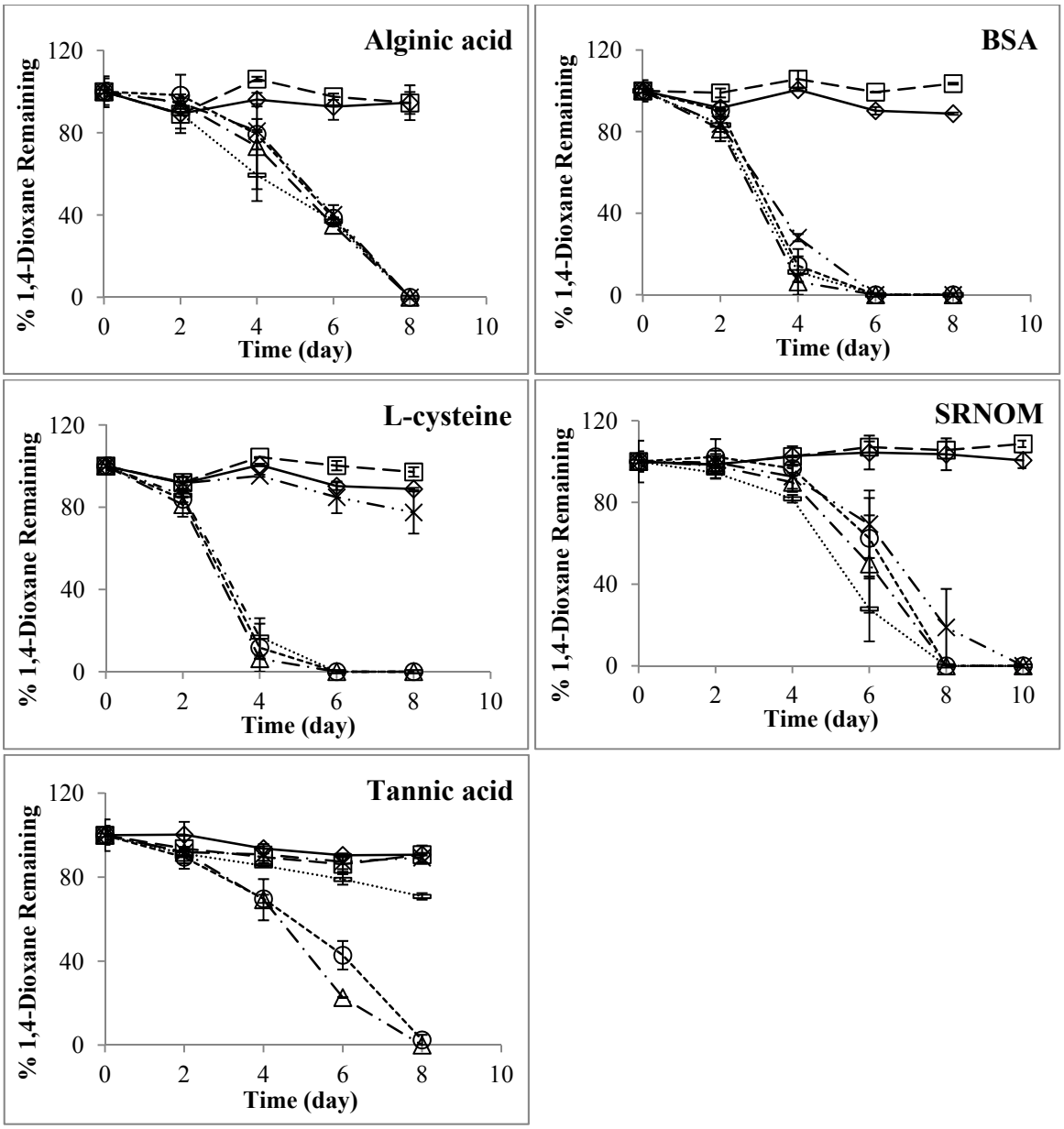

Figure 3.5. Biodegradation of 1,4-dioxane by *Pseudonocardia dioxanivorans* CB1190 in the presence of five separate organic ligands. Low, medium, and high concentrations are indicated by circles, dash signs, and X signs, respectively. Diamonds, open squares, and triangles represent abiotic control as negative control, ligand-amended abiotic control, and ligand-free control as positive control, respectively. Alginic acid, L-cysteine, and tannic acid were added at 0.005, 0.05, and 0.5 mM, whereas BSA and SRNOM were added at 0.01, 0.1 and 1 mg L^{-1}. 1,4-Dioxane biodegradation was hindered by 0.05 and 0.5 mM tannic acid and 0.5 mM L-cysteine. The error bars correspond to the range of duplicate samples.

3.3.4. Metal Speciation in Ammonium Mineral Salt Medium. Speciation of Cd(II), Cu(II), Ni(II), and Zn(II) in the AMS medium with various concentrations of metal salts were predicted using PHREEQC simulation (Table 3.1-3.4). From the model, unmodified AMS contained total soluble Cu(II), Ni(II), and Zn(II) at respective concentrations of 2.93×10^{-8}, 4.21×10^{-8} and 1.40×10^{-6} M, but no total Cd(II) concentration. The pH and pe of the AMS were estimated at 7.10 and 13.51, respectively. Adding metal salts into AMS increased total soluble metal concentrations at approximately the same amount added. Dissolved free ions were determined as the most abundant species of the metals in AMS. Addition of metal salts had minimal effects on pH and pe of the medium.

3.3.5. Gene Abundance Analyses. Transition metals, organic ligands, and metal-ligand complexes affected bacterial population as evidenced by quantification of 16S rRNA and DXMO genes in cultures with different treatments. In this study, % 16S rRNA gene abundances[2] of cultures with 20 mg L^{-1} Cd(II), Cu(II), Ni(II) and Zn(II) at day 4 were estimated at 109 ± 18 %, 53 ± 13 %, 78 ± 16 % and 165 ± 26%, respectively (Figure 3.6a). 16S rRNA gene copy numbers at day 8 in cultures exposed to 2 mg L^{-1} Cu(II) and supplemented with 0.005 mM tannic acid, 0.5 mM alginic acid, or 0.5 mM L-cysteine were estimated at 4.63×10^5, 3.62×10^5 and 4.14×10^6 gene copies mL^{-1}, respectively, whereas those for cultures amended with just the three ligands at the aforementioned concentrations were 3.71×10^8, 1.98×10^8 and 1.14×10^5 gene copies mL^{-1} (Figure 3.6b). It should be noted that the copy numbers of 16S rRNA genes in metal- and ligand-free controls were in the range of $4.71 \times 10^6 - 6.35 \times 10^7$ gene copies mL^{-1}, and those of cultures with 2 mg L^{-1} Cu(II) were nearly two orders of magnitude lower ($2.57 \times 10^4 - 2.28 \times 10^5$ gene copies mL^{-1}) (Figure 3.6b). Comparison of the cell concentration estimates

[2] % Gene abundance = (Gene abundance of the tested condition/Gene abundance of metal-free control) x 100

based on qPCR amplification of DXMO and 16S rRNA genes in tannic acid-free controls, and cultures with addition of 0.005 or 0.05 mM tannic acid was performed after 8 days of incubation (Figure 3.6c). Abundance of genes in the metal-free control at day 8 was estimated at 6.33×10^7 16S rRNA gene copies mL^{-1} and 1.99×10^7 DXMO gene copies mL^{-1}. Gene abundance increased in the presence of 0.005 mM tannic acid (3.69×10^8 16S rRNA gene copies mL^{-1} and 7.42×10^7 DXMO gene copies mL^{-1}), but decreased in the presence of 0.05 mM tannic acid (2.28×10^5 16S rRNA gene copies mL^{-1} and 2.87×10^5 DXMO gene copies mL^{-1}) (Figure 3.6c).

Table 3.1. Chemical Equilibrium Modeling of Cadmium Speciation after Dissolving Various Amounts of Cadmium Sulfate 8/3 Hydrate ($3CdSO_4 \cdot 8H_2O$) in Abiotic AMS.

Species/Parameters	**Concentration (mg L^{-1})**						
Total Cd added	0	0.5	1	5	10	20	50
	Concentration (M)						
Total Cd added	0.00E+00	4.45E-06	8.90E-06	4.45E-05	8.90E-05	1.78E-04	4.45E-04
Total soluble Cd	*0.00E+00*	*4.45E-06*	*8.90E-06*	*4.45E-05*	*8.90E-05*	*1.78E-04*	*4.45E-04*
Cd^{2+}	0.00E+00	3.14E-06	6.28E-06	3.14E-05	6.28E-05	1.26E-04	3.13E-04
$CdSO_4$	0.00E+00	1.14E-06	2.28E-06	1.14E-05	2.29E-05	4.59E-05	1.16E-04
$Cd(SO_4)_2^{2-}$	0.00E+00	9.37E-08	1.87E-07	9.41E-07	1.89E-06	3.83E-06	9.86E-06
$CdCl^+$	0.00E+00	2.52E-08	5.03E-08	2.51E-07	5.02E-07	9.99E-07	2.46E-06
$CdEdta^{2-}$	0.00E+00	2.47E-08	4.86E-08	2.20E-07	4.00E-07	6.92E-07	1.32E-06
$CdHCO_3^+$	0.00E+00	1.12E-08	2.25E-08	1.12E-07	2.24E-07	4.48E-07	1.11E-06
CdCO3	0.00E+00	1.12E-08	2.23E-08	1.11E-07	2.22E-07	4.43E-07	1.10E-06
$CdOH^+$	0.00E+00	1.70E-09	3.40E-09	1.70E-08	3.39E-08	6.77E-08	1.68E-07
CdOHCl	0.00E+00	1.03E-10	2.07E-10	1.03E-09	2.06E-09	4.09E-09	1.01E-08
$CdCl_2$	0.00E+00	1.35E-11	2.69E-11	1.34E-10	2.67E-10	5.30E-10	1.30E-09
Cd_2OH^{3+}	0.00E+00	9.07E-13	1.81E-12	9.06E-12	2.52E-11	1.00E-10	6.23E-10
$Cd(OH)_2$	0.00E+00	6.30E-14	2.52E-13	6.29E-12	1.81E-11	3.61E-11	8.92E-11
$CdCl_3^-$	0.00E+00	1.74E-15	3.47E-15	1.73E-14	3.44E-14	6.81E-14	1.65E-13
$Cd(OH)_3^-$	0.00E+00	1.62E-18	3.24E-18	1.62E-17	3.23E-17	6.42E-17	1.59E-16
$Cd(CO3)_3^{4-}$	0.00E+00	3.01E-21	6.01E-21	3.00E-20	6.00E-20	1.20E-19	2.97E-19
$Cd(OH)_4^{2-}$	0.00E+00	3.50E-25	7.00E-25	3.49E-24	6.98E-24	1.39E-23	3.43E-23
$CdHEdta^-$	0.00E+00	4.28E-29	8.43E-29	3.82E-28	6.94E-28	1.20E-27	2.28E-27
$CdHS^+$	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
$Cd(HS)_2$	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
$Cd(HS)_3^-$	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
$Cd(HS)_4^{2-}$	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
pH	*7.100*	*7.100*	*7.100*	*7.100*	*7.099*	*7.099*	*7.098*
Pe	*13.505*	*13.506*	*13.506*	*13.506*	*13.506*	*13.506*	*13.507*

Table 3.2. Chemical Equilibrium Modeling of Copper Speciation after Dissolving Various Amounts of Copper(II) Chloride Dihydrate ($CuCl_2 \cdot 2H_2O$) in Abiotic AMS.

Species/Parameters	**Concentration (mg L^{-1})**						
Total Cu added	0	0.5	1	5	10	20	50
	Concentration (M)						
Total Cu added	0.00E+00	7.81E-06	1.56E-05	7.81E-05	1.56E-04	3.13E-04	7.81E-04
Total Soluble Cu	*2.93E-08*	*7.84E-06*	*1.57E-05*	*7.82E-05*	*1.56E-04*	*3.13E-04*	*7.80E-04*
Total Cu(I)	*4.72E-20*	*1.75E-17*	*3.78E-17*	*2.11E-16*	*4.30E-16*	*8.79E-16*	*2.67E-15*
Cu^{+}	4.68E-20	1.74E-17	3.74E-17	2.06E-16	4.09E-16	7.77E-16	1.67E-15
$CuCl_2^{-}$	3.80E-22	1.63E-19	4.03E-19	5.16E-18	2.09E-17	1.02E-16	9.92E-16
$CuCl_3^{2-}$	1.83E-25	8.46E-23	2.24E-22	4.38E-21	2.54E-20	1.99E-19	4.10E-18
$Cu(S_4)_2^{3-}$	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
$CuS_4S_5^{3-}$	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
Total Cu(II)	*2.93E-08*	*7.84E-06*	*1.57E-05*	*7.82E-05*	*1.56E-04*	*3.13E-04*	*7.80E-04*
Cu^{2+}	1.38E-08	2.67E-06	5.74E-06	3.12E-05	6.10E-05	1.13E-04	2.52E-04
$Cu(OH)_2$	7.21E-09	2.27E-06	4.90E-06	2.73E-05	5.50E-05	1.08E-04	2.24E-04
$Cu_2(OH)_2^{2+}$	6.10E-09	2.07E-06	3.15E-06	6.68E-06	1.23E-05	2.40E-05	1.05E-04
$CuSO_4$	1.37E-09	5.08E-07	1.10E-06	6.12E-06	8.34E-06	2.25E-05	5.56E-05
$CuCO_3$	4.06E-10	1.50E-07	3.23E-07	1.76E-06	6.24E-06	9.80E-06	1.26E-05
$CuOH^{+}$	3.50E-10	1.30E-07	2.80E-07	1.59E-06	3.43E-06	6.34E-06	1.25E-05
$CuEdta^{2-}$	7.60E-11	2.82E-08	6.07E-08	1.54E-06	3.06E-06	5.81E-06	1.12E-05
$CuHCO_3^{+}$	1.22E-12	1.13E-08	5.23E-08	3.35E-07	6.63E-07	1.26E-06	2.72E-06
$CuCl^{+}$	8.18E-14	4.86E-10	1.12E-09	9.57E-09	2.76E-08	8.63E-08	4.27E-07
$Cu(CO3)_2^{2-}$	4.32E-14	1.59E-11	3.42E-11	1.81E-10	3.43E-10	5.99E-10	1.02E-09
$CuCl_2$	6.92E-15	2.56E-12	5.49E-12	2.95E-11	5.67E-11	1.02E-10	2.49E-10
$Cu(OH)_3^{-}$	8.29E-17	3.57E-14	8.82E-14	1.15E-12	4.72E-12	2.37E-11	1.87E-10
$CuCl_3^{-}$	3.34E-20	1.23E-17	2.64E-17	1.40E-16	2.66E-16	4.63E-16	1.56E-15
$Cu(OH)_4^{2-}$	6.09E-23	2.82E-20	7.46E-20	1.48E-18	8.71E-18	7.00E-17	7.88E-16
$CuCl_4^{2-}$	1.45E-23	2.19E-21	3.35E-21	7.18E-21	9.10E-21	1.10E-20	2.04E-20
$CuHEdta^{-}$	9.30E-29	4.63E-26	1.31E-25	3.96E-24	3.34E-23	4.31E-22	1.36E-20
$Cu(HS)_3^{-}$	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
pH	*7.100*	*7.099*	*7.098*	*7.093*	*7.086*	*7.074*	*7.039*
Pe	*13.505*	*13.506*	*13.507*	*13.513*	*13.519*	*13.532*	*13.566*

Table 3.3. Chemical Equilibrium Modeling of Nickel Speciation after Dissolving Various Amounts of Nickel(II) Chloride Hexahydrate ($NiCl_2 \cdot 6H_2O$) in Abiotic AMS.

Species/Parameters	**Concentration (mg L^{-1})**						
Total Ni added	0	0.5	1	5	10	20	50
	Concentration (M)						
Total Ni added	0.00E+00	8.52E-06	1.70E-05	8.52E-05	1.70E-04	3.41E-04	8.52E-04
Total Soluble Ni	*4.21E-08*	*8.56E-06*	*1.71E-05*	*8.52E-05*	*1.70E-04*	*3.41E-04*	*8.52E-04*
Ni^{2+}	4.15E-08	7.38E-06	1.11E-05	5.31E-05	1.16E-04	2.43E-04	6.25E-04
$NiSO_4$	4.59E-10	8.73E-07	4.44E-06	1.34E-05	2.83E-05	5.86E-05	1.48E-04
$NiCO_3$	1.12E-10	2.14E-07	1.09E-06	1.30E-05	1.35E-05	2.45E-05	6.08E-05
$NiEdta^{2-}$	4.82E-11	9.11E-08	4.62E-07	5.49E-06	1.19E-05	1.36E-05	1.36E-05
$NiHCO_3^{+}$	1.93E-12	3.66E-09	1.86E-08	2.21E-07	4.82E-07	1.00E-06	2.52E-06
$NiCl^{+}$	4.12E-13	7.82E-10	3.97E-09	4.73E-08	1.03E-07	2.19E-07	1.21E-06
$NiOH^{+}$	9.65E-14	1.99E-10	1.09E-09	2.04E-08	6.46E-08	2.14E-07	5.40E-07
$Ni(SO_4)_2^{2-}$	4.53E-14	8.61E-11	4.38E-10	5.22E-09	1.13E-08	2.35E-08	5.87E-08
$Ni(CO_3)_2^{2-}$	7.08E-15	1.33E-11	6.73E-11	7.95E-10	1.72E-09	3.50E-09	8.41E-09
$NiCl_2$	2.97E-15	5.61E-12	2.85E-11	3.38E-10	7.33E-10	1.51E-09	5.17E-09
$Ni(OH)_2$	4.50E-17	1.00E-13	5.91E-13	1.73E-11	7.98E-11	4.37E-10	3.74E-09
$Ni(OH)_3^{-}$	4.72E-19	8.89E-16	4.51E-15	5.33E-14	1.15E-13	2.37E-13	5.77E-13
$NiHEdta^{-}$	2.90E-24	5.17E-22	7.77E-22	9.40E-22	9.54E-22	9.64E-22	9.81E-22
pH	*7.100*	*7.099*	*7.098*	*7.097*	*7.095*	*7.093*	*7.085*
Pe	*13.505*	*13.507*	*13.507*	*13.509*	*13.510*	*13.513*	*13.520*

Table 3.4. Chemical Equilibrium Modeling of Zinc Speciation after Dissolving Various Amounts of Zinc(II) Sulfate Heptahydrate ($ZnSO_4 \cdot 7H_2O$) in Abiotic AMS.

Species/Parameters	**Concentration (mg L^{-1})**						
Total Zn added	0	0.5	1	5	10	20	50
	Concentration (M)						
Total Zn added	0.00E+00	7.69E-06	1.54E-05	7.69E-05	0.000154	0.000308	0.000769
Total Soluble Zn	*1.40E-06*	*9.09E-06*	*1.68E-05*	*7.83E-05*	*1.55E-04*	*3.09E-04*	*7.70E-04*
Zn^{2+}	1.03E-06	6.68E-06	1.23E-05	5.77E-05	1.15E-04	2.29E-04	5.73E-04
$ZnSO_4$	3.24E-07	2.10E-06	3.88E-06	1.81E-05	3.58E-05	7.10E-05	1.74E-04
$Zn(SO4)_2^{2-}$	1.97E-08	1.28E-07	2.36E-07	1.10E-06	2.17E-06	4.29E-06	1.04E-05
$ZnEdta^{2-}$	1.28E-08	7.98E-08	1.42E-07	5.38E-07	9.01E-07	1.74E-06	4.32E-06
$ZnOH^+$	7.84E-09	5.09E-08	9.40E-08	4.39E-07	8.71E-07	1.44E-06	2.48E-06
$ZnHCO_3^+$	3.93E-09	2.55E-08	4.71E-08	2.20E-07	4.37E-07	8.70E-07	2.17E-06
$ZnCO_3$	3.11E-09	2.02E-08	3.72E-08	1.74E-07	3.44E-07	6.84E-07	1.69E-06
$Zn(OH)_2$	8.97E-10	5.82E-09	1.07E-08	5.02E-08	9.94E-08	2.21E-07	1.17E-06
$ZnCl^+$	2.51E-10	1.75E-09	3.45E-09	2.45E-08	6.94E-08	1.98E-07	4.89E-07
ZnOHCl	3.04E-11	2.12E-10	4.18E-10	2.96E-09	8.38E-09	2.67E-08	1.41E-07
$Zn(CO_3)_2^{2-}$	5.61E-12	3.64E-11	6.73E-11	3.14E-10	6.21E-10	1.23E-09	3.04E-09
$Zn(OH)_3^-$	4.51E-14	2.93E-13	5.40E-13	5.68E-12	2.29E-11	1.17E-10	1.31E-09
$ZnCl_2$	3.33E-14	2.50E-13	5.27E-13	2.52E-12	4.99E-12	9.91E-12	2.45E-11
$ZnCl_3^-$	7.74E-18	6.23E-17	1.41E-16	2.30E-15	1.33E-14	1.08E-13	2.57E-12
$Zn(OH)_4^{2-}$	1.73E-19	1.13E-18	2.08E-18	9.69E-18	1.92E-17	6.55E-17	3.31E-15
$ZnCl_4^{2-}$	1.18E-21	1.02E-20	2.46E-20	6.12E-19	5.03E-18	3.81E-17	9.38E-17
$ZnHEdta^-$	1.91E-23	1.19E-22	2.12E-22	8.04E-22	1.35E-21	2.15E-21	3.72E-21
$Zn(HS)_2$	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
$Zn(HS)_3^-$	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
pH	*7.100*	*7.1*	*7.1*	*7.099*	*7.099*	*7.098*	*7.097*
Pe	*13.505*	*13.506*	*13.506*	*13.506*	*13.506*	*13.507*	*13.509*

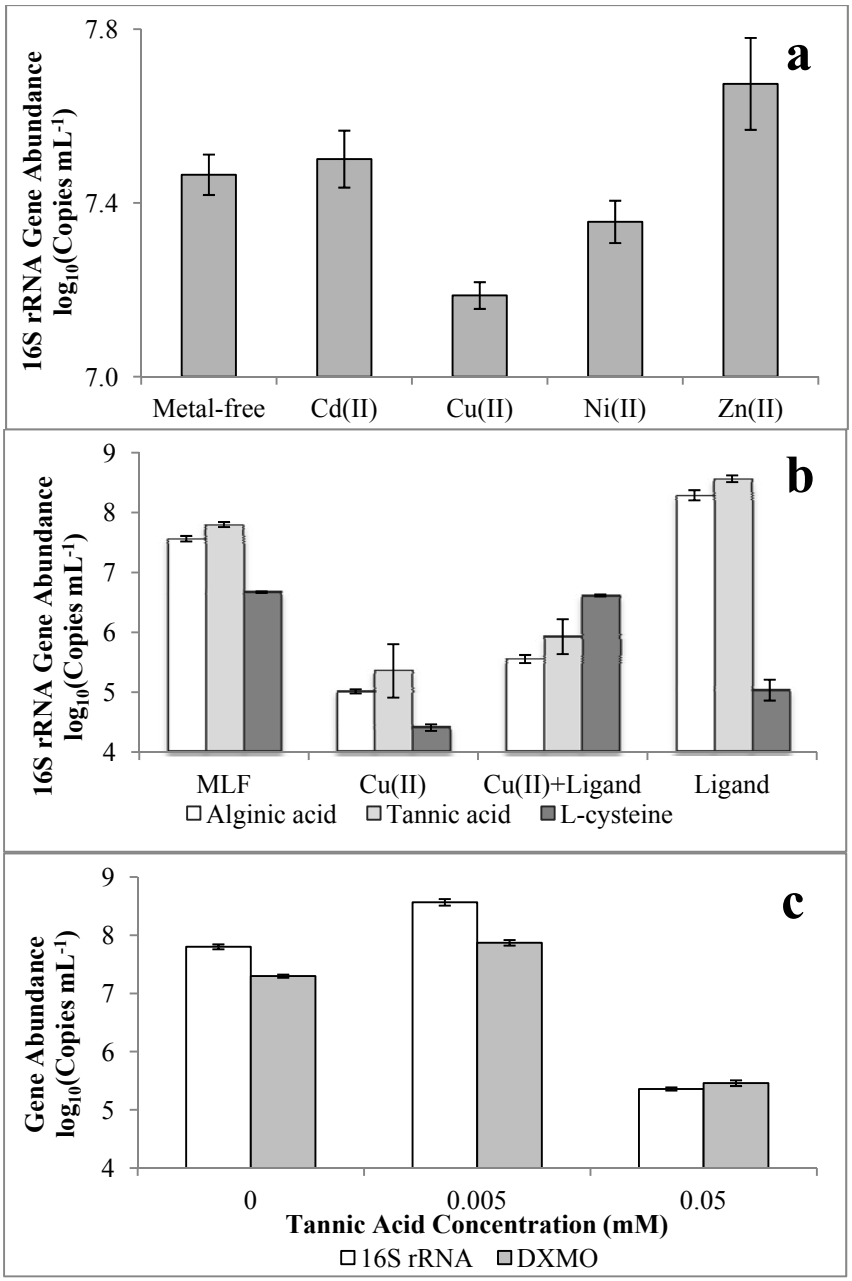

Figure 3.6. Gene abundance in cultures. (a) In the presence of 20 mg L^{-1} transition metals on day 4 of the experiment, 16S rRNA gene copy numbers followed the same order as 1,4-dioxane biodegradation rates, i.e., Cu(II) < Cd(II) < Ni(II) < Zn(II). (b) 16S rRNA copy numbers were lowest for Cu(II)-exposed cells but increased when 0.005 mM tannic acid, 0.5 mM alginic acid, and 0.5 mM L-cysteine were added together with 2 mg L^{-1} Cu(II). The results imply that organic ligands decreased bioavailability and toxicity of Cu(II) resulting in enhanced bacterial growth. MLF is metal/ligand-free controls. (c) 16S rRNA and DXMO gene abundance decreased with the addition of 0.05 mM tannic acid, suggesting that tannic acid was inhibitory to *P. dioxanivorans* CB1190 growth and metabolism. The error bars correspond to the standard deviation of three measurements of duplicate samples (n = 6).

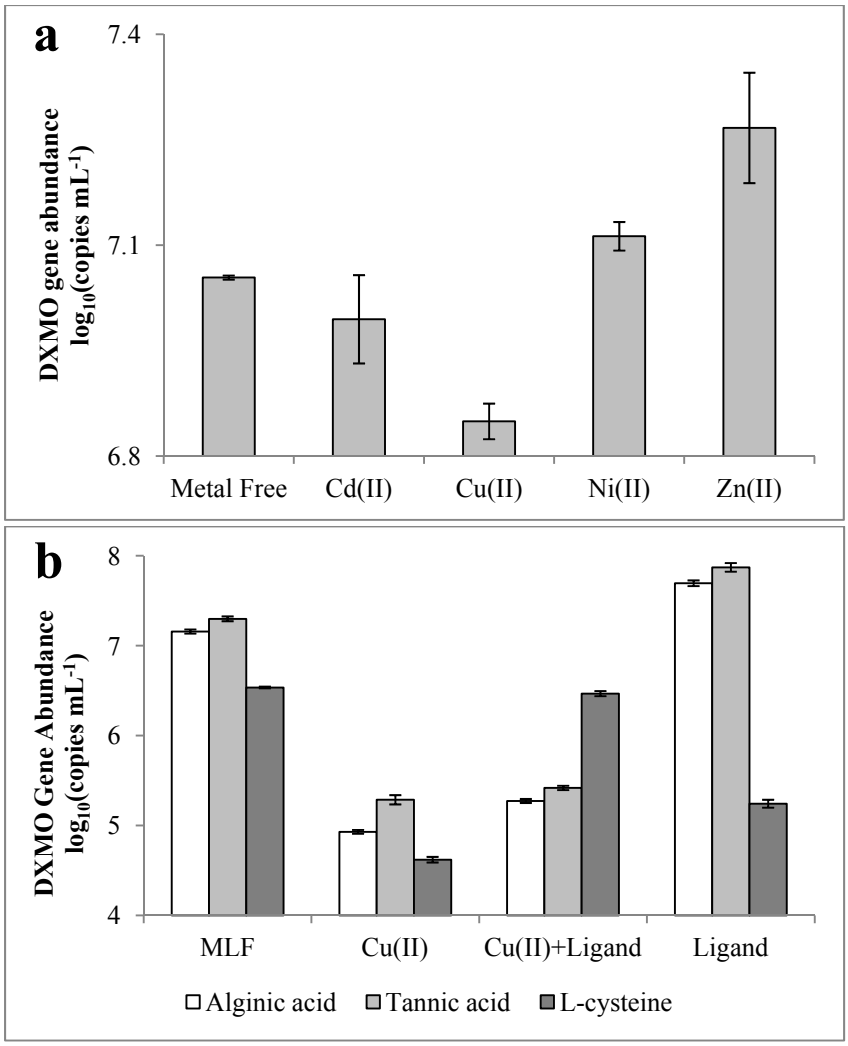

Figure 3.7. Dioxane monooxygenase (DXMO) gene abundance in *Pseudonocardia dioxanivorans* CB1190 cultures. (a) In the presence of 20 mg L^{-1} transition metals after 4 days incubation, DXMO copy numbers followed the same order as 1,4-dioxane biodegradation rates, i.e., Cu(II) < Cd(II) < Ni(II) < Zn(II). (b) Like 16S rRNA, DXMO copy numbers were lowest for Cu(II)-exposed cells but generally increased when 0.005 mM tannic acid, 0.5 mM alginic acid, and 0.5 mM L-cysteine were added together with 2 mg L^{-1} Cu(II). This implies that organic ligands decreased bioavailability and toxicity of Cu(II) resulting in enhanced bacterial growth. MLF stands for metal/ligand-free controls.

3.4. Discussion. Bacterial multicomponent monooxygenase enzymes are greatly influenced by the availability of transition metals, which serve as cofactors (*167, 168*). Dioxane monooxygenase is responsible for catalyzing the initial step of 1,4-dioxane biodegradation (*82*). This study revealed key associations between transition metals and 1,4-dioxane biodegradation catalyzed by dioxane monooxygenase, ranging from no effect to complete inhibition of 1,4-dioxane biodegradation at certain concentrations. On the basis of biodegradation rates, *P. dioxanivorans* CB1190 would be considered most sensitive to Cu(II) among tested transition metals. Addition of 10 or 20 mg L^{-1} Cu(II) resulted in nearly complete stoppage of 1,4-dioxane biodegradation, but addition of mg L^{-1} Cu(II) only increased the time required for 1,4-dioxane concentrations to fall below the detection limit. Addition of 10 or 20 mg L^{-1} Cd(II) and Ni(II) reduced biodegradation rates by 27 - 32% as compared to those of metal-free controls, and delayed complete biodegradation of 1,4-dioxane by two days. Lower concentrations of Cd(II) and Ni(II) (i.e., 1 mg L^{-1}) and all concentrations of Zn(II) tested in this study had no effect on biodegradation rate of 1,4-dioxane (Figure 3.2). Interestingly, the activity of the 1,4-dioxane monooxygenase of *P. dioxanivorans* CB1190 was highly influenced by addition of Cu(II) at concentrations as low as 1 mg L^{-1} and Ni(II) at 10 mg L^{-1}, but was not affected by exposure to Zn(II) up to 20 mg L^{-1} (Figure 3.1) which was in agreement with Riis *et al* (*182*). A statistically significant difference ($p < 0.05$) was found between 16S rRNA copy numbers in metal-free controls and cultures containing 20 mg L^{-1} Cu(II), indicating that Cu(II) suppressed bacterial growth. It should be noted that 16S rRNA gene abundance, which has been used for determining cell concentrations in bacteria, is highly correlated to that of DXMO gene ($r^2 = 0.92$). This suggests that abundance of DXMO gene, which is a functional gene associated with 1,4-dioxane biodegradation (*84*), can also be applied to estimate cell population of the cultures (Figure 3.6

and Figure 3.7). Despite the fact that 20 mg L^{-1} Cd(II) and Ni(II) did not suppress bacterial growth, biodegradation of 1,4-dioxane to below detection limit still experienced a lag. These results suggested that biodegradation capability of *P. dioxanivorans* CB1190 might be affected by more than one mechanism. In contrast, Zn(II) at 20 mg L^{-1} appeared to promote the growth of the bacterium. This result is in agreement with previous studies which demonstrated that Zn(II) is a growth stimulator for various microorganisms (*183-185*). Indeed, it was previously determined that the cell yield of *P. dioxanivorans* CB1190 increased while substrate uptake rates remained similar for cultures grown in AMS and basal salts medium containing 90 mg L^{-1} and 7 mg L^{-1} Zn(II), respectively (*18, 20, 186*). These studies support our findings that addition of 20 mg L^{-1} Zn(II) increased cell abundance without significantly affecting the biodegradation rate.

This study determined that 1,4-dioxane biodegradation rates measured in *P. dioxanivorans* CB1190 cultures exposed to transition metals followed the order: Cu(II) < Cd(II) ≤ Ni(II) < Zn(II), and this trend was similar to those established in previous studies which evaluated the effects of transition metals on aerobic biodegradation of methyl *tertiary*-butyl ether (MTBE) (*187*). It was reported that MTBE biodegradation rates in the presence of metals were in the following sequence: Cu(II) < Cr(III) < Zn(II) < Mn(II) tested at concentrations of 10 or 50 mg L^{-1} (*187*). Furthermore, transition metals also influenced microbial biodegradation of diesel fuel, and the order of toxicity was found to be Zn(II) < Pb(II) < Ni(II) < Cd(II) < Cu(II) < Cr(VI) < Hg(II) (*182*). 16S rRNA genes quantified on day 4 in cultures individually amended with 20 mg L^{-1} of each transition metal relative to metal-free control (Figure 3.6a) were in the following order: Cu(II) (53 %) < Ni(II) (78%) < Cd(II) (109%) < Zn(II) (165%), suggesting that the bacterial cell density was negatively impacted by Cu(II) and Cd(II). These results are consistent

with the 1,4-dioxane biodegradation rates measured using those cultures: Cu(II) (2.78 mg L^{-1} d^{-1}) < Ni(II) (19.26 mg L^{-1} d^{-1}) < Cd(II) (20.56 mg L^{-1} d^{-1}) < Zn(II) (30.17 mg L^{-1} d^{-1}).

Transition metals, including iron, copper, cadmium, mercury, nickel, and vanadium, produce energetic radicals, resulting in oxidative stress, nucleic acid damage, lipid peroxidation, depletion of protein thiols, and other effects (*188*). These reactive radicals include a wide range of oxygen-, carbon-, nitrogen-, sulfur-radicals, originating from the superoxide radical, hydrogen peroxide, and lipid peroxides (*188*). Transition metals can also produce toxic effects on microorganisms through interactions with proteins such as those involved in transport and respiration. Certain transition metals may replace essential metal cofactors of key enzymes altering their functions (*189*). For example, copper ions have a high affinity for methionine, cysteine, and histidine, and thus block the active sites of proteins, containing these three amino acids (*190*). It was demonstrated that Cu(II) and Ni(II) inhibit sMMO activity in purified enzyme assays (*167*). While none of the tested concentrations of Zn(II) decreased 1,4-dioxane biodegradation rates, 16S rRNA gene copy numbers in the presence of 20 mg L^{-1} Ni(II) and Zn(II) were significantly higher than the control ($p < 0.05$). These results diverged from previous studies which demonstrated a negative influence of Zn(II) and Ni(II) on sMMO, one of the enzymes that can cometabolize 1,4-dioxane biodegradation (*167*, *168*). It should be noted that 65.4 mg L^{-1} Zn^{2+} ions were used in experiments described by Grosse and colleagues (*27*), which was higher than the concentrations tested in this study. In addition, the previous studies utilized purified cell-free extracts of the sMMO enzyme (*167*, *168*). Purified extracts may be more sensitive to metal ions because of the absence of potential binding sites located on alternative proteins or other components in whole cells. These findings suggest that the availability of free metal ions is a key factor in determining their influence on enzyme activity.

The dominant species of each transition metal in AMS medium was independently predicted using PHREEQC geochemical software version 3 (*180*) in order to confirm the bioavailability of free ions in solution (Table 3.1-3.4). The model suggested that all added metal salts nearly completely dissolved in AMS and that free metal ions were the most abundant species of each transition metal. In other words, precipitation of these transition metals was unlikely in the medium under the tested conditions. The pH and pe of the abiotic medium, which was equilibrated with oxygen and carbon dioxide in the air, were predicted as 7.10 and 15.05, and adding metal salts would not change these values. These results suggested that interaction of transition metals with AMS components would be negligible and adding transition metal salts in AMS does not alter oxidation-reduction equilibrium of the medium. Therefore, the bioavailability of free metal ions would be primarily influenced by competitive interactions with cell membrane-bound active metal transport proteins and other ligands in the medium.

The mitigation of metal toxicity could be described by metal ion-ligand complex interactions. It is likely that some organic ligands bind with transition metals reducing solution-phase metal concentrations and subsequently alleviating toxicity towards bacteria. This conclusion agrees with previous reports of formation of metal complexes in the presence of alginic acid, cysteine, and tannic acid (*191-195*). In this study, biodegradation rates in the presence of 2 mg L^{-1} Cu(II) with addition of L-cysteine to 0.05 and 0.5 mM, and tannic acid to 0.005 mM were significantly higher than the 2 mg L^{-1} Cu(II) treatment alone. L-cysteine and tannic acid at those concentrations exhibited an antagonistic interaction with Cu(II), which improved biodegradation of 1,4-dioxane with respect to 2 mg L^{-1} Cu(II) (Figure 3.3). L-cysteine, which contains sulfhydryl functional group in the molecule, was reported to bind with toxic metals, reducing their bioavailabilty. For example, it can form complexes with Cu(II) which precipitate cysteine

and cuprous cysteine (*193*). It should be noted that little precipitation was observed in the cultures into which 2 mg L^{-1} or higher amounts of Cu(II) and L-cysteine were supplemented. Belcastro and coworkers (*194*) theoretically described cysteine-metal ion complexes, and proposed the order of metal affinity to cysteine as Cu(II) > Zn(II) > Hg(II) > Cd(II) (*194*). L-cysteine, which is an antioxidant, can also remove reactive species that may be produced by transition metals. Tannic acid is a phenolic compound also capable of forming complexes with transition metals. A previous study characterized dissolved tannic acid and its metal-ion complexes using electrospray ionization mass spectrometry (*195*). It was found that tannic acid can form complexes with many transition metals including Cu(II), Mn(II), Ni(II), Zn(II), Cd(II), Pb(II), Co(II), Cr(III), Al(III), and Fe(III) (*195*). Another study reported that a tannin-based flocculant at concentrations between 100 – 150 mg L^{-1} was successful in removing Cu(II), Ni(II) and Zn(II) from polluted water (*196*). Furthermore, the abundance of DXMO gene targets in cultures with addition of 0.005 mM tannic acid, 0.5 mM alginic acid, or 0.5 mM L-cysteine in the presence of 2 mg L^{-1} Cu(II) were greater than those of 2 mg L^{-1} Cu(II) treatment in the absence of organic ligands (Figure 3.6b). The results suggested that bacterial growth was better when tannic acid, alginic acid, or L-cysteine was added in the presence of Cu(II) at specific concentrations, thus producing higher rates of 1,4-dioxane biodegradation. In general, a positive trend was observed with increasing alginic acid and L-cysteine concentrations, which was supported by increase in the number of 16S rRNA gene copies in those samples (Figure 3.6b).

Alginic acid is a biopolymer extracted from brown seaweed, which carries carboxyl groups capable of forming complexes with metal ions. Alginic acid was previously found to remove cobalt, copper, cadmium, lead, and zinc (*191, 192, 197*). It was also reported that copper-resistant strains of *Pseudomonas aeruginosa* produced more extracellular polymeric substance

containing alginic acid as a major component than copper-sensitive strains to alleviate Cu(II) toxicity (*198*). However, *P. dioxanivorans* CB1190 exposed to 2 mg L^{-1} Cu(II) along with alginic acid exhibited biodegradation rates not appreciably different from 2 mg L^{-1} Cu(II) treatment. Similar results were also found when adding BSA or SRNOM at all concentrations, 0.05 or 0.5 mM tannic acid, or 0.005 mM L-cysteine along with 2 mg L^{-1} Cu(II) into *P. dioxanivorans* CB1190 cultures. Large proteins, such as BSA did not alleviate toxicity of Cu(II) in the present study even though BSA was found to mitigate bacterial toxicity due to the weathered quantum dot nanoparticles containing transition metals in their core/shell structures (*199*). SRNOM, another large organic molecule, did not mitigate Cu(II) toxicity.

In attempts to correct rates of 1,4-dioxane biodegradation by *P. dioxanivorans* CB1190 concurrently exposed to ligands mitigating metal toxicity, the effects of individual organic ligands on biodegradation of 1,4-dioxane were also investigated. Unexpectedly, the results revealed that addition of tannic acid (0.05 or 0.5 mM) and L-cysteine (0.5 mM) could independently affect biodegradation of 1,4-dioxane (Figure 3.5). As compared with ligand-free controls, addition of tannic acid at 0.05 mM and 0.5 mM decreased 1,4-dioxane biodegradation rate by 69 and 89 %, respectively, while 0.5 mM L-cysteine decreased the rate by 83 % (Figure 3.5). These results are in agreement with 16S rRNA gene abundance data, which demonstrated that the bacterial cell numbers in the presence of 0.5 mM L-cysteine and 0.05 mM tannic acid were lower than those in ligand-free controls (Figure 3.6b and 3.6c, respectively). Consequently, biodegradation rates were lower because bacterial growth was slowed down. As evidenced in previous studies, tannic compounds affect the physiology and growth of microorganisms (including methanogens, proteolytic bacteria, and various pathogens), and the inhibition likely results from hydrogen bonding of the acid with vital proteins (*200-203*). Thus, inhibition of 1,4-

dioxane biodegradation in the presence of tannic acid was possibly due to the suppression of the monooxygenase activity or change in its affinity for 1,4-dioxane. Inhibitory effects of L-cysteine on *P. dioxanivorans* CB1190 might result from the suppression of threonine deaminase, which is the enzyme responsible for the synthesis of various amino acids including leucine, isoleucine, threonine and valine (*204*). It is also possible that at high concentrations, the amino acid can chelate metal ion cofactors essential for microbial growth since the thiol group in L-cysteine can strongly bind metal ions (*205*) limiting bioavailability of even the essential metals. The deficiency of essential metals can subsequently trigger lipid peroxidation and produce thiyl, which affects overall number and function of microorganisms (*206*). Moreover, high level of intracellular L-cysteine was reported to promote oxidative DNA damage via the Fenton reaction in *Escherichia coli* (*207*). DXMO copies in cultures grown in the presence of 0.005 mM tannic acid and 0.5 mM alginic acid were higher than ligand-free control at day 8, suggesting that growth of *P. dioxanivorans* CB1190 can be promoted by certain organic ligands, which may enhance biodegradation of 1,4-dioxane.

Bacterial and fungal degradation of 1,4-dioxane under laboratory-controlled conditions has been well established, but biodegradation of 1,4-dioxane is rarely observed in the field. In addition to limitations caused by inadequate microbial communities, dissolved oxygen, and aquifer biogeochemistry, the inhibitory effects of co-contaminants might be responsible for hampering 1,4-dioxane biodegradation. A recent study demonstrated that chlorinated solvent co-contaminants, including 1,1,1-trichloroethane and 1,1-dichloroethene, inhibited 1,4-dioxane biodegradation by various monooxygenase-expressing bacteria (*88*). However, the effects of transition metals, which are prevalent at one-third of solvent-impacted sites (*208*) are not yet characterized. To our knowledge, this study is the first to investigate the effects of transition

metals and organic ligands on biodegradation of 1,4-dioxane. Our results endorse that strategies for intrinsic or engineered bioremediation of 1,4-dioxane in the environment should take into consideration the effects of transition metals, organic ligands, and their interactions on 1,4-dioxane-degrading microorganisms. An understanding of the potentially inhibitory effects of transition metals and organic ligands on bacterial communities and enzyme activities will be valuable to environmental microbiology and biochemistry researchers, and also benefit practitioners interested in implementing bioremediation systems involving monooxygenase-catalyzed transformation of xenobiotic compounds in contaminated environments.

CHAPTER 4

MOLECULAR BIOLOGICAL TOOLS TO EVALUATE SITE-SPECIFIC 1,4-DIOXANE BIODEGRADATION

4.1. Introduction. Biomarkers serve a wide range of applications as indicators of pharmacological responses to therapeutic intervention, pathogenic processes, and even biological processes involving biodegradation of organic contaminants. Previous studies demonstrated that biomarkers targeting conserved region of 16S-rRNA genes were useful to identify the presence or absence of specific microorganisms capable of biodegrading compounds (*209-213*). However, this approach in complex microbial communities can be difficult for closely related microorganisms.

Alternatively, biomarkers targeting functional genes can be used to target biodegradation. Bacterial multicomponent monooxygenases (BMMs) were identified as the group of enzymes catalyzing biotransformation of various environmental contaminants (*214, 215*). Due to their capability of catalyzing a broad range of organic pollutants including aliphatic and aromatic hydrocarbons, tetrahydrofuran (THF), and other contaminants of concern, many studies have focused on characterizing and identifying nucleic acid biomarkers coding for this functional group of enzymes (*209-213*). For example, *pmoA* gene coding the alpha subunit of particulate methane monooxygenase (pMMO) was found to be associated with a mixed community of methanothrophs in soil capable of cometabolizing trichloroethene (TCE) (*216*). Moreover, quantification of the cytochrome P450 monooxygenase-encoding gene (*ethB*) was directly related to biodegradation of methyl *tert*-butyl ether (MTBE) in environmental samples (*217*). Biotransformation of organic pollutants was also related to alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). For example, aldehyde and semialdehyde hydrogenase in *Pseudonocardia tetrahydrofuranoxydans* K1 were identified as integral components for THF biological degradation (*218*). These studies suggested that functional genetic biomarkers can be

used to target biodegradation with broad substrate specificities, and may be useful for indicating potential biodegradation in the environment.

In recent years, there have been many attempts to consider bioremediation as a treatment strategy for emerging water contaminants of concern, such as 1,4-dioxane. Biodegradation of 1,4-dioxane has been well established in laboratory studies. It was confirmed that at least six genera of monooxygenase-expressing bacteria could biotransform 1,4-dioxane via growth supporting or co-metabolic pathways. It was proven that tetrahydrofuran (THFMO), propane (PrMO), phenol (PHE), methane (MMO) and toluene (TOL, T4MO, or RMO) are capable of 1,4-dioxane biodegradation (*18, 83, 219-221*). It was also demonstrated that THFMO in *Pseudonocardia* sp. strain ENV478 was associated with THF and 1,4-dioxane breakdown (*83*).

Recently, the genome of *Pseudonocardia dioxanivorans* CB1190, a bacterium able to use 1,4-dioxane for growth, was sequenced and annotated (*80*). The analysis of *P. dioxanivorans* CB1190's genome revealed eight putative BMM clusters encoding at least 17 monooxygenases, including the entire THF degradation gene cluster similar to *Pseudonocardia* sp. strain ENV478 (*80*). In fact, it was reported that expression of THFMO gene cluster was upregulated during biodegradation of 1,4-dioxane in *P. dioxanivorans* CB1190 (*81*). Thus, the gene cluster within the microorganism is also referred to as dioxane monooxygenase (DXMO).

Monitored natural attenuation and engineered bioremediation may be promising as alternative remedial strategies for 1,4-dioxane impacted sites. However, direct evidence to confirm 1,4-dioxane biodegradation at the site is still lacking. Pornwongthong et al. (*222*) demonstrated that DXMO could be used as an indicator of 1,4-dioxane biodegradation by *P. dioxanivorans* CB1190. This suggested that functional genetic biomarkers might be valuable for monitoring

1,4-dioxane biodegradation in the environment. To confirm this hypothesis, this study was conducted to validate the usefulness of genetic biomarkers in environment samples. The specific objective of this study were to (i) determine the feasibility of intrinsic biodegradation of 1,4-dioxane in contaminated industrial activated sludge from a polyester-manufacturing facility and *in situ* bioremediation of 1,4-dioxane in polluted groundwater from Air Force Plant 44 (AFP44), (ii) demonstrate application of quantitative polymerase chain reaction (qPCR) assays to determine abundance of putative monooxygenase genes in environmental samples and (iii) relate biomarker gene abundance with biodegradation of 1,4-dioxane in microcosms.

4.2. Materials and Methods

4.2.1. Chemicals. 1,4-Dioxane from Sigma-Aldrich, Saint Louis, MO, ACS grade hydrochloric acid, methylene chloride, sodium hydroxide, and sodium sulfate, all from Fisher Scientific, Fair Lawn, NJ, and 99.97% methane from Matheson Gas Inc., Joliet, IL were used in this study.

4.2.2. Bacterial Strains and Culture Conditions. *Pseudonocardia dioxanivorans* CB1190 was grown in nitrate mineral salt (NMS) (*223*) with 100 mg L^{-1}1,4-dioxane, while *Methylosinus trichosporium* OB3b was grown in 250 mL Boston round glass bottles (Wheaton, Millville, NJ) capped with screw-cap Mininert® valves (Alltech Associates, Inc., Deerfield, IL) containing NMS with 50% v/v methane in the head space as a growth supporting substrate (*23*). Bacterial stocks were maintained for at least five transfers to assure robust growth and enzyme activity. All cultures were incubated at 30˚C while shaking on an incubator shaker at 150 rpm under aerobic condition, and aseptic techniques were used to prevent contamination throughout the study. The culture medium was always less than 20% of the container to prevent mass transfer

limitations of oxygen or methane. Culture purity was periodically examined by plating cultures onto NMS agar plate and sequencing of 16S rRNA genes.

4.2.3. Environmental Samples.

4.2.3.1. Industrial Activated Sludge Samples. Activated sludge samples were collected from an industrial wastewater treatment plant of a polyester manufacturing facility. Samples were collected in 3.8 L semirigid polyethylene containers with screw caps (Hedwin Cubitainer®; Thermo Fisher Scientific Inc., Wilmington, DE). Activated sludge samples were placed on ice and delivered on the same day to the laboratory.

4.2.3.2. Field Samples from Air Force Plant 44. Air Force Plant 44 (AFP44) is southwest of Tucson, Arizona as a part of the Tucson International Airport Area (TIAA). The groundwater at the site is contaminated with 1,4-dioxane and TCE, both of which are contaminants of concern (*224*). Three groundwater samples from monitoring wells EPA-03, M-69, and M-101, soil sample B-125, and Biotrap® samplers (Microbial Insights Inc., Knoxville, TN) equilibrated with groundwater in each monitoring well were collected in June 2011 at AFP44 site in Tucson, AZ (Figure 4.1). Samples were then placed on ice and shipped on the same day to the laboratory for microcosm studies.

4.2.4. Biodegradation of 1,4-Dioxane by Microbial Consortia from Environmental Samples

4.2.4.1. Activated Sludge Experiment. Fifty milliliters of original activated sludge samples were incubated in triplicate sterile 250 mL boston round glass bottles capped with screw-cap Mininert® valves (Alltech Associates, Inc., Deerfield, IL) at 30˚C while shaking on an incubator shaker at 150 rpm. Samples were collected over time for analysis of 1,4-dioxane. Analysis of gene abundance was performed right after 1,4-dioxane was completely removed in the microcosms.

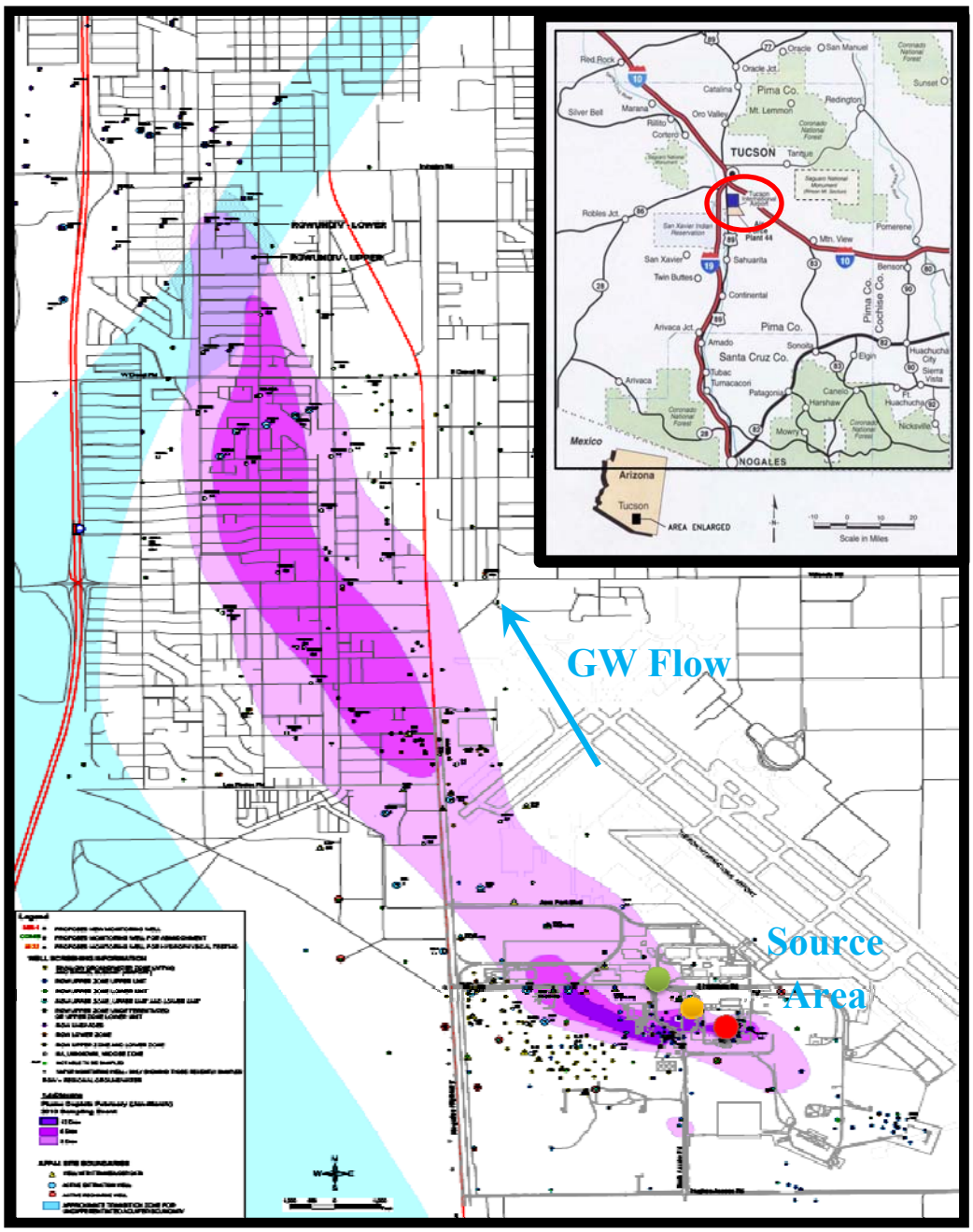

Figure 4.1. Map of the Air Force Plant 44 Super Fund Site in Tucson, AZ. Groundwater of the site has been contaminated with 1,4-dioxane and chlorinated solvents. The plume size of 1,4-dioxane is 6 miles long from southeast to northeast. Red, yellow and green dot represent M-69, M101 and EPA-03 monitoring wells, respectively.

4.2.4.2. AFP44 Microcosm Experiment. Microcosms were prepared using groundwater collected from monitoring wells EPA-03, M-69, and M-101, and soil sample B-125. Various treatments were chosen to investigate the feasibility for 1,4-dioxane degradation through different bioremediation strategies including natural attenuation, biostimulation, and bioaugmentation (Table 4.1). A total of 63 microcosms (7 different treatments with triplicate for three sources of groundwater) were established and incubated in autoclaved 250 mL boston round glass bottles capped with screw-cap Mininert® valves (Alltech Associates, Inc., Deerfield, IL) at 30˚C while shaking on an incubator shaker at 150 rpm (Figure 4.2). Microcosms including group C and E in Table 5.1 were amended with methane for the first 60 days of the experiment. Samples were collected over time for analysis of 1,4-dioxane (all bottles) and methane (only microcosms group C and E in Table 4.1). Functional gene abundance was analyzed at the beginning and end of the experiment.

Figure 4.2. Microcosms constructed from AFP44 groundwater and soil on incubator shaker

Table 4.1. Initial ingredients for various microcosms*

Treatment	Initial Ingredient
AC. Autoclaved Control	10 g B-125 soil + 50 mL groundwater (Sterilized at 121˚C for 15 minutes)
BC. Bead Control	10 g B-125 soil + 50 mL groundwater + 1 mL extract from sterile BioTrap® beads
N. Natural Attenuation	10 g B-125 soil + 50 mL groundwater (no amendment) + 1 mL biomass extract from BioTrap® beads
B. Biostimulated with NMS + O_2: BS (Oxygen)	10 g B-125 soil + 25 mL groundwater + 25 mL NMS + 1 mL biomass extract from BioTrap® beads + flushing with O_2 10 min at initial point of experiment
C. Biostimulated with NMS + CH_4: BS (Methane)	10 g B-125 soil + 25 mL groundwater + 25 mL NMS + 1 mL biomass extract from BioTrap® beads + 50% v/v methane in gaseous phase
D. Bioaugmented with *P. dioxanivorans* CB1190 + NMS: BA (CB1190)	10 g B-125 soil + 20 mL groundwater + 25 mL NMS + 1 mL biomass extract from BioTrap® beads + 5 mL *P. dioxanivorans* CB1190 in NMS
E. Bioaugmented with *M. trichosporium* OB3B + CH_4 + NMS: BA (OB3b)	10 g B-125 soil + 20 mL groundwater + 20 mL NMS + 1 mL biomass extract from BioTrap® beads + 5 mL *M. trichosporium* OB3b in NMS + 50% v/v methane in gaseous phase

* Since the accessibility of soil in each monitoring well is limited, B-125 soil which is located nearby all monitoring wells and accessible was chosen to be added into the microcosms. Biomass in each monitoring well was added in form of BioTrap® beads extract.

4.2.5. Biotrap® Bead Preparation (for AFP44 Microcosm Study Only). For autoclaved controls, 120 beads from BioTrap® Samplers extracted from locations EPA-03, M-69, and M-101 were collected in sterile 50 mL Falcon Conical Centrifuge tubes (Becton Dickinson, Franklin Lakes, NJ) and autoclaved sterilized at 121°C for 30 minutes. For sterile bead control, 360 sterile beads were collected in three sterile 50 mL Falcon Conical Centrifuge tubes for a total of 120 sterile beads per tube. For active biomass, 600 beads from BioTrap® samples extracted

from locations EPA-03, M-69, and M-101 were collected in five sterile 50 mL Falcon conical centrifuge tubes for a total of 120 beads per tube. Autoclaved beads, sterile beads, and beads with active biomass were suspended with 5 mL TE buffer and stored at 4°C until ready for processing.

4.2.6. Biomass Extraction from Biotrap® Samplers (for AFP44 Microcosm Study Only). Biomass was extracted from the BioTrap® beads using a Q125 Ultrasonic Liquid Processor (Qsonica, Newtown, CT) equipped with a 1/8" probe. Samples were sonicated at 50% amplitude for 90 seconds (10 s intervals with 2 s rest). Sonicated solutions were collected in new sterile 15 mL Falcon conical centrifuge tubes. Beads were washed 2 times in 5 mL TE buffer with each rinse transferred to the 15 mL Falcon tube. Samples were centrifuged at 18,000 x *g* for 3 minutes and the supernatant was discarded. Biomass pellets were resuspended in 3 mL groundwater sample from the appropriate location (EPA-03, M-69, or M-101) and treatment (Autoclaved, sterile, or non-amended). The biomass extract were used in further microcosm experiments according to Table 4.1.

4.2.7. Geochemistry (for AFP44 Microcosm Study Only). Geochemical characteristics of microcosms, including temperature, pH, oxidation-reduction potential (ORP), and dissolved oxygen (DO) were determined at day 0 and day 158 of the experiment. All parameters were measured by a benchtop multiparameter meter (Orion Star Series, Thermo Fisher Scientific, Waltham, MA).

4.2.8. Gas Chromatography.

4.2.8.1. Analysis of Methane by GC-FID. Concentrations of methane were analyzed by manually injecting 100 µL head space samples into a Hewlett-Packard 6890 Chromatograph

(GC) equipped with a Flame Ionization Detector (FID) (Hewlett-Packard, Atlanta, GA) and a Restek® Stabilwax-DB capillary column (30 m length x 0.53 mm id x 1 μm film thickness; Restek, Bellefonte, PA). The injector was set at 220 °C in splitless mode, while the detector was set at 250 °C. The oven program was isothermally set at 40 °C (2 min). The retention time for methane was 1.42 minutes. Concentrations of methane in microcosms were reported as mg L^{-1} methane in gaseous phase.

4.2.8.2. Analysis of TCE by GC-FID. Analyses of TCE were perform by manually injecting 100 μL gaseous headspace samples into Hewlett-Packard 6890 Chromatograph (GC) equipped with a Flame Ionization Detector (FID) and a Restek Stabilwax-DB capillary column (30 m x 0.53 mm id x 1 μm). The inlet and detector were maintained at 220˚C and 250 ˚C, respectively. The oven was programmed as 45 °C (1 min) at 100 °C min^{-1} to 200 °C (3 min). The retention time for TCE was 3.07 minutes. Concentrations of TCE in gas and liquid phase were calculated according to:

$$M = CwVw + CgVg = Cw(Vw + HcVg), \qquad \text{(Eq. 4.1)}$$

where M is the total TCE mass (unit of mole), Cw is the concentration of TCE in the aqueous phase (μM), Cg is the concentration of TCE in gaseous phase (μM), Vw is the volume of liquid phase in media bottles (in liters), Vg is the volume of gaseous head space (in liters) and Hc is dimensionless Henry's constant (*225, 226*). Limit of detection was determined as 5 μg L^{-1} with this method.

4.2.8.3. Analysis of 1,4-Dioxane. Hewlett-Packard 6890 Chromatograph (GC) equipped with a Flame Ionization Detector (FID) and a Restek Stabilwax-DB capillary column (30 m x 0.53 mm id x 1 μm) was used for 1,4-dioxane quantification at higher concentrations (mg L^{-1} range).

Samples were filtered through 0.2-μm-pore nylon syringe filters. Two microliters of filtered samples was manually injected into the GC-FID. The injector and detector were maintained at 220˚C and 250 ˚C, respectively. The oven program was set as 80 °C (3 min) then ramped at 20 °C min^{-1} to 140 °C (1 min). The retention time for 1,4-dioxane was 3.7 minutes. The detection limit was determined at 0.8 mg L^{-1} and the accuracy of 1,4-dioxane concentration measurement was better than ± 4% with this method.

For quantification of lower 1,4-dioxane concentrations (μg L^{-1} range), aliquots of samples (200 μL) were collected and transferred to screw top vials. Biodegradation of 1,4-dioxane in samples was terminated by acidifying with 12.1 N hydrochloric acid to pH less than 1 prior to being stored at -20˚C for later extraction. Preserved samples were extracted using a frozen-microextraction method described in Li et al. (*19, 227*). Briefly, methylene chloride (200 μL) as extracted solvent was added into samples with the spike of 200 μg L^{-1} 1,4-dioxane-d8 internal standard. After mixing vigorously, the vials were placed in a vial tray under the temperature of -80 ˚C at a 45˚ angle for 45 minutes. Methylene chloride extracts were removed from the vial and transferred to a clean instrumental vial with an insert. Sodium sulfate (0.05 g) was added into the vial to remove water residue in the extract. Extracted samples (5 μL) were injected into a Hewlett-Packard 6890 Plus Gas Chromatograph (GC) coupled with a mass spectrometry detector (MSD) (Hewlett-Packard, Atlanta, GA) and Supelco SPB-1 column (30 m x 0.53 mm id x 1 μm; Supelco, Bellefonte, PA) by a 7693A ALS autosampler (Agilent Technologies, Westlake Village, CA) under pulse splitless mode. The oven program was set as 40 °C (1 min) at 10 °C/min to 120 °C (1 min). Other GC-MSD parameters and method details were derived from Isaacson et al. (*228*). The monitored ions for quantification (from preliminary run) are listed in Table 4.2.

Table 4.2. Retention times and selection ions for GC-MSD determination

Compound	Molecular ions (m/z)	Retention time (min)
1,4-dioxane	43, 58, 88	6.75
1,4-dioxane-d8	46, 64, 96	6.73

4.2.9. Biomass Quantification

4.2.9.1. Biomass in Groundwater Samples. Biomass in groundwater samples was quantified as total protein concentration by using a modified Bradford method previously described (*19, 229*). Measurement of 595-nm absorbance was immediately conducted in cuvettes using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

4.2.9.2. Biomass in Soil Sample. Biomass was extracted from 1 g B-125 soil by soaking samples with 1 mL of nuclease free water. Soaked samples were then vigorously mixed by a vortex for 1 minute and supernatants were immediately collected. The collected samples were extracted and analyzed for total soluble protein by using the same method used for quantification of biomass in groundwater samples.

4.2.10. Quantitative Polymerase Chain Reaction (qPCR). Abundance of a dioxane monooxygenase (DXMO), propane monooxygenase (PrMO), and phenol-2-monooxygensae (PHE) in microcosms was determined using qPCR. Primers were designed targeting DXMO genes (forward primer 5'-CCAAACGGGCGTCAGTCAT-3' and reverse primer 5'-AGAACGTGCGCTCCCAAAG-3'), PrMO genes (forward primer 5'-GAAGAGTCGTGGAAGCAGATC-3' and reverse primer 5'-GTACTTGTACTCGAACCACTCG-3'), and PHE genes (forward primer 5'-TGGTCCGGCGAGCCCTTGTA-3' and reverse primer 5'-CACACCTGCTCCGACGGCTG-3'), and were used to amplify the genes using qPCR (*84*). Briefly, each 20- μL reaction mixture

containing 1X Luminaris Color HiGreen High ROX qPCR Master Mix (Thermo Scientific, Waltham, MA), 0.3 μM of each primer (IDTDNA, Coralville, IA), and 2 μL of template DNA. The standards for qPCR analysis were obtained from genomic DNA extracted from pure cultures of *P. dioxanivorans* CB1190 with known quantity. The cycling parameters for qPCR for all gene targets were 50˚C for 2 min and 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 s and annealing at 60˚C for 45 s. The gene abundances were reported as copies of gene per milliliter of samples. The minimum detection limit for the primer sets used in this study was determined as 1.3×10^3 copies mL^{-1}.

4.3. Results

4.3.1. Characteristics of the Environmental Samples. Average 1,4-dioxane concentrations in activated sludge samples were measured at 81.97 ± 1.56 mg L^{-1}. 1,4-Dioxane concentrations in groundwater collected from EPA-03, M-69, and M-101 were determined as 3.83 ± 0.98, 730.73 ± 8.01 and 20.83 ± 4.43 μg L^{-1}, respectively (Table 4.3). In contrast, the analysis of TCE in groundwater samples yielded concentrations below the detection limit (Table 4.3). Protein concentrations of 21.36 ± 3.77, 12.22 ± 4.76, and 11.20 ± 1.18 mg L^{-1} were established for EPA-03, M-69, and M-101 groundwater samples, respectively, while biomass in sandy soil samples from B-125 were determined as 4.72 ± 0.76 μg total soluble protein g^{-1} soil.

Table 4.3. Background Concentrations of Toxic Chemicals in Groundwater Samples

Groundwater Sample	Dioxane (μg L^{-1})		TCE (μg L^{-1})	
	Historical Value	June 2011	Historical Value	June 2011
EPA-03*	9.4	3.83 ± 0.98	55	LOD
M-69*	340	730.73 ± 8.01	390	LOD
M-101[&]	10	20.83 ± 4.43	240	LOD

* Historical values for both compounds were determined in groundwater samples collected in February 2009.
[&] Historical values for both compounds were determined in groundwater samples collected in June 2009.

4.3.2. Activated Sludge Experiment. Biodegradation of 1,4-dioxane in microcosms prepared from the activated sludge samples is illustrated in Figure 4.3. Microorganisms in the sludge were able to remove 1,4-dioxane to below detection limit in 10 days after an initial lag period of approximately 5 days. Note that no removal of 1,4-dioxane was observed in heat-sterilized activated sludge samples.

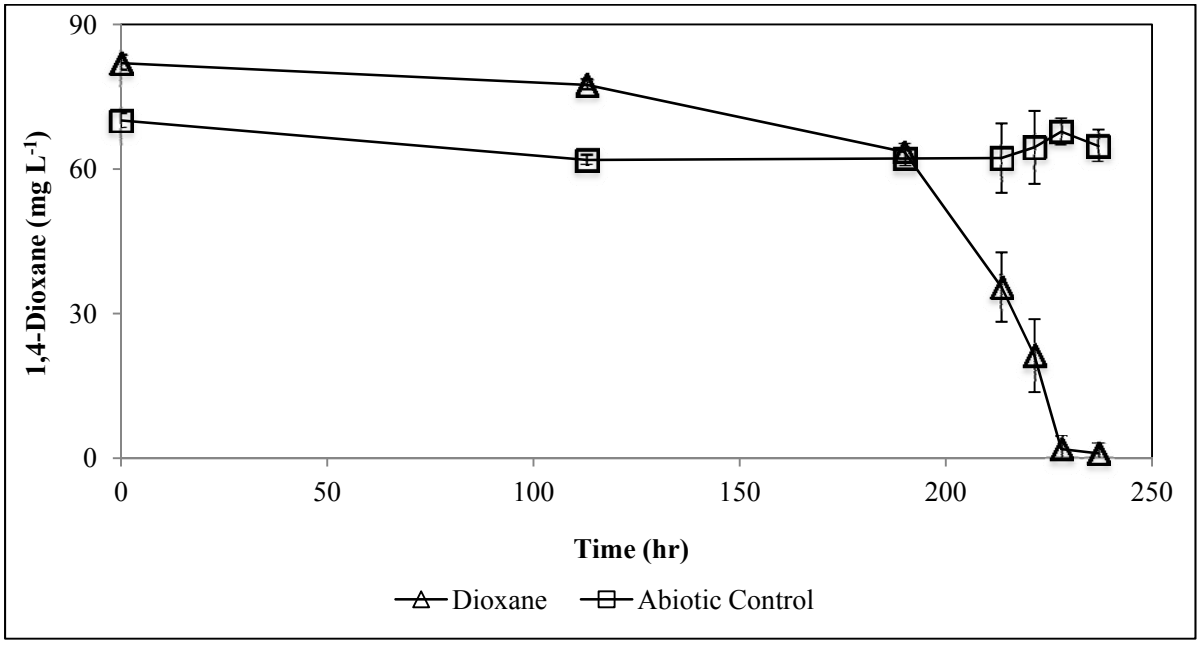

Figure 4.3. 1,4-Dioxane degradation in microcosms prepared from industrial activated sludge. Indigenous bacteria in the microcosms could remove 1,4-dioxane, while heat-sterilized microcosms could not. Error bars represent standard deviation of triplicate samples.

4.3.3. AFP44 Microcosm Experiment.

4.3.3.1. Biodegradation of Methane. Biodegradation of methane was observed in microcosms with stimulation of methane and those with bioaugmentation of *M. trichosporium* OB3b (group C and E in Table 4.1). Methane in methane-amended microcosms was clearly removed within 60 days of observation (Figure 4.4).

4.3.3.2. Biodegradation of 1,4-Dioxane. 1,4-Dioxane biodegradation in microcosms including killed control, bead control, natural attenuation, biostimulation with oxygen, biostimulation with methane and oxygen, and bioaugmention with *M. trichosporium* OB3b prepared from groundwater high (M-69) and low (EPA-03, M-101) 1,4-dioxane concentration are illustrated in Figure 4.5 and 4.6, respectively. 1,4-Dioxane biodegradation in microcosms bioaugmented with *P. dioxanivorans* CB1190 are shown in Figure 4.7. The results revealed that 1,4-dioxane in groundwater samples could be removed by microcosms simulating biostimulation or bioaugmentation (Figure 4.5-4.7). The biodegradation rates determined for each microcosm are presented in Figure 4.9. While 1,4-dioxane biodegradation rates were highest in microcosms bioaugmented with *P. dioxanivorans* CB1190 (466.40 – 512.81 $\mu g\ L^{-1}\ d^{-1}$), the biodegradation rates were much lower in oxygen-biostimulated microcosms (0.07 – 8.08 $\mu g\ L^{-1}\ d^{-1}$), methane-biostimulated (0.08 – 7.68 $\mu g\ L^{-1}\ d^{-1}$), and *M. trichosporium* OB3b-bioaugmented microcosms (0.08 – 9.34 $\mu g\ L^{-1}\ d^{-1}$). Interestingly, 1,4-dioxane was not degraded in the methane-amended microcosms (both biostimulation with methane and bioaugmentation with *M. trichosporium* OB3b) until the methane feed was stopped, after which dioxane was degraded by the next sampling event for all three wells with the most significant degradation in well M-69. Note that no removal of 1,4-dioxane was observed in heat-sterilized controls, bead controls and natural attenuation microcosms.

Methane-biostimulated and *M. trichosporium* OB3b-bioaugmented microcosms from monitoring well M-69 were further analyzed to improve the resolution of 1,4-dioxane degradation. After day 150, these microcosms were re-spiked with methane and 1,4-dioxane, and monitored for an additional 60 days (Figure 4.8). In contrast to our previous results, significant 1,4-dioxane degradation was not observed in these samples even after methane was depleted. In fact, 1,4-dioxane concentrations remained relatively constant for the duration of the extended study period.

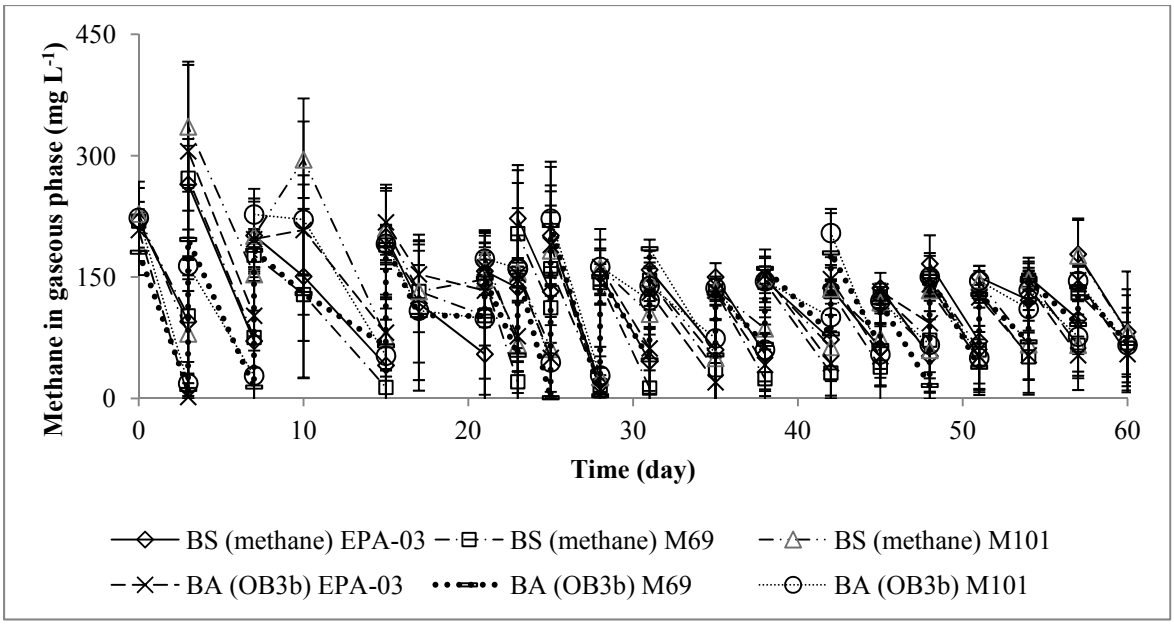

Figure 4.4. Methane degradation in methane-amended microcosms. The result revealed the capability of the microcosms to remove methane. Error bars represent standard deviation of triplicate samples.

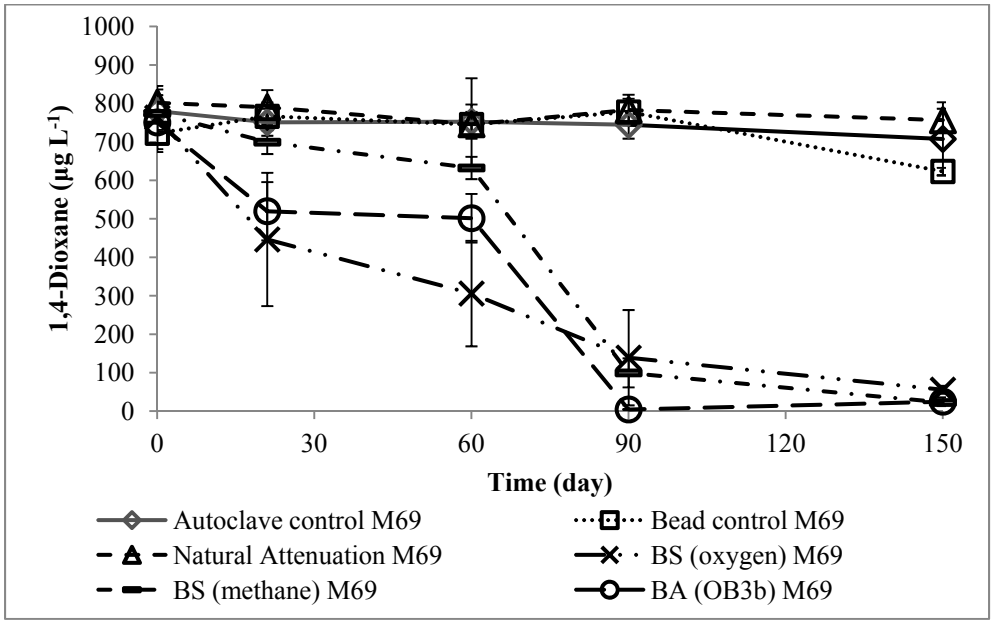

Figure 4.5. 1,4-Dioxane degradation in microcosms prepared from AFP44 groundwater with high 1,4-dioxane concentrations (well M-69). Microcosms biostimulated with oxygen and methane and those bioaugmented with *M. trichosporium* OB3b could remove 1,4-dioxane in groundwater, while natural attenuation could not. Error bars represent standard deviation of triplicate samples.

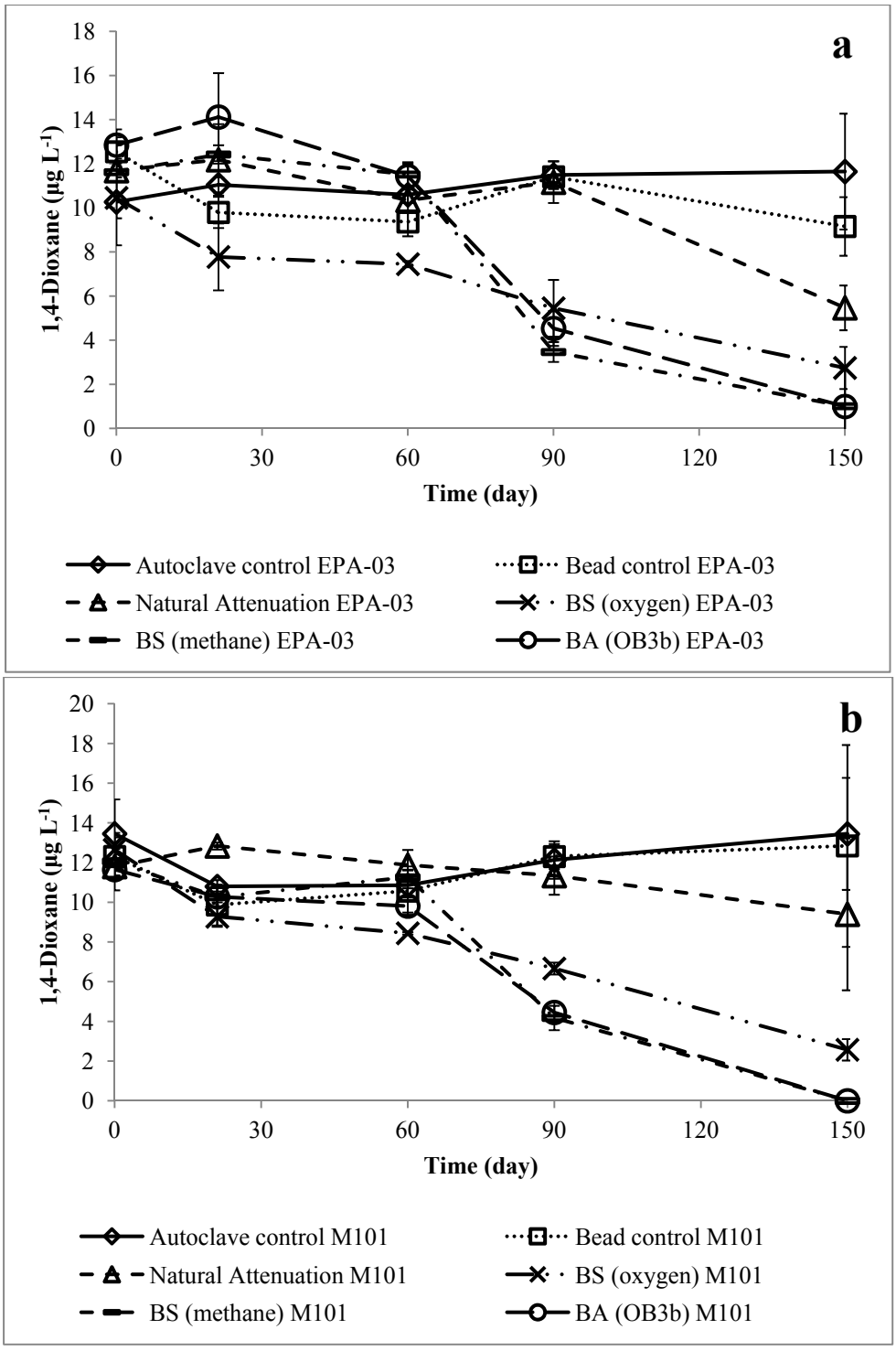

Figure 4.6. 1,4-Dioxane degradation in microcosms prepared from groundwater with low 1,4-dioxane concentrations: (a) microcosms prepared from EPA-03 groundwater; (b) microcosms prepared from M-101 groundwater. Natural attenuation, microcosms biostimulated with either oxygen or methane, and microcosms bioaugmented with *M. trichosporium* OB3b could remove 1,4-dioxane in groundwater. Error bars represent standard deviation of triplicate samples.

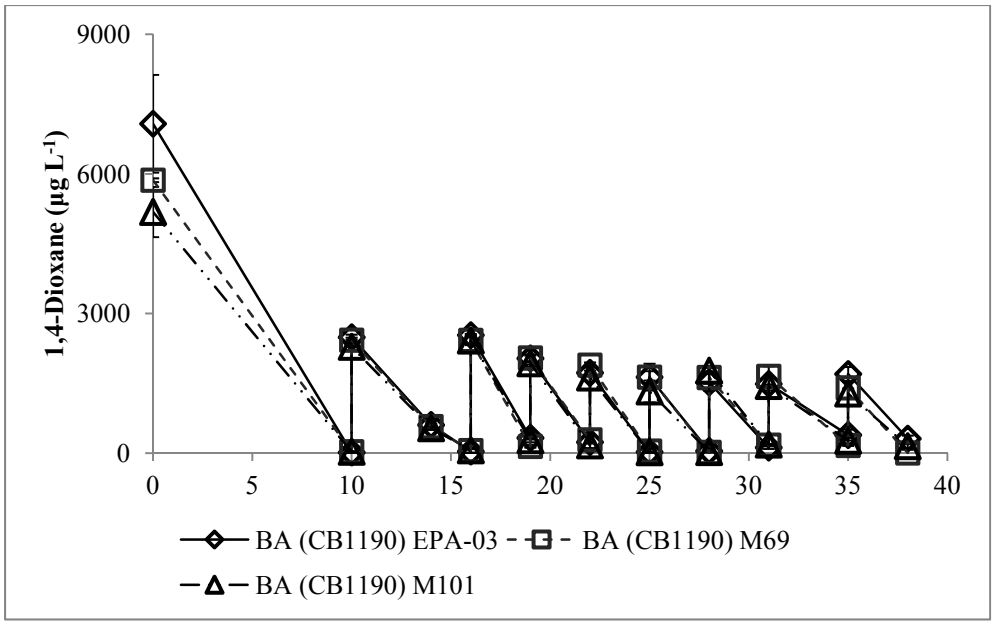

Figure 4.7. 1,4-Dioxane degradation in microcosms bioaugmented with *P. dioxanivorans* CB1190. All microcosms bioaugemented with *P. dioxanivorans* CB1190 could remove 1,4-dioxane in groundwater. Error bars represent standard deviation of triplicate samples.

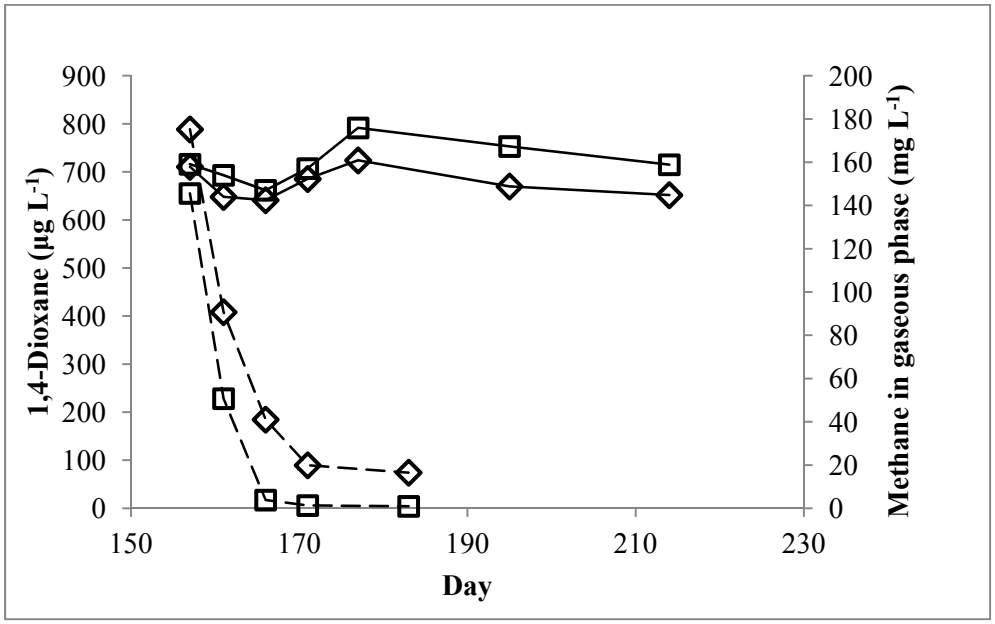

Figure 4.8. Degradation of methane (dashed line) and 1,4-dioxane (solid line) in microcosm number 42 (methane-amended microcosm; diamond) and microcosm number 58 (microcosm bioaugmented with *M. trichosporium* OB3b; square) prepared from groundwater with high 1,4-dioxane concentrations (M-69) after day 150. Microcosms could remove methane but not dioxane.

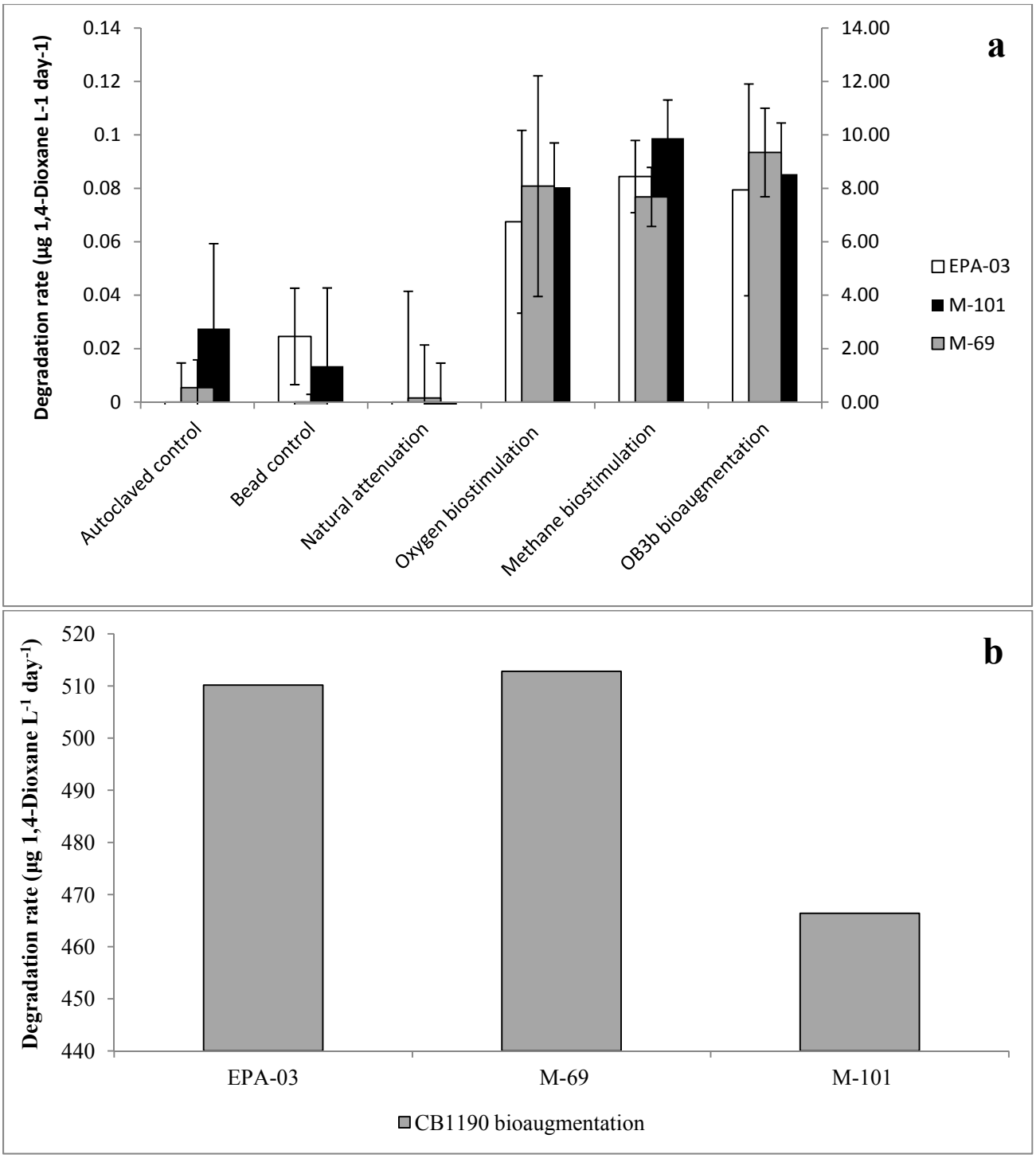

Figure 4.9. 1,4-Dioxane biodegradation rates determined for each microcosm. (a) Biodegradation rates determined for all tested conditions excluding bioaugmentation with *Pseudonocardia dioxanivorans* CB1190. Data for M-69 groundwater are presented using secondary axis. (b) Biodegradation rates determined for microcosms bioaugmented with *P. dioxanivorans* CB1190. Error bars represent standard deviation of triplicate samples.

4.3.3.3. Geochemistry in microcosms. The geochemistry of microcosms is summarized in Table 4.4. At day 0, microcosms' pH ranged between 6.8 and 8.2, ORP between 264 and 362 mV, and DO between 4.9 and 7.4 mg L^{-1}. At day 158, microcosms' pH was between 6.6 and 8.2, ORP between 171 and 455 mV, and DO between 3.7 and 7.8 mg L^{-1}.

Table 4.4. Geochemistry of microcosms. The results indicated that microcosms were maintained under aerobic conditions throughout the study.

Samples	Initial (day 0)				Final (day 158)			
	Temp	pH	ORP	DO	Temp	pH	ORP	DO
Autoclave control EPA-03	26	8.2	338.1	6.8	26.8	7.7	455.7	4.7
Autoclave control M69	24.7	8.1	349.3	6.63	24.6	7.7	419.9	3.7
Autoclave control M101	23.5	8.2	278.3	5.68	25.2	7.6	416.3	4
Bead control EPA-03	23.5	8.0	266.3	6.82	27.3	7.7	407.3	4.1
Bead control M69	23.5	8.0	263.8	6.37	26.8	7.1	421.7	4.2
Bead control M101	23.6	8.0	334.5	6.22	28.8	7.3	412.4	3.8
Natural Attenuation EPA-03	23.6	8.2	267.3	6.58	27.9	7.9	394.1	4
Natural Attenuation M69	23.6	7.9	288.3	6.78	28.1	7.9	399.5	4.3
Natural Attenuation M101	23.6	7.9	293.3	7.32	27.7	7.7	380	5.5
BS (oxygen) EPA-03	23	7.0	349.6	6.37	25.2	7.1	392.4	5.9
BS (oxygen) M69	23	7.0	341.4	6.13	25.5	6.9	398.8	7.8
BS (oxygen) M101	23	7.0	334.1	6.49	25.2	6.9	398.5	5.9
BS (methane) EPA-03	22.6	7.0	361.6	6.26	25.8	6.9	390.6	6.6
BS (methane) M69	22.6	7.0	343.3	6.99	26	8.2	286.5	5.6
BS (methane) M101	22.6	7.0	331	7.41	25.1	7.8	270.6	4.8
BA (CB1190) EPA-03	23.2	6.9	339.8	5.89	24.8	6.6	354.1	5.2
BA (CB1190) M69	23.5	6.9	326.9	5.62	25.1	6.7	350.5	5.4
BA (CB1190) M101	23.4	6.9	330.1	5.83	26.3	6.7	352.4	4.9
BA (OB3b) EPA-03	23	6.9	335.1	5.71	25	8.7	171.4	5.4
BA (OB3b) M69	23	6.9	335.8	4.86	24.4	8.4	184.7	6.3
BA (OB3b) M101	23.1	6.8	339.7	5.67	26.9	6.7	294.5	4.6

4.3.3.4. Biomass. Total soluble protein concentrations were determined at day 0 and day 158 of the experiment in each microcosm (Figure 4.10). An increase in biomass was observed in all amended microcosms from all three wells. According to a Pearson's correlation, no statistically significant correlation between either biomass concentration at day 150 or biomass production rate and biodegradation rate of 1,4-dioxane in biostimulated and bioaugmented microcosms could be established in this study (Table 4.5).

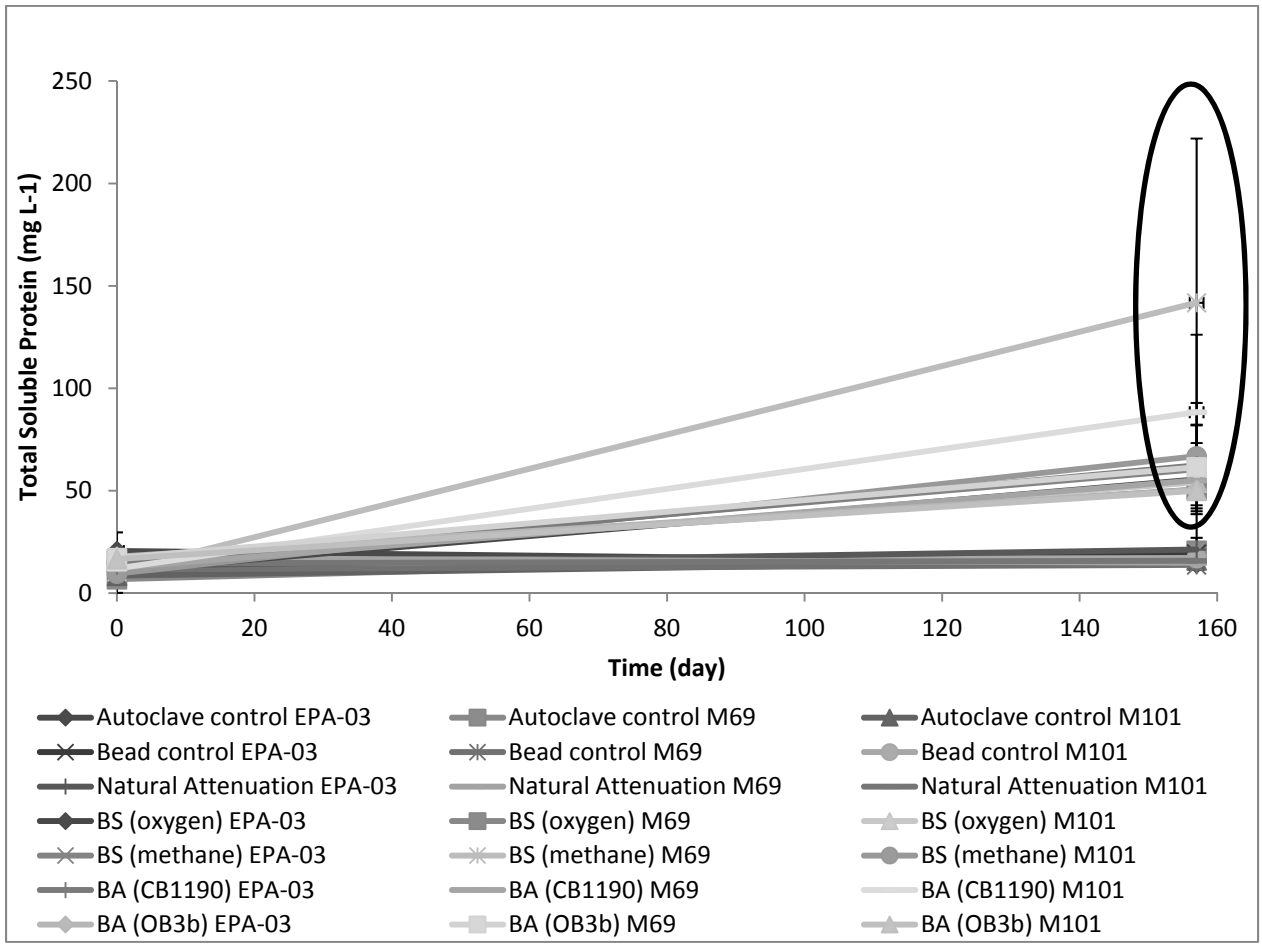

Figure 4.10. Total soluble protein in microcosms. Increase in total soluble protein was observed in all amended microcosms for all three wells. Error bars represent standard deviation of triplicate samples.

Table 4.5. Correlation between either protein production rate or protein concentration at day 150 and 1,4-dioxane biodegradation rates in microcosms. No correlation between either biomass production rate or biomass concentration and biodegradation rate was observed.

Microcosms	Comparison with degradation	Correlation	Degree of freedom	t-value	p-value	r^2
BA+BS	Protein production rate	0.09	11	0.30	0.77	0.01
	Protein concentration	0.07	11	0.24	0.82	0.01
BA only	Protein production rate	0.64	5	1.68	0.15	0.41
	Protein concentration	0.55	5	1.32	0.24	0.30
BS only	Protein production rate	0.49	5	1.12	0.31	0.24
	Protein concentration	0.52	5	1.22	0.28	0.27

4.3.4. Analysis of Gene Abundance. Copy numbers of gene targets were determined for activated sludge microcosms (AS) and AFP44 microcosms with biostimuation and bioaugmentation after 10 and 150 day of the experiment, respectively (Figure 4.11). The results revealed that abundances of the gene targets were highest in microcosms bioaugmented with *P. dioxanivorans* CB1190 and activated sludge microcosms. Correlations between abundance of the gene targets vs. biodegradation rate are summarized in Table 4.6. Statistical analyses combining data from the two microcosm studies revealed a statistically significant correlation between DXMO, PHE and PrMO abundance and 1,4-dioxane biodegradation rate (r^2 = 0.83 to 0.88; $p < 0.01$). In AFP 44 bioaugmented microcosms, DXMO and PHE gene abundances were statistically related to 1,4-dioxane biodegradation rates (r^2 = 0.93 and 0.96, respectively: $p <$ 0.01), while PrMO gene abundance was not significantly related to the rate ($p = 0.14$). In AFP44 biostimulated microcosms, abundances of these genes did not correlate to 1,4-dioxane biodegradation rate ($p = 0.11 – 0.74$).

Table 4.6. Correlation between abundances of gene targets and 1,4-dioxane biodegradation rates in industrial activated sludge and AFP44 microcosms. Gene abundance alone was not sufficient to predict 1,4-dioxane biodegradation in some circumstances.

Microcosms	Comparison with biodegradation rate	Correlation	Degree of freedom	t-value	p-value	r^2
AS + AFP44 BA and BS	DXMO abundance	0.91	16	8.67	<0.01	0.83
	PrMO abundance	0.93	16	9.68	<0.01	0.86
	PHE abundance	0.91	16	8.68	<0.01	0.83
AFP44 BA and BS only	DXMO abundance	0.81	11	4.44	<0.01	0.66
	PrMO abundance	-0.43	11	-1.50	0.16	0.18
	PHE abundance	0.49	11	1.78	0.10	0.24
AFP44 BA only	DXMO abundance	0.96	5	7.06	<0.01	0.93
	PrMO abundance	0.65	5	1.72	0.15	0.43
	PHE abundance	0.98	5	11.35	<0.01	0.97
AFP44 BS only	DXMO abundance	0.17	5	0.35	0.74	0.03
	PrMO abundance	0.20	5	0.42	0.69	0.04
	PHE abundance	-0.70	5	-1.95	0.11	0.49

4.4. Discussion.

4.4.1. Intrinsic Biodegradation of 1,4-Dioxane in Activated Sludge. Biosludge samples from the wastewater treatment plant of a polyester-manufacturing facility were used in this study to determine intrinsic biodegradation of 1,4-dioxane. It should be noted that 1,4-dioxane was a by-product of polyester manufacturing and not associated with chlorinated solvents which could affect biodegradation of 1,4-dioxane (*88*). It was demonstrated that 1,4-dioxane was removed in activated sludge samples from 81.97 ± 1.56 mg L^{-1} to below detection limit within 10 days (Figure 4.3). Biodegradation was identified as the dominant removal mechanism as no 1,4-dioxane removal was observed in heat-sterilized biosludge. This suggested that activated sludge samples already contained microorganisms capable of actively biodegrading 1,4-dioxane.

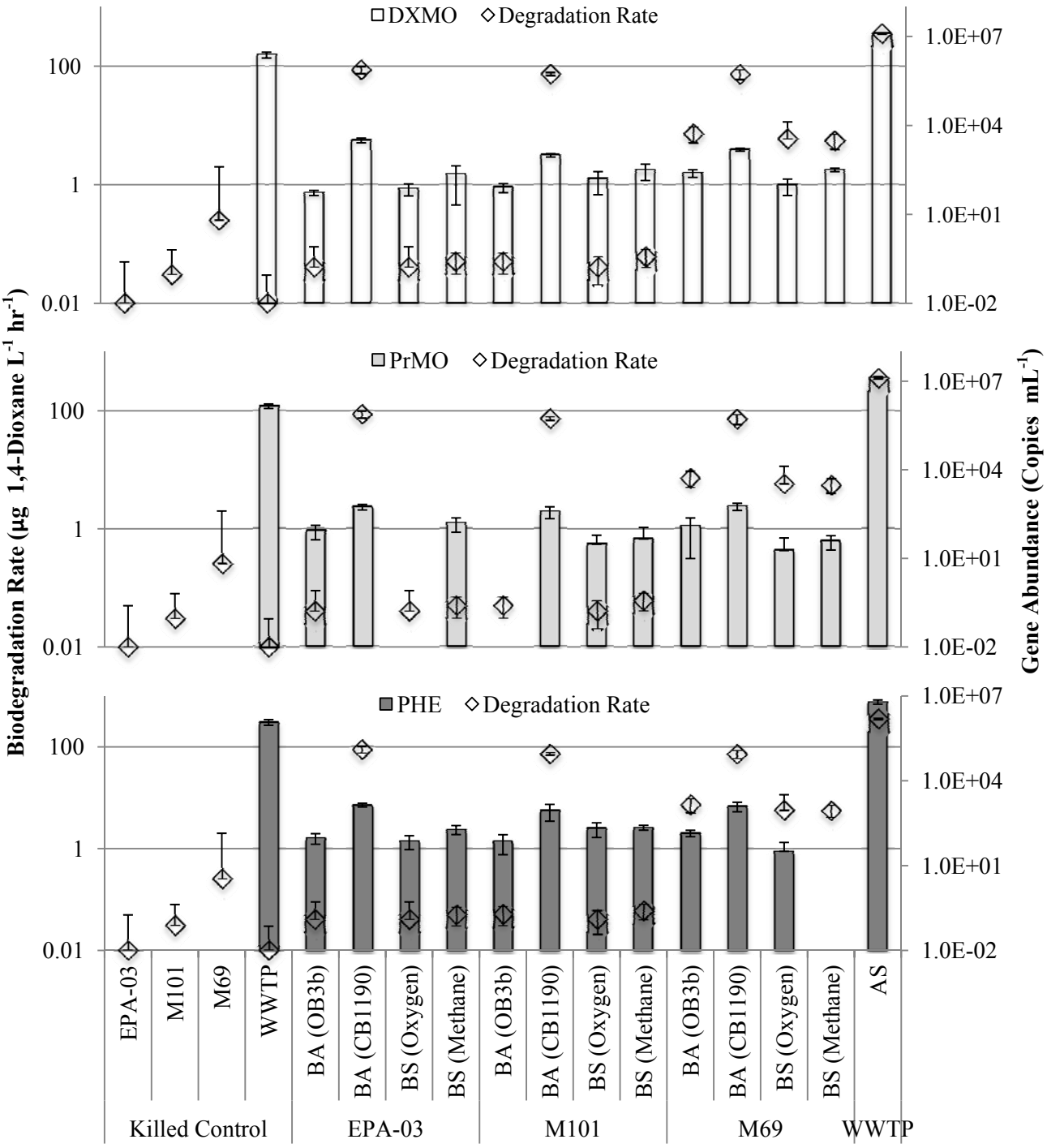

Figure 4.11. Abundance of DXMO, PrMO and PHE vs. 1,4-dioxane biodegradation rate in different microcosms. Biomarkers were successfully applied to correlate biodegradation in a wide range of 1,4-dioxane-contaminated environmental samples (10 µg/L to 100 mg/L). High correlation was observed between abundance of these gene targets vs. 1,4-dioxane biodegradation rate ($r^2 = 0.83$ to 0.88; $p < 0.01$). Error bars represent standard deviation of triplicate samples.

4.4.2. Biodegradation of 1,4-dioxane in AFP44 microcosms. In this study, groundwater samples collected from three monitoring wells were analyzed for 1,4-dioxane and TCE (Table 4.3). 1,4-Dioxane concentration in groundwater collected from EPA-03, M-69, and M-101 was determined as 3.83 ± 0.98, 730.73 ± 8.01, and 20.83 ± 4.43 μg L^{-1}, respectively. The concentration of 1,4-dioxane in M-69 and M-101 groundwater samples was higher than USEPA Preliminary Remediation Goal of 6.1 μg L^{-1}, while that of EPA-03 was a pristine well not impacted by 1,4-dioxane. The results are also supported by previous analyses by AECOM, Inc. in which M-69 groundwater contained highest concentration of 1,4-dioxane, followed by M-101 and EPA-03, respectively. Note that the 1,4-dioxane concentration in M-69 groundwater in this study was higher than that obtained from a previous measurement in February 2009 by AECOM, Inc. (340 μg L^{-1}), while 1,4-dioxane concentration of EPA-03 groundwater sample is comparable to 9.4 μg L^{-1} 1,4-dioxane in the same study. In contrast, the analysis of TCE in groundwater samples yielded concentrations below the detection limit of 5 μg L^{-1} (Table 4.3). It is possible that losses of TCE in these groundwater samples could have occurred by volatilization during collection, shipment and/or preparation processes of groundwater samples. Because TCE was not detected in groundwater, the subsequent microcosm study primarily focused on evaluation of 1,4-dioxane biodegradation rates.

Concentrations of total soluble protein in each groundwater sample were analyzed in this study. Protein concentrations of 21.36 ± 3.77, 12.22 ± 4.76 and 11.20 ± 1.18 mg L^{-1} were established for EPA-03, M-69 and M-101 groundwater samples, respectively. The statistical analysis confirmed that the amount of biomass in M-69 groundwater sample, the dirtiest sample among three groundwater samples, is significantly lower than that of EPA-03, a pristine groundwater

sample ($p < 0.05$, CI = 95%). The results indicate that other chemical contaminants in M-69 groundwater sample might be toxic to microorganisms causing the lower yield of biomass.

Results for the natural attenuation microcosms for wells M-69 and M-101 did not show evidence of intrinsic 1,4-dioxane biodegradation (Figures 4.5 and 4.6b, respectively). This finding is not consistent with the previous field study, which provided evidence of intrinsic 1,4-dioxane degradation, especially in well M-69 (*139*). The discrepancy can be attributed to a number of factors including selection of 1,4-dioxane-degrading bacteria as a result of high 1,4-dioxane amendments to *in situ* microcosms, differences in 1,4-dioxane and oxygen delivery, or changes in the biogeochemistry of the samples. Differences in 1,4-dioxane delivery presents an interesting point of discussion as the stable isotope probing (SIP) study conducted at the same site (*139*) relied on bacteria to colonize 1,4-dioxane amended BioSep® beads, whereas 1,4-dioxane was a part of the aqueous fraction in the microcosm study. Previous research has demonstrated that biofilm communities are resistant to antibiotics and disinfection (*230-232*) with resistance usually associated with protection of the sessile cells by exopolysaccharides. The increased protection provided by biofilms might have contributed to different findings in our natural attenuation microcosms. In addition, high concentrations of 1,4-dioxane were used to amend BioSep® beads in the previous SIP study while each microcosm was prepared with native contaminant concentrations (*139*). Indeed, the loss of TCE in our samples is a prime example of changing conditions from the field to the laboratory, and each of these factors are likely to contribute to the differences in our study and the stable isotopic probing study (*139*). This difference in experimental design may have contributed to the different findings in our study.

Microcosms constructed using B-125 soil and groundwater from EPA-03 that contained low levels of 1,4-dioxane and TCE contamination demonstrated a decreasing trend after 90 days

(Figure 4.6a). This would suggest that natural attenuation might be occurring at that site; however, further evaluation is necessary to confirm this trend since the concentration of 1,4-dioxane was near the limit of detection resulting in high variation of the results. In addition, while microcosm bottles were set up to be representative of site conditions, possible differences between site conditions and microcosms over 150 days (e.g., nutrient, air, 1,4-dioxane bioavailability/delivery, temperature or biomass levels) could result in the different results.

Continuous/steady decreases in 1,4-dioxane concentrations were observed in microcosms subjected to biostimulation with oxygen for all three wells with decreases being the most significant in well M-69 (Figure 4.5-4.6). Preliminary degradation rates were at 0.04 µg 1,4-dioxane L^{-1} day^{-1} for wells EPA-03 and M-101, and approximately 5.4 µg 1,4-dioxane L^{-1} day^{-1} for well M-69. 1,4-Dioxane concentrations did not decrease in the methane-amended microcosms until the methane feed was stopped at day 60 of the experiment, after which 1,4-dioxane was rapidly degraded. These results suggested that methanotrophs, microorganisms capable of cometabolizing 1,4-dioxane, were present in those monitoring wells.

Cometabolic 1,4-dioxane degradation was eventually lost in methane-biostimulated and *M. trichosporium* OB3b-bioaugmented microcosms after 150 days and efforts to recover 1,4-dioxane degradation were unsuccessful (Figure 4.8). Differences in 1,4-dioxane degradation kinetics may be linked with the observed differences in primary substrate utilization. Methane utilization occurred at a much faster rate in the first 60 days of incubation with an average of 39.15 mg methane L^{-1} d^{-1} (S.D. = 15.17) compared to after day 150 when rates were 15.75 mg methane L^{-1} d^{-1}. This suggests that methanotrophic populations and the enzymes responsible for cometabolic degradation of 1,4-dioxane may be present, but at insufficient concentrations to significantly remove 1,4-dioxane in these environmental microcosms. Furthermore, trace

elements essential for cometabolic 1,4-dioxane degradation may be limiting in the microcosms analyzed after day 150 due to the extended period of incubation.

1,4-Dioxane degradation in microcosms amended with *P. dioxanivorans* CB1190 was the most rapid and complete of all test conditions (Figure 4.7). Biodegradation rates for all three wells ranged from 460 to just over 520 µg dioxane L^{-1} day^{-1} (Figure 4.9). Given that *P. dioxanivorans* CB1190 requires high 1,4-dioxane concentrations on a regular basis to support its growth, bioaugmentation with *P. dioxanivorans* CB1190 is only considered promising for 1,4-dioxane source areas.

4.4.3. Biomarkers for 1,4-Dioxane Biodegradation in Complex Environments. This study evaluated the possibility of using total soluble protein to indicate biodegradation of 1,4-dioxane. Total soluble protein was used as a proxy to estimate biomass production during biodegradation of 1,4-dioxane. Correlation between total soluble protein (both production rate and concentration) and 1,4-dioxane biodegradation rates suggested that there was little to no association between either protein production rate or protein concentration at day 150 and 1,4-dioxane biodegradation rate in AFP microcosms with bioaugmentation and biostimulation (Table 4.4). The results suggested that protein production rate and protein abundance was not a good indicator for predicting biodegradation of 1,4-dioxane.

It was shown that 16S rRNA biomarker specific to *P. dioxanivorans* CB1190 is useful to indicate potential biodegradation of 1,4-dioxane. *P. dioxanivorans* CB1190 has three copies of 16S rRNA based on draft genomic annotation (*80*). According to NCBI database (NCBI Reference Sequence: NR_042850.1), the size of 16S rRNA of the bacterium is 1422 bp. Phylogenetic tree analysis demonstrated that 16S rRNA of *P. dioxanivorans* CB1190 is similar to that of other

strains in the *Pseudonocardia* genus which have not been validated for 1,4-dioxane biodegrading ability, some bacteria in the order *Actinomycetales*, and a species of *Streptomyces* sp. (Figure 4.12). Hence, a primer set for the 16S rRNA is likely to provide false positive results and uncertainty in determining the potential to biodegrade 1,4-dioxane in the environment. In addition, a 16S rRNA-based biomarker does not definitively reveal the functional activities of those organisms under site-specific conditions. To overcome this limitation and reduce uncertainty, development of qPCR assays targeting functional gene biomarkers that are specific to 1,4-dioxane is necessary.

It was demonstrated that monooxygenases are associated with 1,4-dioxane biodegradation in many microorganisms (*18, 24, 233*). DXMO was proven to catalyze 1,4-dioxane and THF biodegradation in *Pseudonocardia* stain ENV478 and *P. dioxanivorans* CB1190 (*81-83*). Based on an NCBI blast comparison, the genes in the putative DXMO cluster in *P. dioxanivorans* are nearly identical (98-100% similarity; figure 4.13) with the cluster in *Pseudonocardia tetrahydrofuranoxydans* K1 (*24, 233*). PrMO was found in propanotrophs capable of cometabolizing 1,4-dioxane (*18, 219, 234*). The putative PrMO cluster of *P. dioxanivorans* CB1190 compared to the PrMO cluster in *Rhodococcus jostii* RHA1 (a versatile organic-degrading strain and a 1,4-dioxane degrader) showed 35-85% similarity. PHE was found in many bacteria capable of transforming 1,4-dioxane (*18*) (*80*) and was used as an indicator for biodegradation of 1,4-dioxane and TCE in groundwater (*139*). This evidence suggested that genes encoding DXMO, PrMO, and PHE could be candidates for biomarkers for indicating 1,4-dioxane biodegradation.

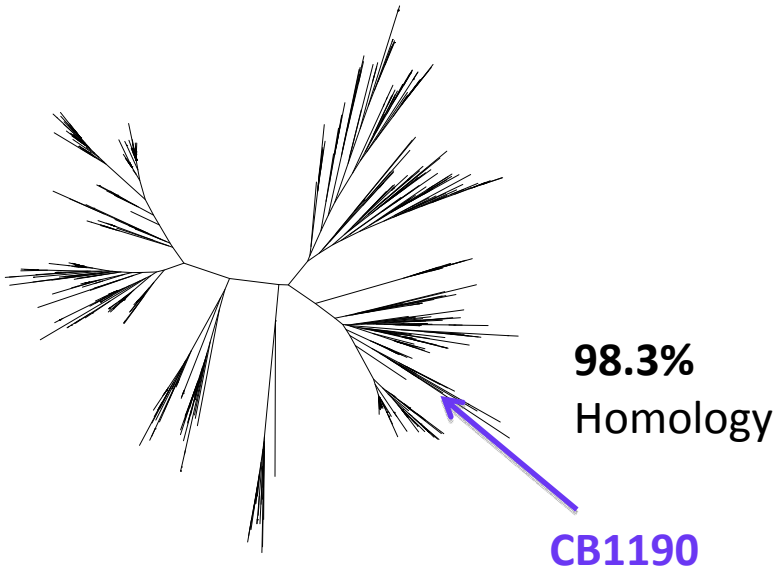

Figure 4.12. Unrooted 16S rRNA phylogenetic tree showing that *P. dioxanivorans* CB1190 is grouped in the same cluster as other bacterial strains unable to biodegrade 1,4-dioxane.

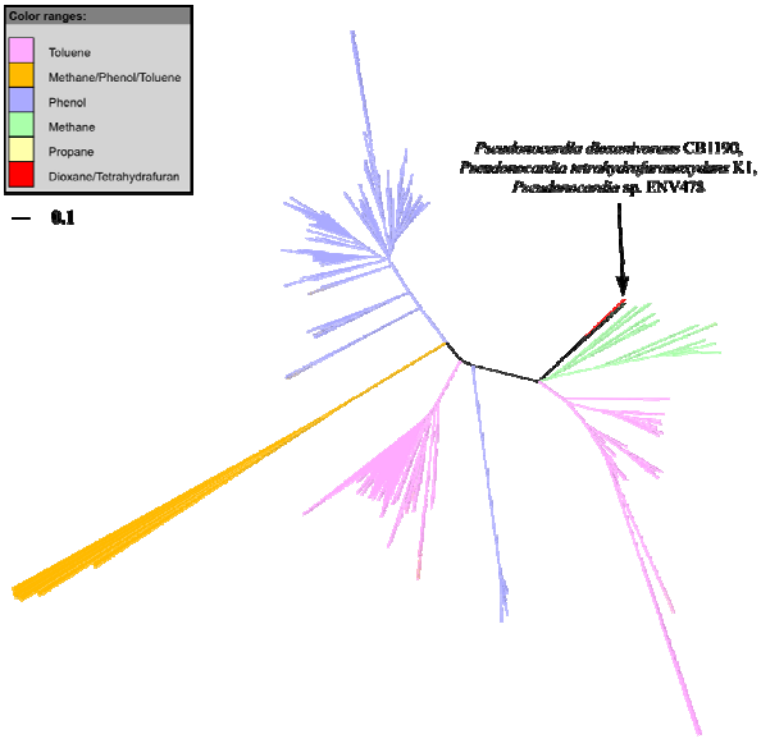

Figure 4.13. Phylogenetic tree generated based on protein sequence similarity of *dxmB* which encodes the beta subunit of the multicomponent dioxane/tetrahydrofuran monooxygenase (DXMO) of *Pseudonocardia dioxanivorans* CB1190 (Accesssion# AEA29039).

The influence of sample complexity on gene abundance was performed to emphasize the benefit of quantifying functional gene biomarkers associated with 1,4-dioxane biodegradation. In this study, industrial activated sludge microcosms and AFP44 microcosms with biostimulation and bioaugmentation were used as models for mixed bacterial consortia since the microcosms confirmed biodegradation of 1,4-dioxane. The results from gene abundance analysis for DXMO, PrMO, and PHE in all microcosms demonstrate that these genes can be applied for determining presence or absence of 1,4-dioxane-degrading microorganisms. However, the abundance could not be used to indicate viability of bacterial community in the samples. This was confirmed by results of heat-sterilized activated sludge samples that exhibited high gene abundance, but no 1,4-dioxane biodegradation. This suggests that using gene abundance to predict viability of 1,4-dioxane-degrading bacteria might produce a false positive result.

The correlation between abundance of the gene targets and biodegradation of 1,4-dioxane was demonstrated in Table 4.6. Using all data from the two microcosm studies, abundance of DXMO, PrMO and PHE was highly correlated to 1,4-dioxane biodegradation (r^2 = 0.83 to 0.88; $p < 0.01$) suggesting that DXMO, PrMO, and PHE primer sets might be useful in predicting 1,4-dioxane biodegradation. However, the abundance of these gene targets in some circumstances could not predict 1,4-dioxane biodegradation. For example, abundances of DXMO, PrMO, and PHE were not good proxies to predict the biodegradation in AFP44 biostimulated microcosms, which was indicated by poor correlations between the two parameters (p = 0.11 – 0.74). In bioaugmented microcosms, copy numbers of PrMO could not be used to associate 1,4-dioxane biodegradation (p = 0.14), but copy numbers of DXMO and PHE correlated well with the biodegradation rates (r^2 = 0.93 and 0.96, respectively: $p < 0.01$). These results suggested that analysis of gene abundance is insufficient to accurately predict 1,4-dioxane biodegradation.

These tools should be applied together with other molecular biological tools such as monitoring the expression of these genes, enzyme activity assays or stable isotope probing for more reliable site characterization.

CHAPTER 5

DEVELOPMENT AND APPLICATIONS OF COMPOUND SPECIFIC CARBON ISOTOPE ANALYSIS METHOD FOR VALIDATING 1,4-DIOXANE BIODEGRADATION

5.1. Introduction. Compound specific isotope analysis (CSIA) has been used as a monitoring tool to assess the biodegradation of various organic contaminants by pure cultures (*32, 33, 146, 147*) and mixed consortia (*139, 148, 149*). CSIA provides evidence for breaking of chemical bonds in reactions such as hydrolysis, oxidation, reduction, and biochemical reactions, which results in a change in the ratio of stable isotopes reflecting an increase in the heavy isotope in the residual compound (*31, 150*). CSIA provides a measurement of isotope enrichment factors (ε), which are valuable for characterizing degradation mechanisms as well as quantifying the contaminant degradation based on stable isotope data from field sites (*31, 150*). As a tool for monitoring biodegradation, CSIA provides valuable information in judging whether bioremediation might be feasible to clean up a contaminated site (*158*).

Pseudonocardia dioxanivorans CB1190 and *Mycobacterium austroafricanum* JOB5 (previously *Mycobacterium vaccae* JOB5) are monooxygenase-expressing microorganisms that can degrade 1,4-dioxane in laboratory cultures (*17, 20*). *P. dioxanivorans* CB1190 uses 1,4-dioxane as its only source of carbon and energy for supporting growth; in contrast, *M. austroafricanum* JOB5 degrades 1,4-dioxane via a cometabolic mechanism after growth on propane or toluene. While these bacteria possess a low specific growth rates, low biomass and high half-saturation constants, recent paper reported the abundance of monooxygenase-expressing bacteria as well as several monooxygenase genes at a site where degradation of multiple groundwater contaminants was concomitantly measured, indicating that these bacteria may be suitable for remediation of 1,4-dioxane at contaminated sites (*139*). However, direct evidence for supporting degradation of 1,4-dioxane and validating the success of engineered bioremediation is particularly limited. This study will be the first to develop 1,4-dioxane-specific carbon stable isotope analysis as a tool for monitoring *in situ* biodegradation.

5.2. Materials and Methods

5.2.1. Chemicals. 1,4-Dioxane was procured from five providers including Fisher Chemical (Lot # 102732; Fair Lawn, NJ), Alfa Aesar (Lot # G08W024; Ward Hill, MA), Sigma Aldrich (Lot # 59096MK; Saint Louis, MO), Acros Organics (Lot # B0518954; Fair Lawn, NJ) and Mallinckrodt (Lot # E15H23; Phillipsburg, NJ). Methyl decanoate isotope reference material was purchased from Biogeochemical Laboratories, Indiana University (Bloomington, IN). ACS grade hydrochloric acid, methylene chloride, sodium hydroxide, and sodium sulfate, all from Fisher Scientific, Fair Lawn, NJ, and toluene (Sigma Aldrich, St. Louis, MO), were used.

5.2.2. Bacterial Strains and Culture Conditions. *P. dioxanivorans* CB1190 was grown in ammonium mineral salt medium (AMS) with 100 mg L^{-1}1,4-dioxane (*20*), while *M. austroafricanum* JOB5 was grown in 250 mL Boston round glass bottles (Wheaton, Millville, NJ) capped with screw-cap Mininert® valves (Alltech Associates, Inc., Deerfield, IL) containing Whittenbury medium (*223*) with 200 μg L^{-1} toluene as a growth supporting substrate (*23*). Bacterial stocks were maintained for at least five transfers to assure robust growth and enzyme activity. Culture purity was examined by plating cultures onto AMS agar plate with 440 mg L^{-1} 1,4-dioxane (for *P. dioxanivorans* CB1190), LB plate (for *M. austroafricanum* JOB5), or R2A agar plate (both), and sequencing of 16S rRNA genes.

5.2.3. Industrial Wastewater Samples. Wastewater samples from the influent, effluent, and activated sludge from 1,4-dioxane-containing industrial wastewater treatment plant (WWTP) were procured for stable carbon isotopic analyses and biodegradation experiments. Samples were collected in 3.8 L semirigid polyethylene containers with screw caps (Hedwin Cubitainer®;

Thermo Fisher Scientific Inc., Wilmington, DE). Wastewater samples were placed on ice and shipped on the same day to the laboratory.

5.2.4. Experimental Approach. Commercial 1,4-dioxane and industrial wastewater samples were analyzed for stable carbon isotopic ratio to obtain the isotopic fingerprint of each sample type. Biodegradation of 1,4-dioxane by *P. dioxanivorans* CB1190, *M. austroafricanum* JOB5, activated sludge, 1,4-dioxane-amended activated sludge, and CB1190-augmented activated sludge was carried out in 250 mL Boston round glass bottles (Wheaton, Millville, NJ) capped with screw-cap Mininert® valves (Alltech Associates, Inc., Deerfield, IL). The culture medium was always less than 20% of the bottle volume (i.e., 50 mL in 250 mL bottle) to prevent oxygen mass transfer limitations. Cultures were incubated at 30 °C and 150 rpm agitation, and aseptic conditions were maintained to prevent contamination. Sigma Aldrich 1,4-dioxane (100 mg L^{-1}) and toluene (200 µg L^{-1}) were used as growth substrate for *P. dioxanivorans* CB1190 and *M. austroafricanum* JOB5 cultures as the growth substrate. Before the biodegradation experiments, 1% (v/v) inocula of *P. dioxanivorans* CB1190 or *M. austroafricanum* JOB5 were cultured to obtain approximately 15 and 60 mg protein L^{-1}, respectively. The residual toluene in the headspace of *M. austroafricanum* JOB5 cultures was removed by flushing with filter-sterilized air for 10 min prior to adding 1,4-dioxane (a non-growth substrate). Sigma Aldrich 1,4-dioxane was spiked into the pure cultures of *P. dioxanivorans* CB1190 and *M. austroafricanum* JOB5 and activated sludge samples. Initial 1,4-dioxane concentration of about 100 mg L^{-1} was used in pure culture studies. Mixed culture experiments were conducted with and without amendment of 1,4-dioxane. For experiments with 1,4-dioxane-amended and CB1190-augmented activated sludge, 1,4-dioxane was spiked to make the final concentration of 300 mg L^{-1} to assure the sufficient substrate for supporting the growth of mixed cultures. The ratio of original 1,4-

dioxane in the sludge to Sigma Aldrich 1,4-dioxane at the beginning of the experiments was at 1:4. The concentrations of 1,4-dioxane were monitored over time in pure and mixed cultures. Water samples were collected periodically, and biodegradation of 1,4-dioxane was suspended by the addition of 10 N sodium hydroxide to raise pH above 10.5. Samples were stored at -20 ˚C for later extraction. Preserved samples were frozen and micro-extracted as previously described (*227*) prior to isotopic analysis. Cellular biomass was measured over time in pure cultures to differentiate biodegradation mechanisms. All experiments were conducted using triplicate cultures in three different bottles. Abiotic controls (n = 3) were used throughout in pure culture studies, while killed (autoclaved) controls (n=3) were used in mixed culture experiments. The loss of 1,4-dioxane in abiotic controls was less than 10% of the initial concentrations.

5.2.5. Biomass Quantification. Biomass in aqueous samples was quantified as total protein concentration by using a modified Bradford method (*19, 229*). Briefly, samples (0.5 mL) were digested by adding 200 g L^{-1} sodium hydroxide and boiled at 98°C for 10 min. After centrifugation at 13,200 rpm for 15 min, the supernatant was collected. Analysis of total soluble protein in the supernatant was performed using Coomassie Plus protein assay kit (Pierce Chemical Company, Rockford, IL) with bovine serum albumin standard following the kit instructions. Absorbance at 595 nm was measured immediately in cuvettes using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

5.2.6. Gas Chromatography. Concentrations of 1,4-dioxane were analyzed by manually injecting 2 µL liquid samples, which were filtered from liquid culture samples by 0.45 µm syringe filters, into a Hewlett-Packard 6890 Chromatograph (GC) equipped with a Flame Ionization Detector (FID) (Hewlett-Packard, Atlanta, GA) and a Restek® Stabilwax-DB capillary column (30 m length x 0.53 mm id x 1 µm film thickness; Restek, Bellefonte, PA). The injector

was set at 220 °C in splitless mode, while the detector was set at 250 °C. The oven program was initially set at 80 °C (3 min), then ramped at 20 °C min^{-1} to 140 °C, then maintained for 1 additional minute. The retention time for 1,4-dioxane was 3.7 minutes. Calibration curves were prepared by diluting neat 1,4-dioxane in deionized water to various concentrations that covered the range of the experiments. The detection limit was 0.8 mg L^{-1} and the accuracy of 1,4-dioxane measurement was better than ± 4% with this method.

5.2.7. Carbon Stable Isotope Analysis. 1,4-Dioxane in methylene chloride-extracted (for analyses of water samples) or -diluted samples (for determination of signatures of the pure compound and method optimization) was analyzed using a Finnigan Delta XP isotope ratio mass spectrometer (IRMS) coupled with the Finnigan Trace gas chromatograph (GC) and GC Combustion III (GCC) peripherals (Thermo Finnigan, Bremen, Germany). The mixtures of compounds in the methylene chloride extract prepared in certified 200-µL GC vial inserts (National Scientific Company, Rockwood, TN) were injected into the GC using a CTC Analytics Combi PAL autosampler (CTC Analytics, Zwingen, Switzerland). An aliquot of 1 µL extract was injected into the GC, and the mixture was separated using a fused silica Omegawax 320 capillary column, 30 m length, 0.32 mm ID, 0.25 µm film thickness (Supelco Inc., Bellefonte, PA). Splitless injection with an injection temperature of 250 ˚C, 0.5 minute splitless time followed by 40 mL min^{-1} split flow was used in this analysis. The flow rate was constant at 2.4 mL min^{-1}. The GC temperature program was 40 ˚C held for 1 minute followed by a temperature ramp of 10 ˚C per minute to 60 ˚C, which was held for 0.1 min. Methylene chloride-diluted standards containing 10, 21, 52, 103, and 196 mg L^{-1} Alfa Aesar 1,4-dioxane were injected into the instrument to determine linearity and detection limit of the method, while the accuracy of the CSIA method was determined based upon the analysis of methyl decanoacte isotopic reference

material. The relative abundance of two stable carbon isotopes (^{13}C and ^{12}C) is expressed in the conventional $\delta^{13}C$ nomination and reported in the unit of per mille (‰). $\delta^{13}C$ for 1,4-dioxane is first calculated against a CO_2 working standard, which has a $\delta^{13}C$ value versus VPDB as follows:

$$\delta^{13}C\ (‰) = ((R_{sample}/R_{standard}) - 1) \times 1000 \qquad (5.1)$$

where R is the ratio of ^{13}C and ^{12}C. The final sample $\delta^{13}C$ versus VPDB was obtained by correcting all data using a direct $\delta^{13}C$ offset obtained by analysis of the isotope reference material methyl decanoate. A log based Rayleigh model was used to describe the isotopic shifts during biodegradation, and Rayleigh enrichment factor (ε) was calculated from:

$$\varepsilon \times (ln f) = 1000 \times ln[(\delta^{13}C+1000)/(\delta^{13}C_0+1000)] \qquad (5.2)$$

where f is the remaining fraction of 1,4-dioxane (C/C_0) at any time during biodegradation. The ε values were estimated from the slope of the linear regression obtained by plotting the expression on the right hand side of the equation 5.2 against *ln*(f) using GraphPad Prism 5·0 (GraphPad Software Inc., San Diego, CA, USA).

5.3. Results

5.3.1. Chromatographic Conditions. A representative GC-IRMS chromatogram is shown in Figure 5.1. Chromatographic separation was achieved by using GC-IRMS providing a well-resolved peak of 1,4-dioxane. The measured retention time was 175.77 sec. $\delta^{13}C$ values in standards before and after applying a direct $\delta^{13}C$ offset were plotted versus the 1,4-dioxane concentration (Figure 5.2a and 5.2b, respectively). Linear relationship between 1,4-dioxane concentration and the peak signal amplitude of mass 44 is demonstrated in Figure 5.2b.

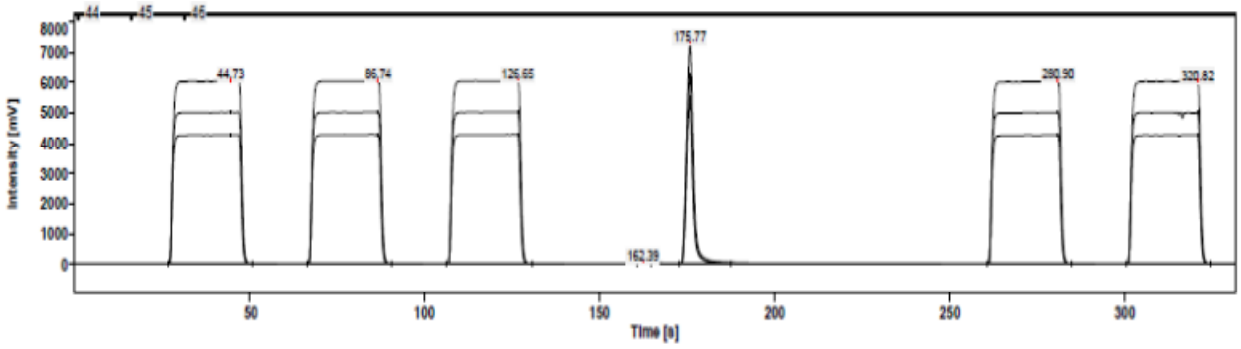

Figure 5.1. Chromatogram of GC-C-IRMS Analysis. 1,4-Dioxane (200 mg L^{-1}) peak is seen in the middle of the chromatogram (RT 175.77). The three first peaks and the last two peaks correspond to the reference CO_2 gas.

5.3.2. $\delta^{13}C$ Fingerprint of Commercial and WWTP 1,4-Dioxanes. The CSIA was applied to experimentally determine the $\delta^{13}C$ signature of 1,4-dioxane obtained from various chemical providers. The $\delta^{13}C$ values of 1,4-dioxane purchased from five providers were in the range of -34.15 to -29.76 ‰, with an arithmetic mean of -31.88 ± 0.25 ‰ (Table 5.1). 1,4-Dioxane from Sigma Aldrich had the lowest $\delta^{13}C$ value (-34.15 ± 0.03 ‰) while that of Mallinckrodt had the highest $\delta^{13}C$ value (-29.76 ± 0.13 ‰). CSIA was also used to determine the isotopic signature of WWTP samples. The isotopic signature of influent and effluent of the WWTP, which contained 333.74 ± 12.03 and 68.80 ± 3.19 mg 1,4-dioxane L^{-1}, respectively, were determined as -43.02 ± 0.18 and -41.59 ± 0.17 ‰, respectively.

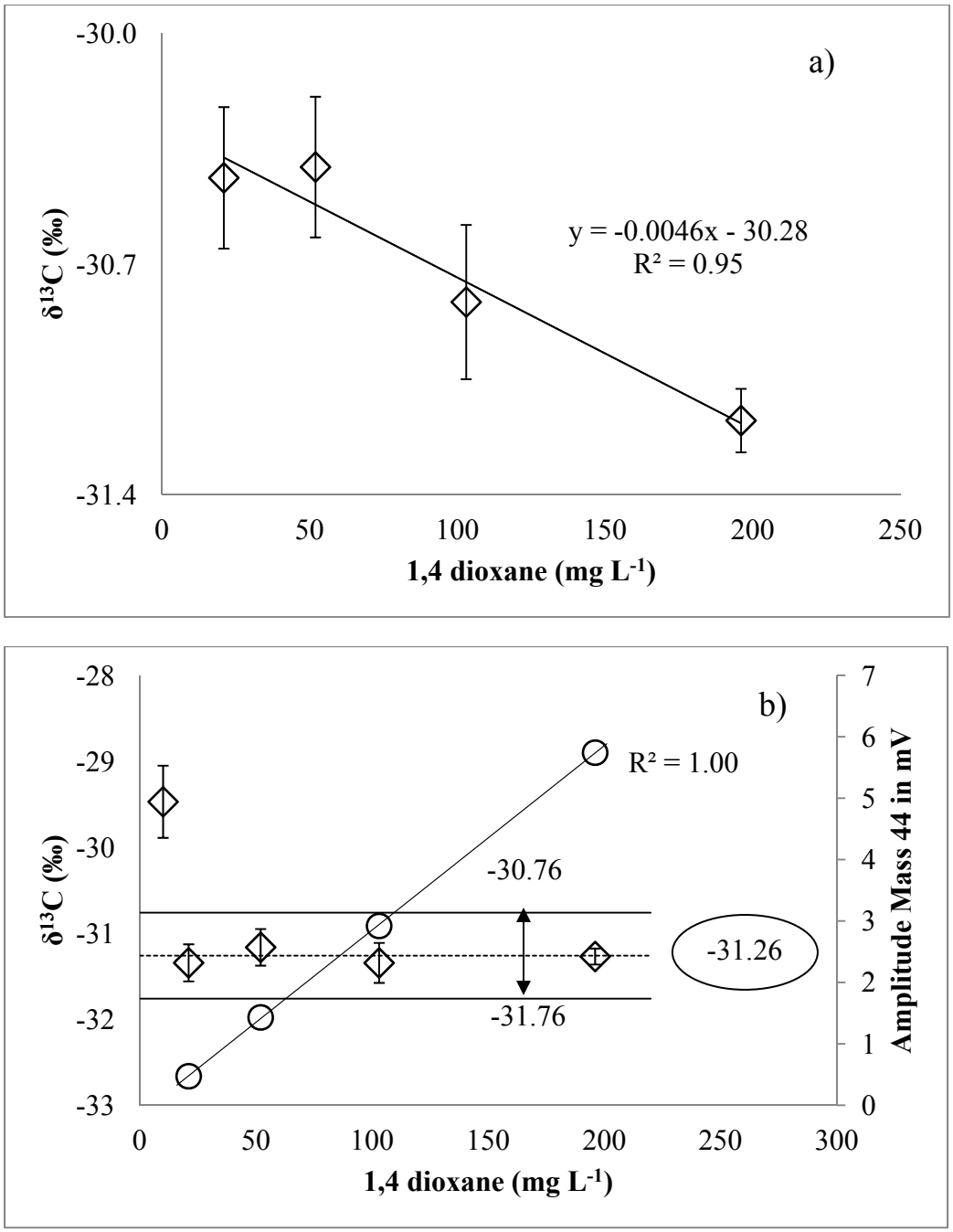

Figure 5.2. Schematic Illustration of Method Optimization (a) The plot of measured $\delta^{13}C$ values (prior normalization) vs. concentration of a 1,4-dioxane standard. An inverse linear correlation was observed between these two variables. (b) The diamonds represent the normalized $\delta^{13}C$ with measured $\delta^{13}C$ value of methyl decanoate isotope reference material. An improvement in precision and accuracy of $\delta^{13}C$ estimates was observed after correction applied measured $\delta^{13}C$. The same plot was also used for determine MDL followed the method described in [30]. The circles show the amplitude of mass 44 in mV. All data points are averaged values from replicates. The error bars correspond to the standard deviations.

Table 5.1. Stable Carbon Isotopic Fingerprint ($\delta^{13}C$) of Various Commercial 1,4-Dioxane. The values are expressed as means of three measurements along with standard deviation (technical error).

Sample	Provider/Type of Sample	$\delta^{13}C$ (‰)
1,4-Dioxane	Fisher Scientific	-31.63 ± 0.20
	Alfa Aesar	-32.17 ± 0.01
	Sigma Aldrich	-34.15 ± 0.03
	Acros Organics	-31.67 ± 0.07
	Mallinckrodt	-29.76 ± 0.13
C_{10} Methyl Decanoate*		-29.67 ± 0.22

* **C_{10} Methyl Decanoate** expected value = -29.67 ± 0.02 ‰

5.3.3. CSIA for Metabolic Biodegradation of 1,4-Dioxane. In cultures of *P. dioxanivorans* CB1190, 1,4-dioxane was biodegraded at the rate of 0.17 ± 0.09 mg 1,4-dioxane $hour^{-1}$ mg $protein^{-1}$ (Figure 5.3), resulting in biomass increase from 14.33 ± 3.65 to 23.59 ± 2.35 mg protein L^{-1}. ^{13}C-1,4-Dioxane was enriched by 2.96 ± 0.37 ‰ (from -34.25 ± 0.04 ‰ to -31.29 ± 0.37 ‰) when over 90 % of 1,4-dioxane was degraded (Figure 5.4), while no change in $\delta^{13}C$ was observed in abiotic controls (Figure 5.7a). An average enrichment factor of -1.4 ± 0.3 (mean ± 95% confidence interval) with r^2 of 0.88 was computed for this mechanism (Figure 5.6).

5.3.4. CSIA for Cometabolic Biodegradation of 1,4-Dioxane. *M. austroafricanum* JOB5 (biomass of 62.38 ± 4.45 mg protein L^{-1}) cometabolized 1,4-dioxane after growth on toluene (Figure 5.3). After 90% degradation of 1,4-dioxane by *M. austroafricanum* JOB5, the observed enrichment of ^{13}C-containing 1,4-dioxane was 4.81 ± 1.75 ‰ (from -34.51 ± 0.15 ‰ to -29.70 ± 1.74 ‰) (Figure 5.4), and the enrichment factor corresponding to this fractionation was determined as -2.0 ± 0.3 (mean ± 95% confidence interval and $r^2 = 0.97$) (Figure 5.6). As expected in cometabolism, there was no change in biomass during biodegradation of 1,4-dioxane.

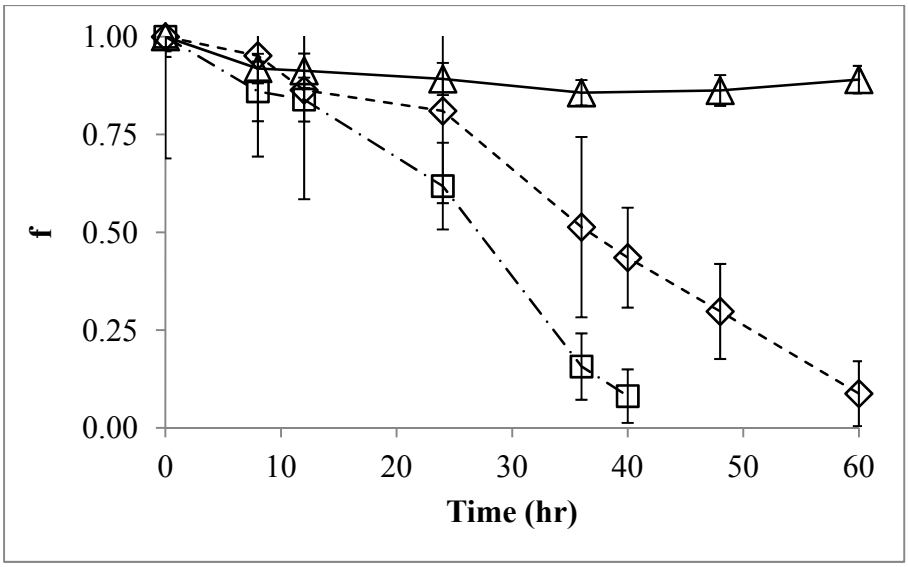

Figure 5.3. 1,4-Dioxane Biodegradation by Pure Cultures. (a) Metabolic biodegradation of 1,4-dioxane by *P. dioxanivorans* CB1190 (diamonds) and cometabolic biodegradation of 1,4-dioxane by *M. austroafricanum* JOB5 (squares) vs. abiotic control (triangles). The data points represent concentrations of 1,4-dioxane averaged from three samples from each bottle of the bacterial culture. The error bars correspond to the standard deviations of the three separate biological replicates.

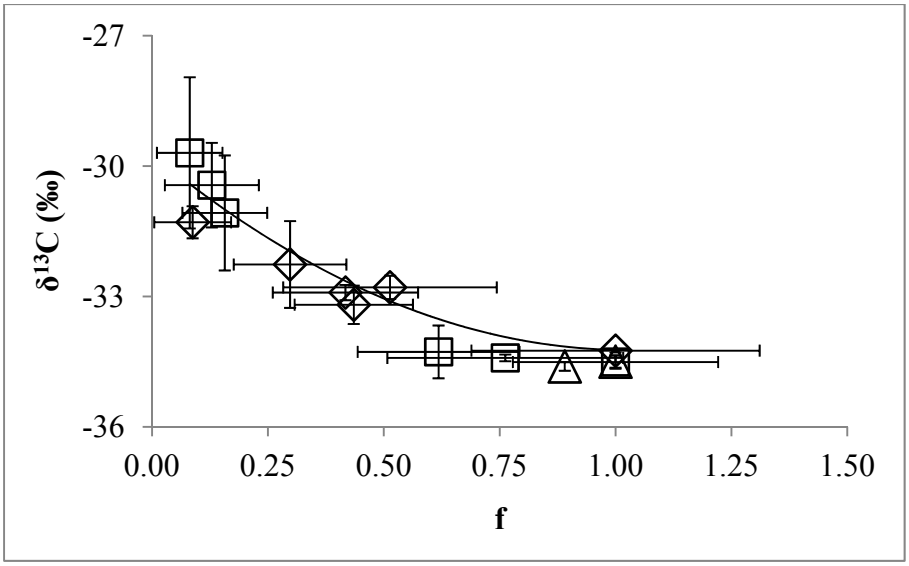

Figure 5.4. Stable Carbon Isotopic Fractionation During Biodegradation of 1,4-Dioxane by Pure Cultures. Carbon stable isotope fractionation during metabolic biodegradation of 1,4-dioxane by *P. dioxanivorans* CB1190 (diamonds) and cometabolic biodegradation of 1,4-dioxane by *M. austroafricanum* JOB5 (squares) vs. abiotic control (triangles). The data points represent $\delta^{13}C$ values averaged from three biological replicates. The error bars correspond to the standard deviations of triplicate samples.

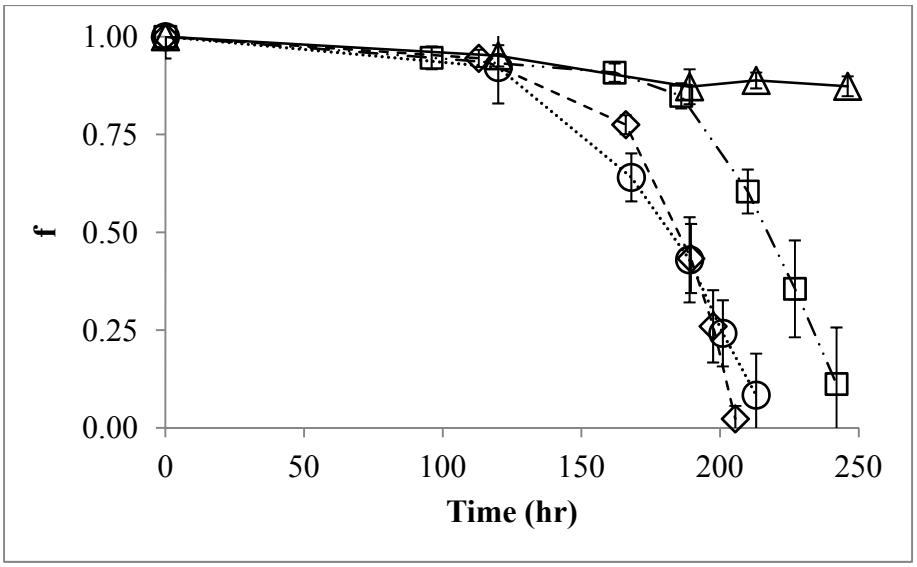

Figure 5.5. 1,4-Dioxane Biodegradation by Mixed Cultures. 1,4-Dioxane biodegradation by activated sludge (diamond), activated sludge with spiked 1,4-dioxane (squares), and activated sludge spiked with CB1190 and 1,4-dioxane (circles) vs. killed control (triangles). The data points represent concentrations of 1,4-dioxane averaged from three samples from each bottle of the bacterial culture. The error bars correspond to the standard deviations of the three separate biological replicates.

5.3.5. CSIA for Biodegradation of 1,4-Dioxane in Activated Sludge. Mixed cultures in activated sludge and in CB1190-augmented activated sludge were able to degrade 1,4-dioxane (Figure 5.5). The indigenous microbes in the activated sludge could degrade 62.35 ± 1.56 mg L^{-1} 1,4-dioxane (original 1,4-dioxane in sludge sample) to below the detection limit, with simultaneous increase in biomass within nine days. In contrast, activated sludge spiked with Sigma-Aldrich 1,4-dioxane to final concentration of 300.14 ± 10.16 mg L^{-1} removed 1,4-dioxane to below the detection limit within twelve days, while sludge amended with Sigma-Aldrich 1,4-dioxane to final concentration of 295.78 ± 5.13 mg L^{-1} and augmented with 1 mL CB1190 (28.18 ± 0.29 mg protein L^{-1}) removed 1,4-dioxane to below the detection limit in about ten days. The unamended activated sludge enriched ^{13}C in 1,4-dioxane by 2.20 ± 0.13 ‰ (from –40.92 ± 0.10 ‰ to –38.72 ± 0.09 ‰ during 75% degradation of 1,4-dioxane), while the

activated sludge spiked with Sigma Aldrich 1,4-dioxane in the ratio of 1 to 4 (v/v) enriched 1,4-dioxane by 1.99 ± 0.54 ‰ (from –36.11 ± 0.14 ‰ to –34.12 ± 0.52 ‰ during 90% degradation of 1,4-dioxane). CB1190-amended activated sludge (also spiked with Sigma Aldrich 1,4-dioxane), which served as a positive biodegradation control, enriched for ^{13}C-1,4-dioxane 2.62 ± 0.23 ‰ (from –36.48 ± 0.15 ‰ to -33.86 ± 0.18 ‰ during 80% degradation of 1,4-dioxane). Again, no change in $\delta^{13}C$ was observed in killed controls (Figure 5.7b). The enrichment factors for 1,4-dioxane degradation by activated sludge, activated sludge spiked with 1,4-dioxane, and CB1190-bioaugmented activated sludge were calculated as -1.8 ± 0.2 (mean ± 95% confidence interval and $r^2 = 0.99$), -1.2 ± 0.1 (mean ± 95% confidence interval and $r^2 = 0.97$), and -1.8± 0.4 (mean ± 95% confidence interval and $r^2 = 0.94$), respectively (Figure 5.6 and 5.8).

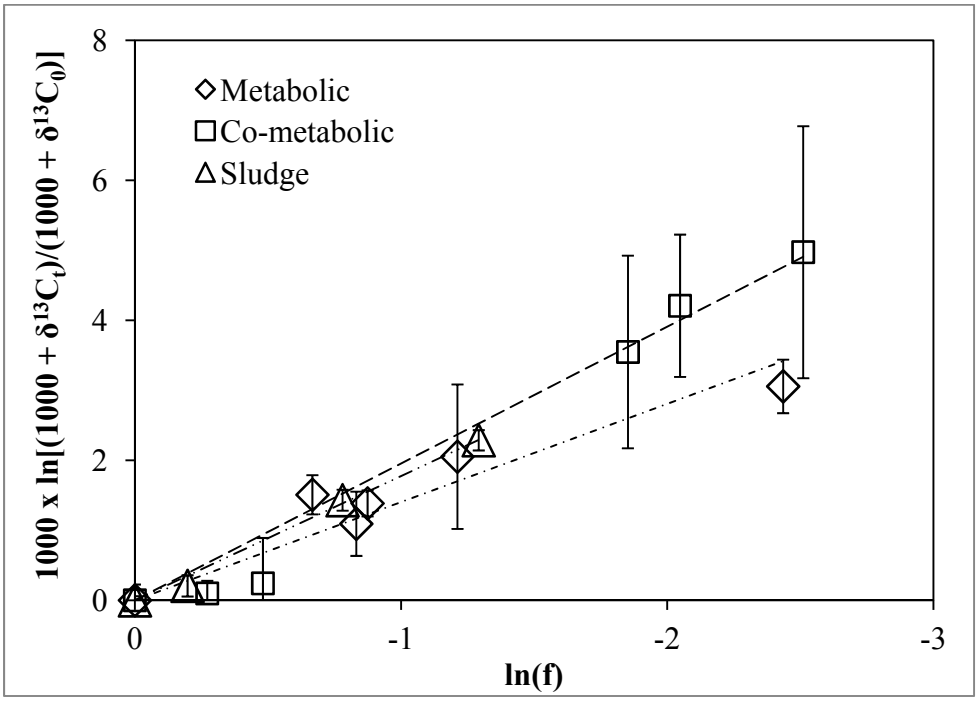

Figure 5.6. Rayleigh Model of ^{13}C-labeled 1,4-Dioxane Enrichment. Linearized Rayleigh model fit of stable carbon isotope fractionation of 1,4-dioxane during metabolic biodegradation by *P. dioxanivorans* CB1190 (diamonds), cometabolic biodegradation by *M. austroafricanum* JOB5 (squares) and biodegradation by indigenous activated sludge (triangles). The slope of each plot represents the enrichment factor (ε) of each experiment. The error bars correspond to the standard deviations.

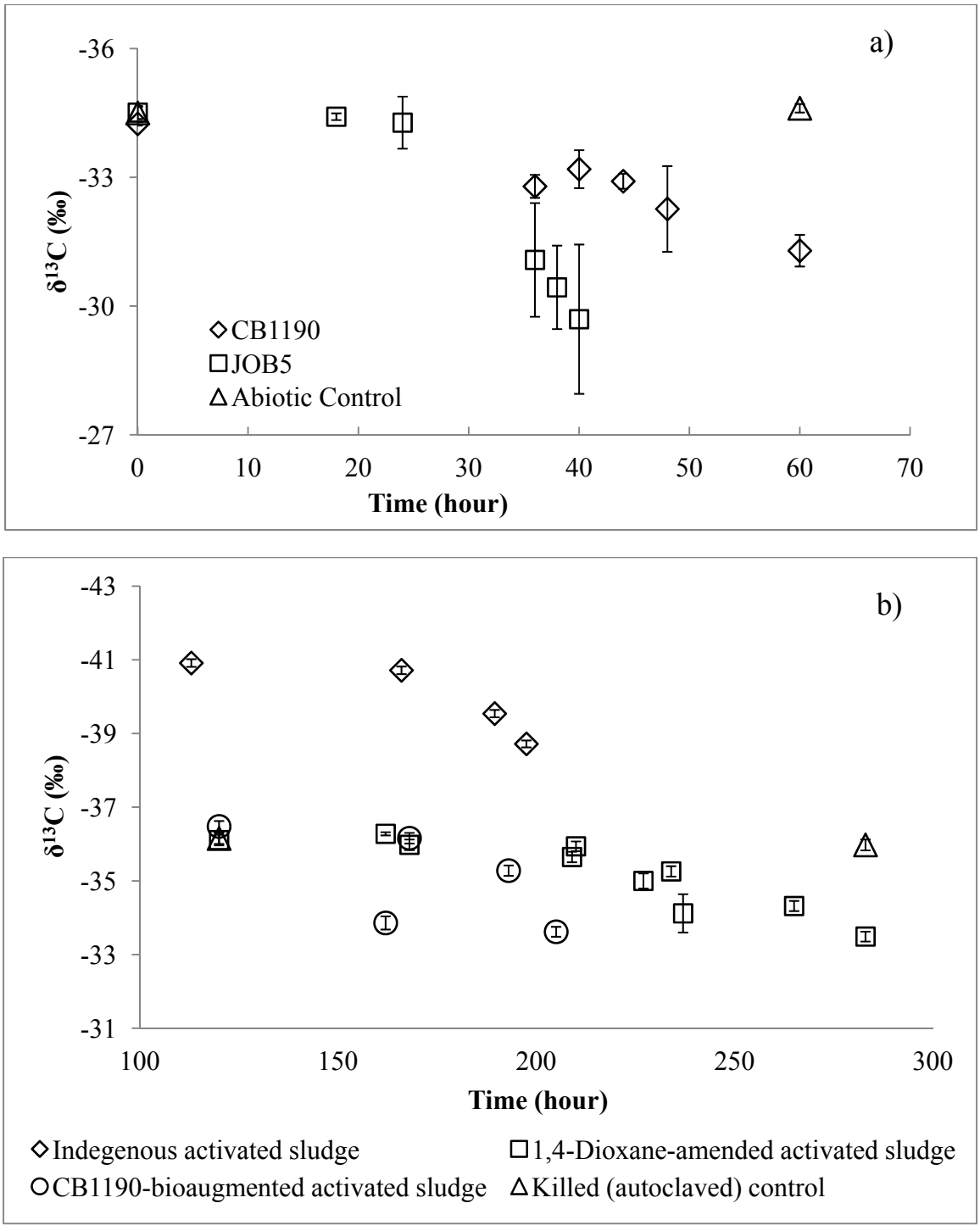

Figure 5.7. Time Course Change of Stable Carbon Isotopic Ratio during Biodegradation of 1,4-Dioxane (a) by pure cultures (b) by mixed cultures. The data points represent $\delta^{13}C$ values averaged from three biological replicates. The error bars correspond to the standard deviations. Note there was no change of $\delta^{13}C$ signatures of abiotic or killed control.

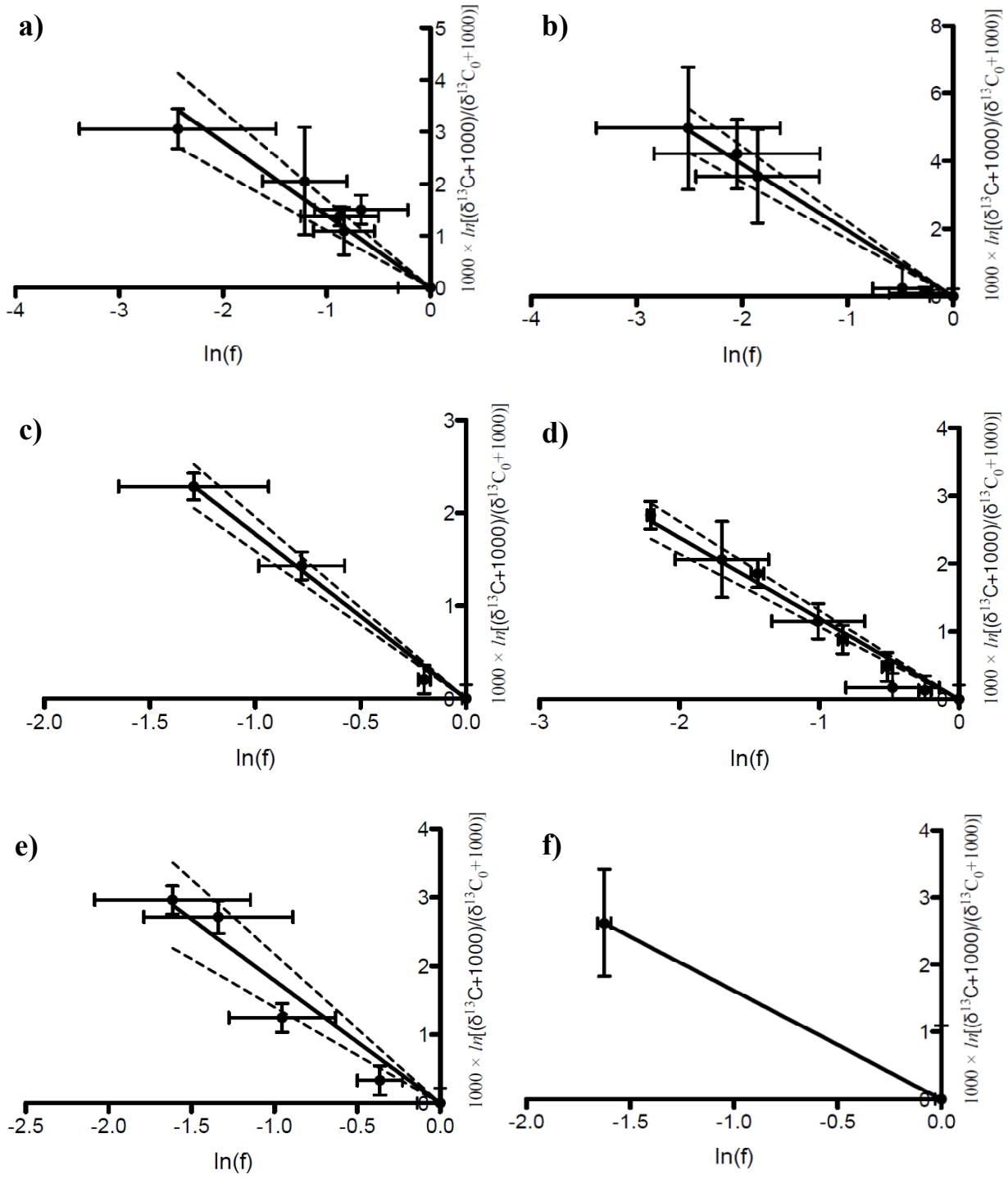

Figure 5.8. Linear Correlations Used for Determining Enrichment Factors in This Study. 1,4-Dioxane biodegradation by (a) *Pseudonocardia dioxanivorans* CB1190, (b) *Mycobacterium austroafricanum* JOB5, (c) activated sludge, (d) activated sludge amended with 1,4-dioxane, (e) activated sludge amended with 1,4-dioxane and *Pseudonocardia dioxanivorans* CB1190 and (f) activated sludge process.

5.4. Discussion. This study is the first to successfully develop a reproducible and reliable carbon stable isotope quantification method for 1,4-dioxane, and to demonstrate its application in confirming biodegradation in a wastewater treatment reactor. 1,4-Dioxane is a hydrophilic compound with low tendency to volatilize or to partition onto solid sorbents from aqueous solutions. These characteristics prohibit direct injection of aqueous samples or headspace into GC-IRMS instrumentation using methods previously used for stable isotopic analysis of various organic contaminants. Recently, a frozen micro-extraction method using methylene chloride was developed for mass spectrometric analysis of 1,4-dioxane (*227*). In our study, that extraction method was successfully applied for extracting 1,4-dioxane from aqueous phase, which allowed subsequent isotopic analysis of the compound.

The analysis of 1,4-dioxane standards at various concentrations yielded varying $\delta^{13}C$ values. Plotting standard 1,4-dioxane concentrations vs. measured $\delta^{13}C$ values revealed the inverse variation between the two variables with a correlation coefficient of 0.95 (figure 5.2a). This implies that the method has an inherent concentration to $\delta^{13}C$ bias. To minimize the bias and improve the analysis, a correction of measured $\delta^{13}C$ with a direct $\delta^{13}C$ offset obtained by analysis of an isotope reference material was applied (*235*). Using isotope reference material to remove the bias relies upon the principle of identical treatment of samples and reference materials, which can be equally affected by possible fractionation processes during chromatographic separation and chemical conversion. Unfortunately, isotope reference material for 1,4-dioxane is not currently available. A selection of an appropriate reference material is thus, required.

In this study, methyl decanoate, a low-molecular-weight C_{10} fatty acid ester with the $\delta^{13}C$ value of -29.67 ± 0.02 ‰, which was available in the laboratory, was chosen as the isotope reference material for 1,4-dioxane. Single point-anchoring using methyl decanoate was applied as the

normalization method to correct all measured $\delta^{13}C$ values. The correction with the isotope reference material resulted in disappearance of the bias due to the analytical method (Figure S3b), and improved the precision of the method (from ± 0.4 ‰ to ± 0.2 ‰ based on the standard deviation of all $\delta^{13}C$ values from isotopic analysis of the four standard concentrations: 21, 52, 103, and 196 mg L^{-1} 1,4-dioxane). The analytical results also revealed no deviation between experimental and certified values of the isotope reference material, suggesting high accuracy of the method (Table 5.1). The plot between the peak amplitude of mass 44 vs. concentration of 1,4-dioxane standard (Figure 5.2b) revealed a linear correlation of the two variables ($r^2 = 1.00$). This indicates that the developed method was applicable for quantification of the 1,4-dioxane (within the tested range of 21 – 196 mg L^{-1} 1,4-dioxane).

Traditional technique used to determine the method detection limit (MDL), in which multiplying standard deviation of the analysis of low level standards by the factor of three results the MDL of 5 mg L^{-1}, was not valid for isotope analysis. However, MDL could be determined by an approach proposed by Jochmann et al. (*236*), who suggested that appropriate MDL could be determined by consecutive calculation of the $\delta^{13}C$ average values and analyzing different concentrations of analyte at low end. The last concentration, for which $\delta^{13}C$ value was within the iterative interval and whose standard deviation was lower than ± 0.5 ‰, could be defined as the MDL for high quality $\delta^{13}C$ estimates. Analysis of 21 mg L^{-1} 1,4-dioxane standard with six replicates yielded the similar precision (± 0.2 ‰ per mil $\delta^{13}C$) and comparable $\delta^{13}C$ value as the estimates for the higher level standards. In contrast, the deterioration of precision (from ± 0.2 ‰ to ± 0.4 ‰) was observed when analyzing the replicates of 10 mg L^{-1} 1,4-dioxane standard (n = 4; Figure 5.2b). From this empirical assessment, MDL was determined as 20 mg L^{-1} 1,4-dioxane for $\delta^{13}C$ estimates.

The newly developed CSIA method was applied for establishing the $\delta^{13}C$ fingerprint of 1,4-dioxane obtained from various commercial providers. The small standard deviation from the analyses of triplicates and methyl decanoate isotope reference material (better than 1 % of the average) indicated the good reproducibility of the method. The variability in the isotopic ratios among 1,4-dioxane samples in this study likely resulted from the differences in manufacturing process and/or $\delta^{13}C$ fingerprint of raw materials used for the chemical synthesis. In other words, the producers of 1,4-dioxane may impart the distinction of ^{13}C isotopic ratio by characteristics of their production processes, raw materials, and geographic locations. Initial $\delta^{13}C$ of 1,4-dioxane from Fisher Scientific (-31.63 ± 0.20 ‰) and Acros Organics (-31.67 ± 0.07 ‰) was comparable because of the fact that 1,4-dioxanes from both providers were synthesized at the same facility in New Jersey, and thus expected to have the same $\delta^{13}C$ signature. These results reveal that the present CSIA method may be used for 1,4-dioxane source differentiation. However, variations among these commercial products, i.e., diversification in the source and isotopic composition of 1,4-dioxane, time course change in isotopic signature and dilution of stable isotope by atoms at non-reactive positions of a compound may intensify the complexity in 1,4-dioxane source differentiation at contaminated sites. This indicated that isotopic data from CSIA alone was not valid for source differentiation. Other information such as source and plume characterization data, historical data on concentration of the contaminants, and geological and hydrogeological data, etc., are required for the interpretation and evaluation (*150*).

It was found that isotopic ratio could be changed when 1,4-dioxane sources were mixed. This was found in the case of 1,4-dioxane-amended activated sludge in biodegradation experiment. The 1,4-dioxane-amended activated sludge contained two 1,4-dioxane sources, original 1,4-dioxane in sludge ($\delta^{13}C$ = -41.59 ± 0.17 ‰) and spiked Sigma Aldrich 1,4-dioxane ($\delta^{13}C$ = -

33.97 ± 0.06 ‰). Mixing original 1,4-dioxane with Sigma Aldrich 1,4-dioxane at the ratio of 1:4 yielded the isotopic fingerprint of -36.11± 0.14 ‰. Fortunately, this observation could be roughly predicted by using mixing linear model, which exhibits mass conservation of a specific isotope and can be described as;

$$\delta^{13}C_M = f_A\delta^{13}C_A + f_B\delta^{13}C_B \quad \text{(Eq. 5.3)}$$

$$1 = f_A + f_B \quad \text{(Eq. 5.4)}$$

where f_A and f_B are proportions of source stable carbon isotopic signatures ($\delta^{13}C_A$ and $\delta^{13}C_B$) and $\delta^{13}C_M$ is the carbon isotopic signatures of the mixture (*237*). Applying mixing linear model with 1,4-dioxane-amended activated sludge, the isotopic signature could be estimated as 35.49 ± 0.18 ‰, which contained a very small error (1.71± 0.63 %) from the actual value. These results revealed the usefulness of isotopic data to predict isotopic fingerprint of 1,4-dioxane mixtures, which may be useful for source identification.

This study also examined the isotopic fractionation of 1,4-dioxane during aerobic biodegradation. Small enrichments in the heavier stable isotope ^{13}C, 2.96 and 4.81 ‰, were observed during metabolic and cometabolic biodegradation, with the Rayleigh enrichment factors of -1.4 and -2.0, respectively. The r^2 values for calculated enrichment factors were correspondingly 0.88 and 0.97 for metabolic and cometabolic degradation, indicating that carbon fractionation during 1,4-dioxane biodegradation could be accurately described by the Rayleigh model. However, the fractionation patterns were statistically indistinguishable between metabolic and cometabolic processes ($p = 0.44$ by Analysis of Covariance (ANCOVA) of enrichment factors). Hence, CSIA might not be sensitive enough to conclusively differentiate the biodegradation mechanisms under aerobic conditions.

Monooxygenase enzymes catalyze 1,4-dioxane degradation (*18*). The same class of enzymes is responsible for aerobic degradation (both metabolic and co-metabolic) of petroleum hydrocarbons and chlorinated ethenes. Interestingly, small differences in isotopic fractionation were also reported in many studies using monooxygenase-expressing bacteria for degrading contaminants under aerobic conditions. For example, ^{13}C enrichment factors for aerobic biodegradation by monooxygenase reactions at the side chain and at the ring of BTEX compounds by bacteria expressing different type of oxygenases ranged from -0.4 to -3.3 (*43*). Similarly, Chu and colleagues (*33*) showed that *M. austroafricanum* JOB5 and *Methylosinus trichosporium* OB3b, which express propane- and methane-monooxygenase, respectively, enriched small extents of the heavy isotope ^{13}C in remaining substrates (ε = no enrichment to -6.7) during the cometabolic biodegradation of chlorinated ethenes under oxic conditions. This implies that the extent of ^{13}C fractionation is generally small during monooxygenase-catalyzed oxidation. 1,4-Dioxane degradation by activated sludge, activated sludge spiked with 1,4-dioxane, and activated sludge amended with both CB1190 and 1,4-dioxane was also observed in this study. Biosludge (50 mL) could degrade 62 mg L^{-1} 1,4-dioxane within nine days, which was the hydraulic retention time of the activated sludge plant. Biosludge spiked with Sigma-Aldrich 1,4-dioxane to final concentration of approximate 300 mg L^{-1} removed 1,4-dioxane to below the detection limit within twelve days, while sludge amended with both Sigma-Aldrich 1,4-dioxane and CB1190 degraded the same amount of 1,4-dioxane faster (in about ten days). The results indicate that the treatment plant efficiently removed 1,4-dioxane from wastewater, and bioaugmentation by *P. dioxanivorans* CB1190 could boost 1,4-dioxane biodegradation rate at the facility.

In laboratory microcosms set up using biosludge samples, 1,4-dioxane biodegradation enriched the remaining 1,4-dioxane in ^{13}C relative to ^{12}C from –40.92 ‰ to –38.72 ‰ yielding the enrichment factor of -1.8 ± 0.2. In contrast, biodegradation of mixed 1,4-dioxane (original 1,4-dioxane in sludge plus the spiked Sigma 1,4-dioxane at the ratio of 1 to 4) by activated sludge and CB1190-augmented sludge enriched ^{13}C from –36.11 ‰ to –34.12 ‰ and from –36.48 ‰ to -33.86 ‰, respectively. The enrichment factors of -1.2 ± 0.1 and -1.8± 0.4 were calculated for 1,4-dioxane degradation by 1,4-dioxane-amended activated sludge and CB1190-augmented sludge, respectively. These ε values are comparable indicating the same isotopic shift pattern in these experiments. This observation supports that the isotopic shift due to biodegradation should be the same regardless of the initial isotopic ratio. Neither degradation nor ^{13}C enrichment of 1,4-dioxane was observed in autoclaved sludge microcosms. Thus, it can be concluded that 1,4-dioxane removal in the plant resulted from aerobic biodegradation.

sludge system were also analyzed and compared in this study (Figure 5.9). Inflow and outflow of activated sludge process contained 1,4-dioxane at 333.74 ± 12.03 and 68.80 ± 3.19 mg 1,4-dioxane L^{-1}, respectively. It should be noted that the 1,4-dioxane was a by-product of polyester manufacturing and not associated with chlorinated solvents such as TCE and 1,1,1-TCA which can affect biodegradation of 1,4-dioxane (*88*). $\delta^{13}C$ signature of -43.02 ‰ in the influent of the activated sludge was different from those determined for laboratory grade 1,4-dioxane samples (-29.57 to -33.97 ‰). It was also shown that $\delta^{13}C$ value in the effluent (-41.59 ‰) was higher (less negative value) than that of the influent confirming that degradation of 1,4-dioxane was present in the activated sludge. The $\delta^{13}C$ value obtained for the effluent (-41.59 ‰) is in the same range of the activated sludge (–40.92 ‰) revealing that activated sludge was a complete mixed flow reactor (CMFR). The larger stable carbon isotopic fingerprint (less negative $\delta^{13}C$

value) of the activated sludge might be due to the further biodegradation of 1,4-dioxane by the concentrated mixed organisms in the sludge leading to the small extent of $^{13}C/^{12}C$ fractionation. Rayleigh enrichment factor (-1.6 ± 0.1) was estimated for 1,4-dioxane biodegradation in that activated sludge process using two data point (based on location) plot. Interestingly, sludge bioaugmented with CB1190 yielded the same order of the enrichment factor obtained from indigenous activated sludge study. This result suggests that aerobic biodegradation of 1,4-dioxane was occurring in the activated sludge plant.

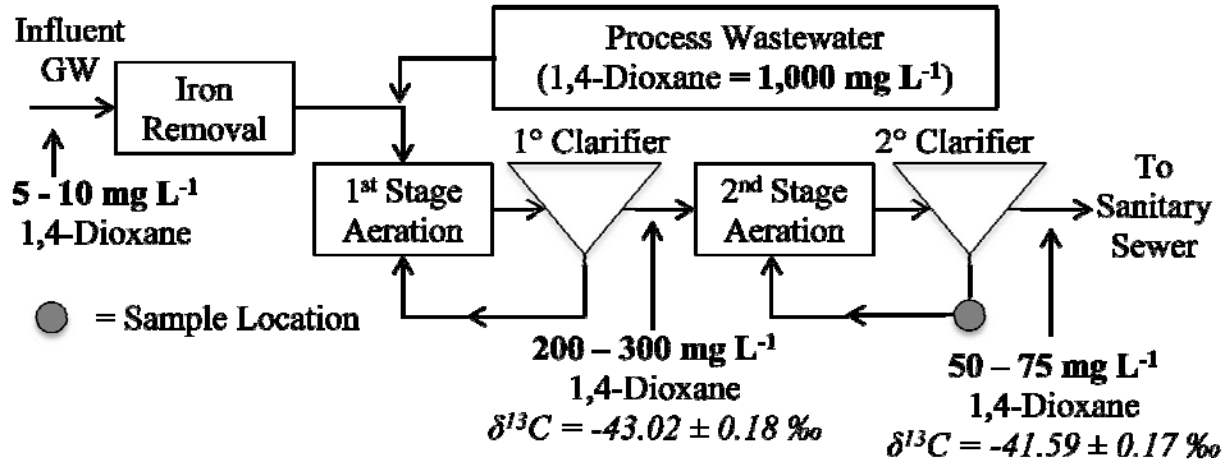

Figure 5.9. The schematic diagram of the industrial wastewater treatment plant and some characteristics of wastewater samples

The analysis of carbon isotopes in this study was performed using liquid samples (<200 µL) containing high concentration of 1,4-dioxane (~100s mg L^{-1}). Since the remediation goal for 1,4-dioxane by the United States Environmental Protection Agency is set at low concentrations (0.94 to 6.1 µg L^{-1}) for many Superfund sites (*238*), our current method will require a considerably larger sample volume and subsequent concentration. Further refinement of the sample extraction method as well as analytical method for µg L^{-1} range in aqueous samples of 1,4-dioxane for stable isotope analysis is underway.

CSIA may not yet be an effective tool to assess biodegradation in the field applications. High variation in $\delta^{13}C$ signatures from different sources of 1,4-dioxane combined with the small isotopic fractionation during biodegradation may result in the complication of field data interpretation. In ideal case that the initial isotopic ratios of the source zone are known, 1,4-dioxane should be entirely biodegraded before differentiation of $\delta^{13}C$ signature can be observed. The small isotopic fractionation may limit the use of CSIA to determine early biodegradation progress. Moreover, it would significantly impact the interpretation of field data where the source and starting composition of 1,4-dioxane would more likely be unknown or questionable. In this case, biodegradation might not be resolvable because of the high variation of $\delta^{13}C$ signature in the source zone and normal variation across a contaminated field site from non-biological effects. Despite these challenges, our method is valuable for measuring $\delta^{13}C$ signature of 1,4-dioxane in water and environmental samples, and monitoring aerobic bioremediation of 1,4-dioxane-contaminated groundwater.

CHAPTER 6

CARBON AND HYDROGEN STABLE ISOTOPE FRACTIONATION DURING BIOTIC AND ABIOTIC DEGRADTION OF 1,4-DIOXANE

6.1. Introduction. To manage the risk associated with contamination of 1,4-dioxane in water resources, it is important to understand the fate of the compound in impacted environments. To assess biotic and abiotic transformation of various organic contaminants, compound specific isotope analysis (CSIA) has been often used as a monitoring tool since it can provide an unequivocal evidence to confirm contaminant degradation (*32, 33, 139, 146-149*). During chemical and biological degradation processes of organic compounds, the bonds containing light isotopes tend to be degraded preferentially due to lower dissociation energies than those containing heavier isotopes. As a result, the change of the ratios of heavy to light isotopes can be used to predict the extent of degradation of the contaminants. CSIA provides a measurement of the isotopic ratios, thus it is valuable as a monitoring tool for characterizing degradation mechanisms as well as quantifying the contaminant degradation based on stable isotope data from field sites (*31, 150*). Moreover, CSIA can be used as a forensic tool for identifying the source of contamination based on the stable isotopic signatures of the compound.

To date, direct evidence for supporting degradation of 1,4-dioxane and validating the success of MNA and engineered bioremediation is particularly limited. This study will provide a valuable monitoring tool, which combines carbon- and hydrogen-isotope ratios to assess degradation of 1,4-dioxane. CSIA of both carbon and hydrogen isotopes were applied to evaluate and compare fractionation patterns associated with (i) biodegradation of 1,4-dioxane by pure and mixed cultures and (ii) abiotic degradation of 1,4-dioxane.

6.2. Materials and Methods

6.2.1. Chemicals. To determine the variation of stable carbon and hydrogen isotopic composition among major providers, pure samples of commercial 1,4-dioxane ACS grade were

obtained from Fisher Chemical (Lot # 102732; Fair Lawn, NJ), Alfa Aesar (Lot # G08W024; Ward Hill, MA), Sigma Aldrich (Lot # 59096MK; Saint Louis, MO), Acros Organics (Lot # B0518954; Fair Lawn, NJ) and Mallinckrodt (Lot # E15H23; Phillipsburg, NJ). The other chemicals used in this study were purchased from Sigma Aldrich.

6.2.2. Biodegradation of 1,4-Dioxane. Biodegradation of 1,4-dioxane by *P. dioxanivorans* CB1190 and activated sludge samples were conducted in 1,000 mL glass media bottles with screw caps (Fisher Scientific, Fair Lawn, NJ). *P. dioxivorans* CB1190 was grown in ammonium mineral salt medium (AMS) (*20*) with 100 mg L^{-1} 1,4-dioxane (Sigma-Aldrich, Saint Louis, MO) as the growth-supporting substrate. To sustain the growth kinetics and enzyme activity, stocks of bacterial pure cultures were maintained for at least five transfers. Inocula of 1% v/v were used with pure culture experiments. *P. dioxanivorans* CB1190 was pre-cultured to obtain biomass of 55 mg protein L^{-1}. Initial Sigma-Aldrich 1,4-dioxane concentration of 1,000 mg L^{-1} was supplied to the culture. The activated sludge sample, which was found to biodegrade 1,4-dioxane in our early work, was used for validating biodegradation and stable isotope enrichment in this study. The sample was collected in 3.8 L semirigid polyethylene containers with screw cap closures (Hedwin Cubitainer®; Thermo Fisher Scientific Inc., Wilmington, DE), then placed on ice and shipped on the same day to the laboratory. In biodegradation experiments using the activated sludge, Sigma-Aldrich 1,4-dioxane was also spiked to the final concentration of 1,000 mg L^{-1}. To prevent oxygen mass transfer deprivations, volume of pure and mixed cultures was always less than 20% of the bottle capacity (i.e., 200 mL in 1,000 mL bottle). Cultures were maintained at 150 rpm agitation and 30 ˚C, and aseptic techniques were used to prevent microbial contamination of the cultures. The concentrations of 1,4-dioxane in pure and mixed cultures were monitored over time by gas chromatography. Water samples (2 mL) were

collected and filter-sterilized by a sterile 0.20 μm nylon filter unit (Fisher Scientific, Fair Lawn, NJ) at different time periods. Samples were stored at -20 ˚C for later liquid-liquid extraction and isotopic analyses. Cellular biomass was quantified over time in the cultures. All experiments were conducted in duplicate. Abiotic controls (n = 2) and autoclaved (killed) control were used for pure and mixed culture experiments, respectively.

6.2.3. Sonolysis of 1,4-Dioxane. Sonolysis of 1,4-dioxane was conducted in a 100 mL reactor using a pen-type sonic dismembrator (model FB120) with 1/8" probe (Fisher Scientific, Hampton, NH). The system was operated at 100% amplitude (180 μm) with fixed frequency of 20 kHz and input power of 19 W. The reactor was temperature-controlled at 25˚C by water circulating in a water bath. 1,4-Dioxane diluted with sterile distilled water to the concentration of 1,000 mg L^{-1} was used for all experiments with the initial volume of 50 mL. The solution with 1,4-dioxane was sonicated with and without aeration by filter-sterilized laboratory air at the rate of 2 L min^{-1}, which was monitored by a gas flow meter. In attempt to correct the effect 1,4-dioxane removal by aeration, a separate experiment with aeration was performed. Samples were periodically collected and quantified for 1,4-dioxane by gas chromatography. At the same time, samples (2 mL) were also collected, filtered-sterilized, and stored at -20 ˚C for subsequent extraction and isotopic analyses. All experiments in this study were conducted in duplicate. Abiotic controls (n=2) were also used in this study.

6.2.4. Analyte Concentrations. Concentrations of 1,4-dioxane were analyzed by a Hewlett-Packard 6890 Chromatograph (GC) equipped with a Flame Ionization Detector (FID) (Hewlett-Packard, Atlanta, GA) and a Restek® Stabilwax-DB capillary column (30 m length x 0.53 mm id x 1 μm film thickness; Restek, Bellefonte, PA). Concentrations of 1,4-dioxane were measured by manually injecting 2 μL liquid samples, which were filtered from liquid culture samples by

0.45 μm syringe filters, into the GC-FID. The injector was set at 220 °C in splitless mode, while the detector was set at 250 °C. The oven program was set at 80 °C (3 min) then ramped at 20 °C min^{-1} to 140 °C (1 min). The retention time for 1,4-dioxane was 3.7 minutes. Calibration curves were prepared by diluting pure 1,4-dioxane in deionized water to various concentrations that covered the range of the experiments. The detection limit was 0.8 mg L^{-1} and the accuracy of 1,4-dioxane measurement was better than ± 4% with this method.

6.2.5. Carbon and Hydrogen isotopes of 1,4-Dioxane. 1,4-Dioxane dissolved in water was extracted with dichloromethane at a ~7:1 analyte to extractant ratio. Carbon and hydrogen isotope ratios of 1,4-dioxane were determined separately using a gas chromatograph and an isotope ratio mass spectrometer interfaced with a combustion reactor (GC-C-IRMS) and a pyrolysis reactor (GC-P-IRMS, Thermo Fisher Scientific, Bremen, Germany). 1,4-Dioxane was separated chromatographically on an HP-5 fused silica capillary column (30 m x 0.25 mm, and 10 μm). For carbon isotopes, after GC separation, the 1,4-dioxane was combusted to CO_2 at 1000 °C in a capillary ceramic tube loaded with Ni, Cu, and Pt wires, water was removed, and the carbon isotope ratio was measured in the IRMS. For hydrogen isotopes, after GC separation, 1,4-dioxane was pyrolyzed in an empty ceramic tube at 1450 °C and the hydrogen isotope ratios were acquired in the IRMS. Carbon and hydrogen isotope ratios are reported in the conventional δ-notation relative to VPDB and VSMOW scales. Absolute calibration of the $\delta^{13}C$ in CO_2 produced from the combustion of 1,4 dioxane and that of the $\delta^{2}H$ from pyrolysis were calibrated relative to C_{16} to C_{30} alkane mixture A3 (Indiana University, USA). The analytical precision was estimated by analyzing different quantities of 1,4-dioxane diluted in hexane and extracted from water with methylene chloride. Repeated injection of 1,4-dioxane diluted in hexane yield values of -32.0 ± 0.4 ‰ (1σ; n=7) for $\delta^{13}C$ and -66.6 ± 6.0 ‰ (1σ; n=7) for $\delta^{2}H$ for standard sizes

containing between 380 and 2277 ng of carbon and 255 and 1915 ng of hydrogen. 1,4-Dioxane standards extracted from water. Repeated methylene chloride extraction experiment of 1,4-dioxane from water yielded $\delta^{13}C$ of -34.04 ± 0.19 ‰ (1 s; n = 4) and $\delta^{2}H$ of -34.6 ± 2.4‰ (1 s; n = 12) for concentrations ranging from 470 to 1175 mg L^{-1}.

6.2.6. Biomass Quantification. Biomass in aqueous samples was quantified as total protein concentration by using a modified Bradford method (*19, 229*). Absorbance measurement was conducted in cuvettes using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

6.3. Results and Discussion.

6.3.1. Hydrogen Isotopic Analysis of 1,4-Dioxane. Measured isotopic ratios for a 1,4-dioxane/hexane binary mixture were compared to 1,4-dioxane extracted from water with methylene chloride. A fractionation of -30.4 ‰ ± 4.6 ‰ (n = 8) was observed for 1,4-dioxane extracted in methylene chloride. This fractionation is compatible with hydrogen isotopic exchange between the methylene chloride extractant and 1,4-dioxane. This interpretation is supported by the fact that there was no variation in $\delta^{13}C$ between 1,4-dioxane extracted with methylene chloride and 1,4-dioxane/hexane binary mixtures.

6.3.2. Stable Hydrogen and Carbon Isotopic Signature of Commercial 1,4-Dioxanes. Hydrogen and carbon isotopic compositions of commercial 1,4-dioxane were found to vary among the different providers. The highest $\delta^{2}H$ was measured for Sigma-Aldrich 1,4-dioxane (-59.6 ± 4.4 ‰, n=5), while Fisher Scientific 1,4-dioxane had the lowest $\delta^{2}H$ value (-68.2 ± 2.4; n=3). Measured $\delta^{13}C$ values ranged from -35.96 ± 0.65 ‰ for Sigma-Aldrich 1,4-dioxane to -31.98 ± 0.40 ‰ for Macllinckrodt 1,4-dioxane (Table 6.1). The differences between $\delta^{2}H$ and

$\delta^{13}C$ of manufactured 1,4-dioxane might result from the differences in isotopic composition of materials used in the manufacturing process of 1,4-dioxane or/and due to different methods of production.

Table 6.1. Stable Carbon and Hydrogen Isotopic Signature of Various Commercial 1,4-Dioxane. The values are expressed as means of n-replicate measurements along with standard deviation (technical error).

Sample	δ^2H_{SMOW} (‰)		$\delta^{13}C_{PDB}$ (‰)	
	Mean ± 1 σ	n	Mean ± 1 σ	n
Acros Organics	-66.4 ± 2.4	4	-34.20 ± 0.32	7
Alfa Aesar	-64.3 ± 0.3	3	-34.80 ± 0.52	2
Fisher Scientific	-68.2 ± 2.4	3	-32.70 ± 0.32	2
Mallinckrodt	-66.6 ± 6.0	7	-31.98 ± 0.40	3
Sigma-Aldrich	-59.6 ± 4.4	5	-35.96 ± 0.65	3

6.3.3. Stable Isotope Fractionation by Pure Cultures. *Pseudonocardia dioxanivorans* CB1190 is among representative strains of six genera of bacteria that was found to biodegrade 1,4-dioxane (*18*). This bacterium is potentially capable of using 1,4-dioxane as a sole carbon and energy source for its growth (*17, 18*). The mineralization of 1,4-dioxane in the pathway whose first step is catalyzed by monooxygenase enzyme involves the addition of oxygen to the molecule of the compound, leading to linearization of the cyclic ether structure (*16*). Biochemical reactions in the 1,4-dioxane biodegradation pathway (*16, 81*) may enrich in heavy hydrogen and carbon isotopes the remaining 1,4-dioxane. Carbon and hydrogen isotope analyses allow monitoring of the changes of the isotopic ratios of 1,4-dioxane. In this study, aerobic biodegradation of 1,4-dioxane by *P. dioxanivorans* CB1190 at the rate of 0.25 ± 0.07 mg day^{-1}

mg protein^{-1} (Figure 6.1) resulted in the enrichment of ^{2}H by 280.8 ‰ (from -22.8 ‰ to 258.0 ‰) and ^{13}C by 2.29 ‰ (from -36.82 ‰ to -34.53 ‰) when over 96 % of 1,4-dioxane was degraded (Figure 6.2a), while no changes in $\delta^{13}C$ and $\delta^{2}H$ were observed in abiotic controls. Enrichment factors of -1.38 ± 0.56 (mean ± 95% confidence interval) for carbon and -45.1 ± 14.2 for hydrogen were computed for 1,4-dioxane biodegradation by *P. dioxanivorans* CB1190 (Figure 6.2b). The magnitude of carbon enrichment factor established for *P. dioxanivorans* CB1190 in this study was similar to that established in the study in Chapter 5 using the same microorganism. It was shown that r^2 values for calculated ϵ_C and ϵ_H were high (> 0.80 for *P. dioxanivorans* CB1190), indicating that carbon and hydrogen fractionation during 1,4-dioxane biodegradation could be accurately computed by the Rayleigh model. It is also likely that hydrogen-CSIA is more sensitive than carbon-CSIA.

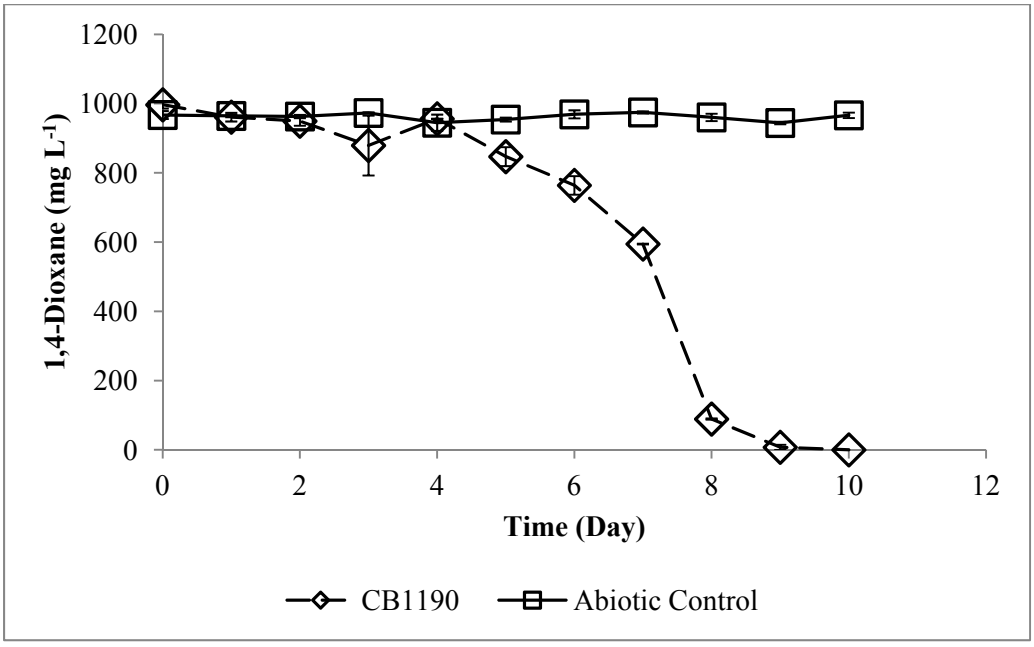

Figure 6.1. Biodegradation of 1,4-Dioxane by Bacterial Pure Culture *Pseudonocardia dioxanivorans* CB1190. 1,4-Dioxane (1000 mg L^{-1}) was removed to below detection limit within nine days of the experiment by the bacterium. The error bars in both plots correspond to the range of analytical values.

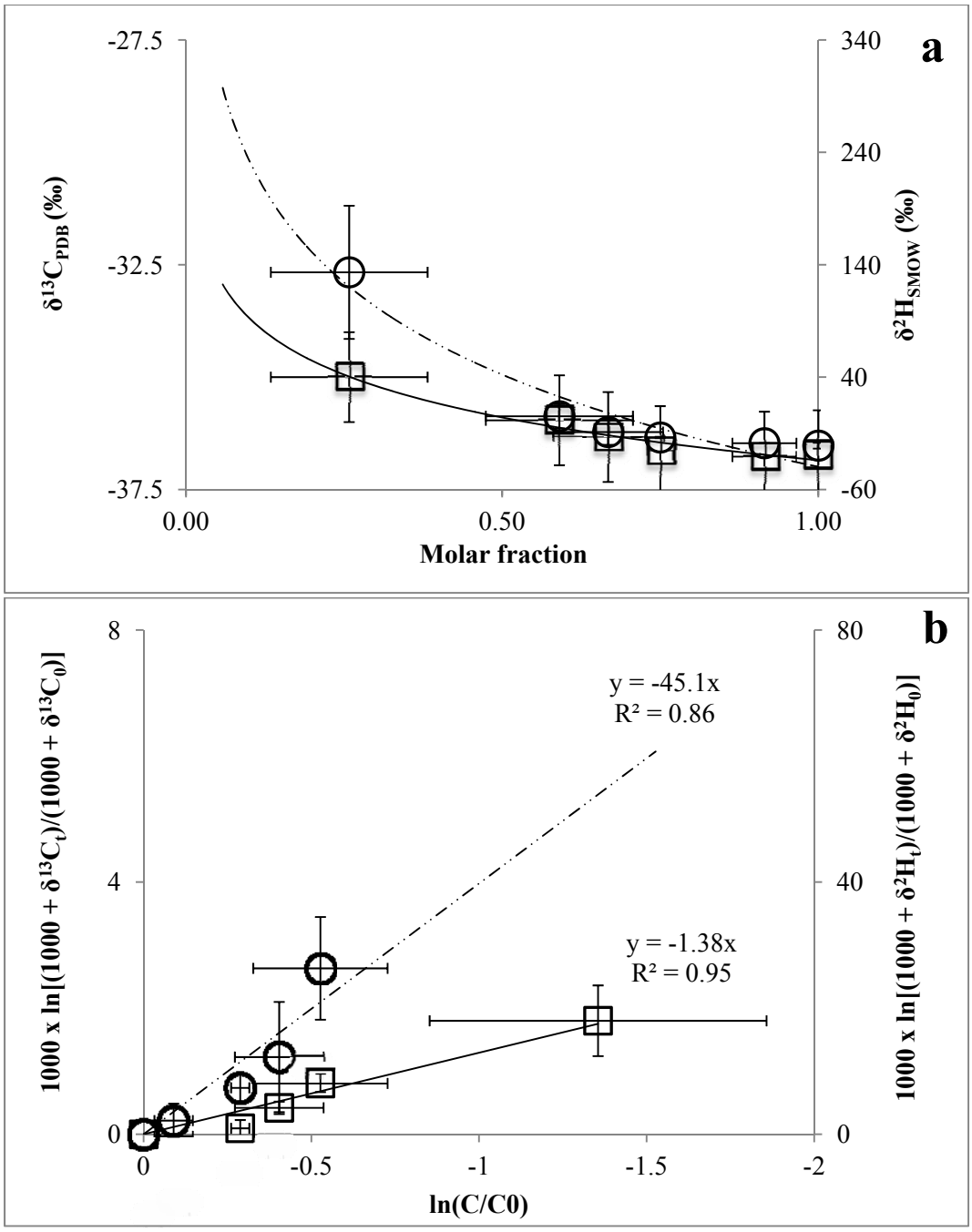

Figure 6.2. Stable Hydrogen and Carbon Isotopic Fractionation During Biodegradation of 1,4-Dioxane by Pure Culture *Pseudonocardia dioxanivorans* CB1190 (a). Biodegradation experiments revealed the fractionation of stable hydrogen and carbon isotope in the remaining 1,4-dioxane. Linearized Rayleigh Model of ^{2}H- and ^{13}C-labeled 1,4-Dioxane Enrichment Established for Biodegradation by *P. dioxanivorans* CB1190 (b). The fractionation of stable hydrogen and carbon isotope by biodegradation could be described by Rayleigh model. Squares and circles represent data from carbon-CSIA and hydrogen-CSIA, respectively. The data points represent averaged values from biological duplicates. The error bars in both plots correspond to the range of analytical values.

High hydrogen fractionation, but very low carbon fractionation pattern established for 1,4-dioxane biodegradation by *P. dioxanivorans* CB1190 in this study is similar to the pattern established for methyl t*ert*-butyl ether (MTBE) biodegradation by *Pseudonocardia tetrahydrofuranoxydans* K1 and *Pseudonocardia* sp. ENV478. Note that *P. dioxanivorans* CB1190, *P. tetrahydrofuranoxydans* K1, and *Psedonocardia* sp. ENV478 were reported to degrade 1,4-dioxane and other organic contaminants by using a very conserved four-component tetrahydrofuran/dioxane monooxygenase as a catalyst of the first step of the reaction (*80, 83, 84*). It is plausible that the monooxygenase enzyme attacks these contaminants using the same mechanism resulting in the similar isotopic fractionation patterns. It was proposed by Elsner et al. (*239*) that intrinsic stable isotope fractionation during aerobic biodegradation by monooxygenase-expressing bacteria is often masked by additional non-fractionating steps, not the rate limiting step where enzyme binds the substrates. This phenomenon may result in the cleavage of C-H bond of organic chemicals, including 1,4-dioxane and MTBE, during oxidation of these compounds.

6.3.4. Stable Isotope Fractionation during Abiotic Degradation Processes. Hydroxyl radicals and ultrasonic waves produced from sonication and aeration can enhance 1,4-dioxane degradation (*11-13*). Beckett and Hua (*12*) demonstrated the use of sonolysis at discrete ultrasonic frequencies to destruct 1,4-dioxane. It was found that during sonolysis at the frequency of 358 kHz, 96% of initial 1,4-dioxane was removed in the first 120 min. In this study, aeration and sonolytical degradation of 1,4-dioxane were investigated. Aeration alone could remove 1,4-dioxane at the rate of 4.15 mg L^{-1} min^{-1}, while sonolytical degradation without aeration remove 1,4-dioxane at much lower rate (0.93 mg L^{-1} min^{-1}) (Figure 6.3). Sonolytical degradation could be enhanced by aeration resulting in higher removal rate of 1,4-dioxane (5.75

mg L^{-1} min^{-1}) (Figure 6.3). Stable carbon and hydrogen isotope fractionations during 1,4-dioxane removal by sonolysis with aeration and aeration alone were observed. It was found that neither sonolysis with aeration nor aeration changed $^{13}C/^{12}C$ ratio, but enriched the remaining 1,4-dioxane in ^{2}H. Sonolytic degradation with aeration could fractionate hydrogen by 62.9 ‰ (from -34.8 ‰ to 28.1 ‰) after 67 % of 1,4-dioxane removed (Figure 6.4a), but aeration at the rate of two liter of air per minute could increased $\delta^{2}H$ values by 56.1 ‰ (from -32.7 ‰ to 23.4 ‰) after 51 % of 1,4-dioxane eliminated (Figure 6.4b). The enrichment factors of -55.1 ± 15.1 and -28.6 ± -32.4 were established for aerated sonolytical degradation and aeration, respectively (Figure 6.4c and 6.4d, respectively). The high r^2 values for calculated ϵ_H values (0.95 for sonolysis and 0.75 for aeration) indicates that stable hydrogen fractionation of 1,4-dioxane by these abiotic processes can be also described by the Rayleigh model.

Beckett and Hua (*11*) proposed a reaction scheme for sonolytical degradation of 1,4-dioxane. Sonolytic reaction in the presence of oxygen can generate reactive oxygen species, e.g., hydroxyl radical (•OH) and hydroperoxyradical (•HOO**)**, etc. These radicals are reactive and can attack molecules of 1,4-dioxane. The reaction between 1,4-dioxane and the radicals starts with an abstraction of hydrogen at any carbons of the compound (*11*). The cleavage of C-H bond may involve with the enrichment of ^{2}H in the remaining molecules of 1,4-dioxane. This is in agreement with other studies revealing that hydrogen fractionation of organic compounds is common for chemical oxidation processes (*239, 240*). Interestingly, sonolysis and aeration could not enrich ^{13}C suggesting that carbon isotope fractionation effects are not related to degradation rates of 1,4-dioxane by these mechanisms. It may be concluded that carbon isotopic analysis of 1,4-dioxane alone will not be sufficient for assessing abiotic degradation by oxidation processes.

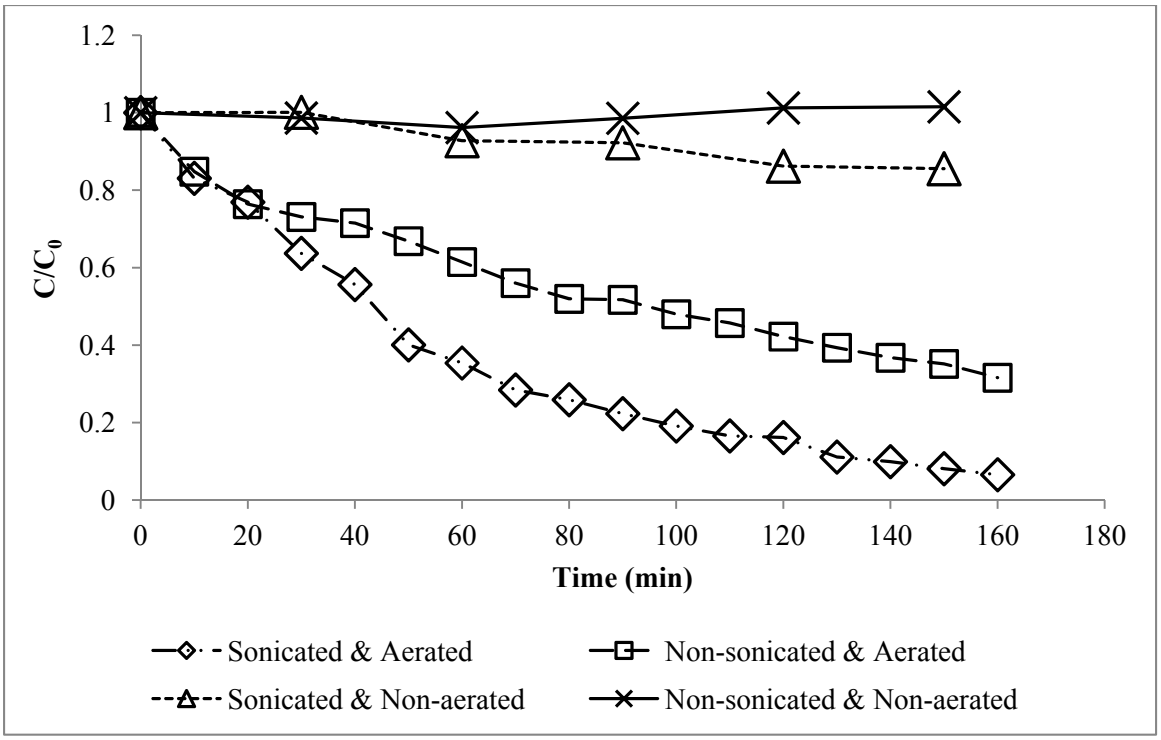

Figure 6.3. Removal of 1,4-Dioxane during Abiotic Processes. Combined sonolysis with aeration could effectively remove 1,4-dioxane. C/C_0 in y-axis represents remaining fraction of 1,4-dioxane in the reactors.

6.3.5. Stable Isotope Fractionation in Activated Sludge. 1,4-Dioxane in the activated sludge sample was a by-product of polyester manufacturing and not associated with chlorinated solvents, such as trichloroethylene and 1,1,1-trichloroethane, or heavy metals, which can affect biodegradation of 1,4-dioxane (*88, 222*). 1,4-Dioxane biodegradation by activated sludge, which removed 94% of spiked 1,4-dioxane within 40 days of experiment (Figure 6.5), exhibited fractionation of both carbon and hydrogen. The observed fractionation of hydrogen and carbon in undegraded 1,4-dioxane was 33.6 ‰ (from -24.4 ‰ to 9.2 ‰) after and 1.06 ‰ (from -36.63 ‰ to -36.10 ‰), respectively (Figure 6.6a). The fractionation was associated with the carbon and hydrogen enrichment factor of -1.06 ± 0.49 and -51.7 ± 30.2 (mean ± 95% confidence interval), respectively (Figure 6.6b). Both ϵ_C and ϵ_H established for biodegradation by the

activated sludge were within the range of error of those determined for 1,4-dioxane biodegradation by *P. dioxanivorans* CB1190.

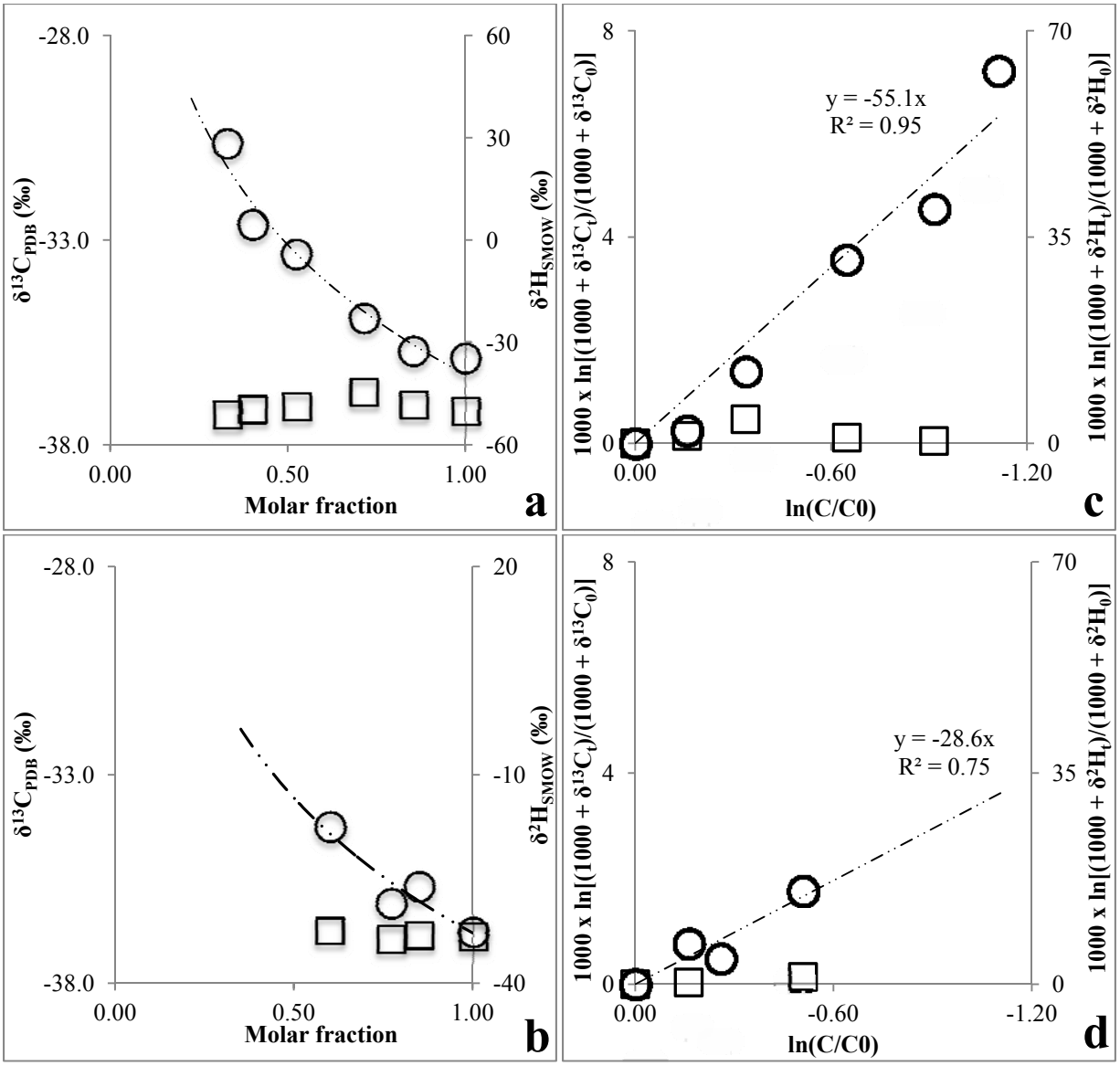

Figure 6.4. Stable Hydrogen Isotopic Fractionation During Abiotic Degradation of 1,4-Dioxane by Sonolysis (a) and Non-Destructive Removal of 1,4-Dioxane by Aeration (b). Both abiotic processes could fractionate stable hydrogen isotope, but could not fractionate stable carbon isotope. Rayleigh Model of ^{2}H-labeled 1,4-Dioxane Enrichment Established for Sonolytical Degradation of 1,4-Dioxane (c) and Removal of 1,4-Dioxane by Aeration (d). The fractionation of stable hydrogen isotope by these abiotic processes could be described by Rayleigh model. Squares and circles represent data from Carbon-CSIA and Hydrogen-CSIA, respectively.

Changes in carbon and hydrogen isotope ratio for biodegradation of 1,4-dioxane by pure and mixed culture, and abiotic removal of 1,4-dioxane were demonstrated (Figure 6.7). It should be noted that isotopic pattern during degradation/removal of 1,4-dioxane by *P. dioxanivorans* CB1190, sonolysis, and aeration could be exhibited by a linear regression curves with r^2 values of 0.87-0.98. Isotopic pattern discovered for activated sludge had similar trend as that established from 1,4-dioxane biodegradation by *P. dioxanivorans* CB1190. This comparison confirmed that removal of 1,4-dioxane in activated sludge experiment was due to aerobic biodegradation.

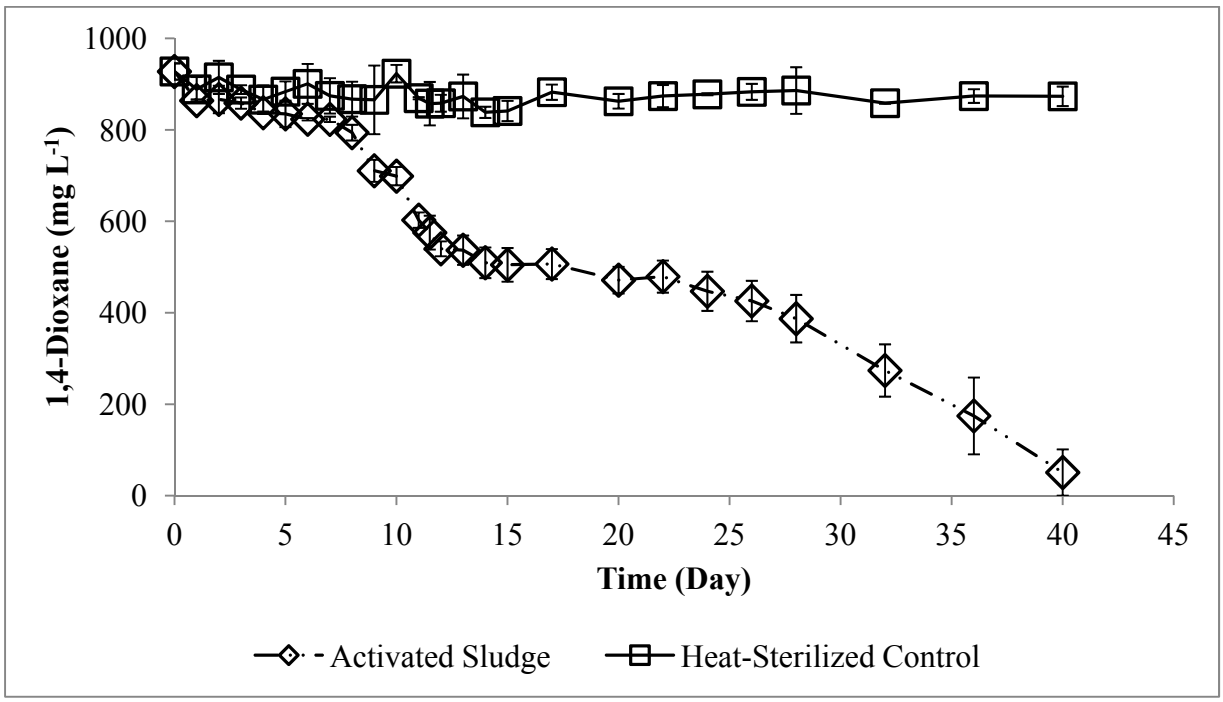

Figure 6.5. Biodegradation of 1,4-Dioxane by Bacterial Mixed Culture in an Industrial Activated Sludge Samples Actively Biodegrading 1,4-Dioxane. 94% of spiked 1,4-dioxane was removed by the bacterial consortium within 40 days of the experiment, while no biodegradation of 1,4-dioxane was observed in heat-sterilized activated sludge. The error bars in both plots correspond to the range of analytical values.

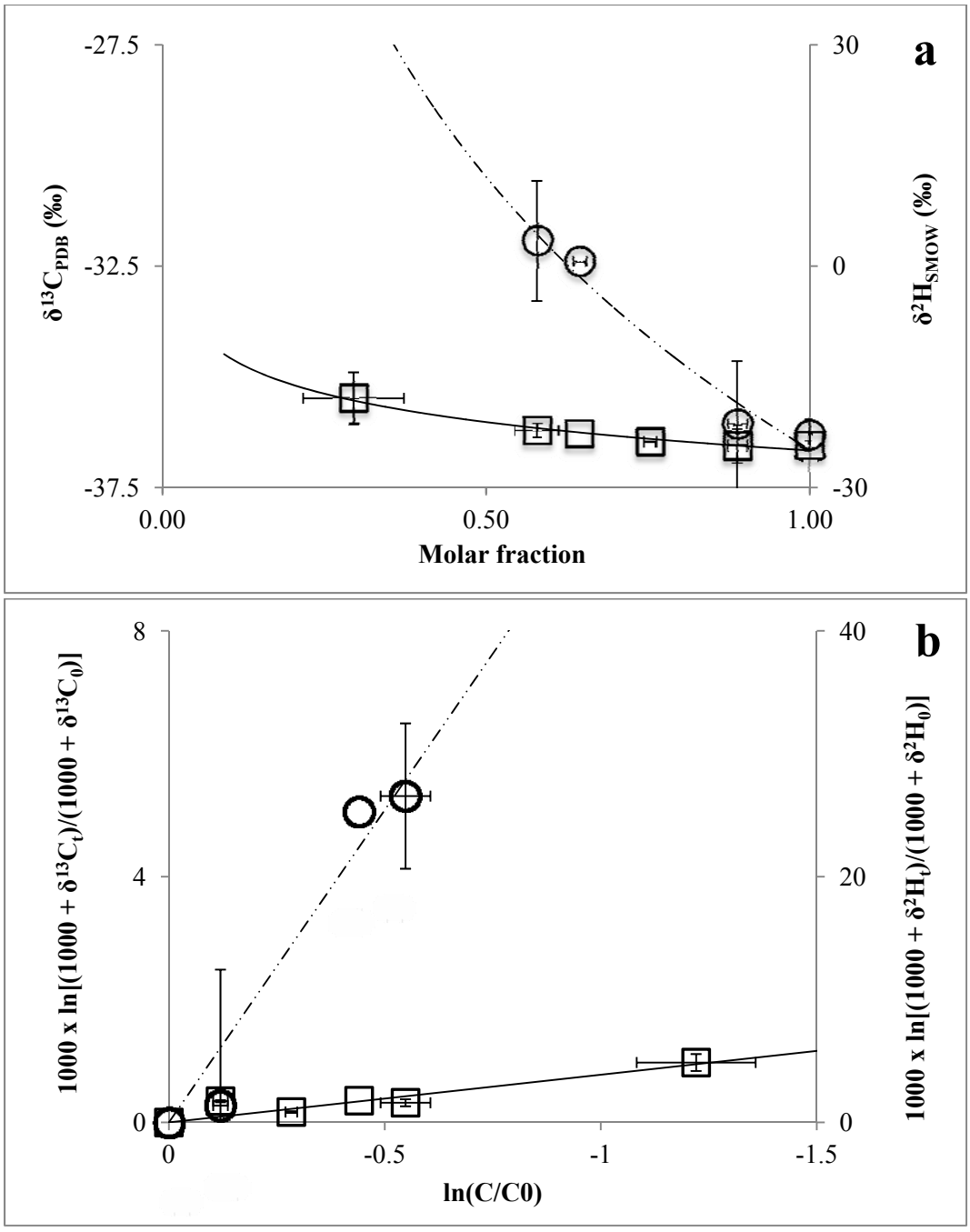

Figure 6.6. Stable Hydrogen and Carbon Isotopic Fractionation During Biodegradation of 1,4-Dioxane by Mixed Consortium in Industrial Activated Sludge Samples (a). Biodegradation experiments revealed the fractionation of stable hydrogen and carbon isotope in the remaining 1,4-dioxane. Linearized Rayleigh Model of ^{2}H- and ^{13}C-labeled 1,4-Dioxane Enrichment Established for Biodegradation by the activated sludge (b). The fractionation of stable hydrogen and carbon isotope by biodegradation could be described by Rayleigh model. Squares and circles represent data from carbon-CSIA and hydrogen-CSIA, respectively. The data points represent averaged values from biological duplicates. The error bars in both plots correspond to the range of analytical values.

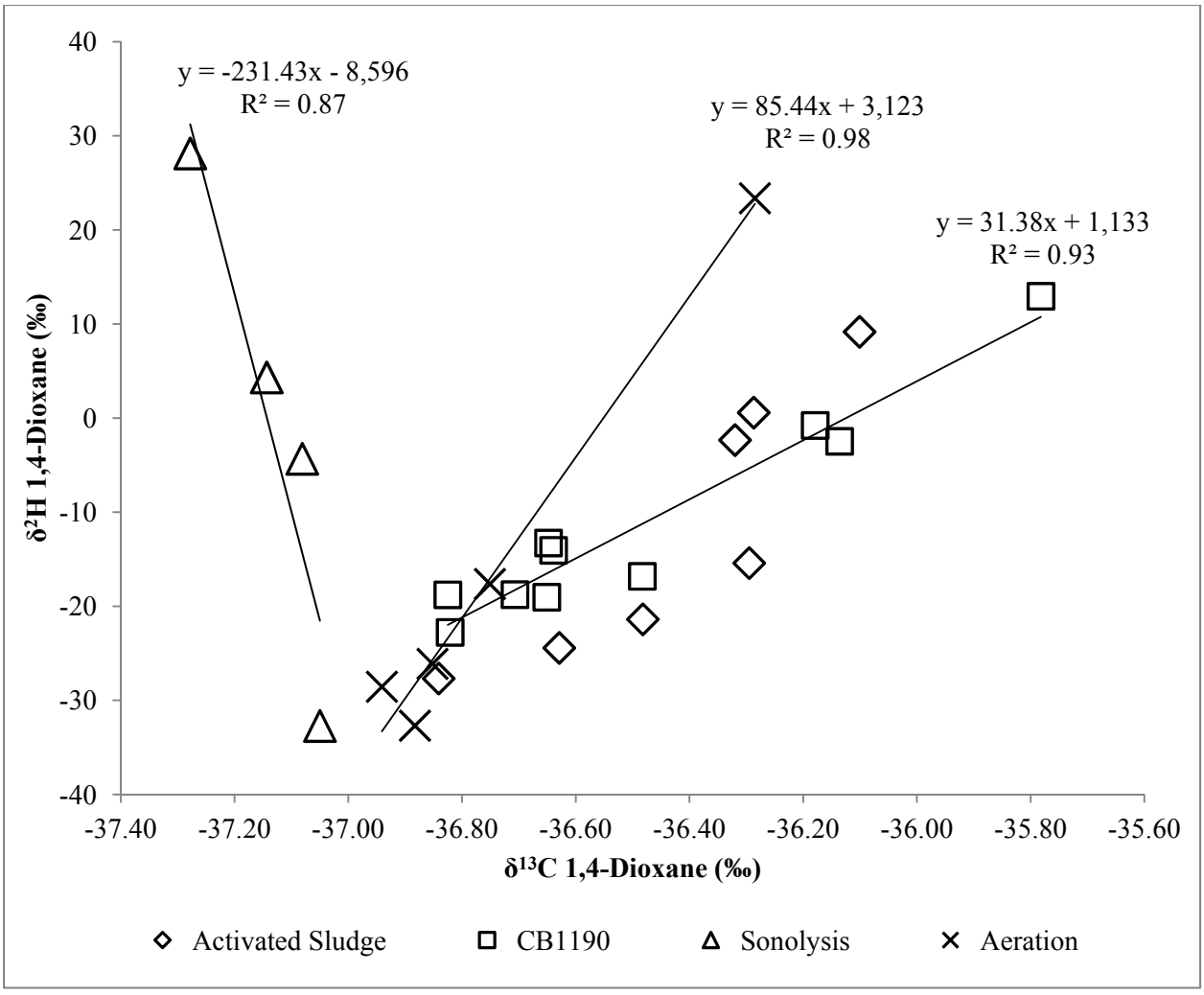

Figure 6.7. Concurrent analysis of stable carbon and hydrogen isotopic signature in the activated sludge sample to relate natural degradation of 1,4-dioxane to biodegradation under aerobic condition.

6.3.6. Environmental Significance and Implications. This study reports the development of a hydrogen-CSIA method, and demonstrates applications of CSIA methods with respect to 1,4-dioxane degradation. CSIA methods were successfully applied to determine isotopic fingerprints of 1,4-dioxane for both carbon and hydrogen. The analyses of isotopic signatures allow the use of CSIA methods as tools to monitor isotopic fractionation during biotic and abiotic transformation of 1,4-dioxane. While no enrichment of ^{13}C in the remaining 1,4-dioxane was

observed during sonolysis and/or aeration, a large enrichment of only stable hydrogen isotopes was observed. In contrast, biodegradation enriched both ^{13}C and ^{2}H in 1,4-dioxane. Based on these results, C-CSIA may be valuable for distinguishing biotic process from abiotic mechanisms. However, the low carbon fractionation in biodegradation and high variation in stable carbon isotopic signatures of 1,4-dioxane might cause the complication of the field data interpretation. It was also found in this study that H-CSIA could not distinguish 1,4-dioxane degradation/removal mechanisms as the enrichment factors established for the investigated processes were in the same order of magnitude. Although large isotopic shifts associated with hydrogen fractionation during aeration are possible for the site with air sparging in unsaturated zone, large isotopic shifts are not likely to be important in most natural systems where mass loss of the compound by aeration is atypical (*241*).

Several laboratory and field studies demonstrated that concurrent compound specific isotope analysis for two elements (two-dimensional stable isotope analysis or 2D-CSIA) in organic compounds can be used for distinguishing degradation mechanisms of various organic contaminants (*36, 38, 41, 44, 242-245*). Combined analyses of carbon isotopes with hydrogen isotopes, which are usually sensitive than carbon isotope analysis, may be the best approach to assess biodegradation especially for organic compounds with small enrichment factors for carbon, including the aromatic hydrocarbons (*39, 245, 246*). Recent development of CSIA methods for 1,4-dioxane allows the use of 2D-CSIA as a sensitive monitoring tool to confirm degradation mechanisms of 1,4-dioxane. In addition, 2D-CSIA can be used in conjunction with molecular biological tools for determining abundance and expression of functional gene biomarkers (*84*) as well as stable isotope probing technologies (*139*) to confirm biodegradation of 1,4-dioxane at contaminated sites.

CHAPTER 7

CONCLUSION AND FUTURE WORK

7.1 Summary and conclusions. Intrinsic and enhanced bioremediation are potential strategies for treatment of organic contaminants in contaminated water resources. Molecular biological and isotopic tools are very important in demonstrating successful bioremediation of organic and inorganic contaminants in various natural and engineered environments. This study demonstrated the development and applications of tools in laboratory pure cultures as well as environmental samples to validate biodegradation of 1,4-dioxane, a probable human carcinogen, as a model water contaminant.

While temporal and spatial monitoring of 1,4-dioxane concentrations helps characterize the nature and extent of contamination, quantification of genetic biomarkers helps determine the population and activity of indigenous microbes in groundwater or wastewater to evaluate the biodegradation potential of 1,4-dioxane. Biodegradation of 1,4-dioxane is catalyzed by monooxygenase enzymes expressed in various actinomycete bacteria (*18, 83*). While these metalloenzymes require trace metals as co-factors in their catalytic sites (*163, 164*), these metals may be toxic at elevated concentrations (*165*). In this study, it was shown that monooxygenase-catalyzed biodegradation rates were subject to interactions among transition metals and natural organic ligands in bacterial culture *P. dioxanivorans* CB1190. The effects of transition metals and organic ligands on bacterial population as well as biodegradation of 1,4-dioxane were successfully confirmed by quantification of a phylogenetic biomarker targeting the 16S rRNA gene and a functional gene biomarker targeting DXMO. These results revealed the usefulness of these biomarkers as indicators for 1,4-dioxane biodegradation potential in pure cultures.

Microcosm studies are valuable for evaluating individual treatment parameters at the bench scale, and this informs selection and optimization of biostimulation and bioaugmentation installations in complex environments. In the present study, microcosms were prepared using

1,4-dioxane-contaminated sludge, or soil and groundwater to assess intrinsic and enhanced 1,4-dioxane biodegradation. While intrinsic biodegradation was observed in activated sludge samples collected from a polyester-manufacturing facility's wastewater treatment plant, microcosms containing only indigenous microorganisms from the groundwater of Air Force Plant 44 (AFP44) did not exhibit 1,4-dioxane biodegradation within 150 days. However, biodegradation of 1,4-dioxane in AFP44 samples could be enhanced by biostimulation with oxygen or methane, and bioaugmentation with bacteria known to metabolize or cometabolize 1,4-dioxane. This indicates that biostimulation or bioaugmentation strategies may be viable alternatives to *ex situ* chemical treatment processes currently in place at AFP44. This study also evaluated functional gene biomarkers for their capability to indicate the potential for biodegradation of 1,4-dioxane. While the abundance of DXMO, PrMO, and PHE could be used to determine presence or absence of 1,4-dioxane-degrading bacteria, they were inconclusive to predict biodegradation rates of 1,4-dioxane in microcosms. These results suggest that quantification of functional gene biomarkers is not sufficient to indicate true biodegradation potential. Other tools should be also applied for a reliable characterization of contaminated sites and provide multiple lines of evidence to support biodegradation.

Compound Specific Isotope Analysis (CSIA) has been previously used as a monitoring tool to validate biodegradation of various organic contaminants (*32, 33, 139, 146-149*). CSIA provides strong evidence for both biological and chemical degradation reactions (including hydrolysis, oxidation, reduction, and enzyme-catalyzed reactions), which result in a change in the ratio of stable isotopes reflecting an increase in the heavy isotope in preference to the light isotope (*31, 150*). A novel method, which combined frozen microextraction of samples containing 1,4-dioxane (*19, 227*) and an analytical method using gas chromatography-combustion-isotope ratio

mass spectrometry (GC-C-IRMS) was applied to determine isotopic signatures ($\delta^{13}C$) in 1,4-dioxane obtained from various chemical providers. The results revealed slight variability in isotopic signatures of 1,4-dioxane, suggesting that CSIA could be applied for 1,4-dioxane source differentiation. In addition, the method was applied to establish kinetic carbon isotopic fractionation factors (ε) during the aerobic biodegradation of 1,4-dioxane by *P. dioxanivorans* CB1190 (grown on 1,4-dioxane), cometabolic culture *Mycobacterium austroafricanum (vaccae)* JOB5 (grown on toluene, cometabolized 1,4-dioxane), and mixed consortia of microorganisms in industrial activated sludge samples. The fraction of ^{13}C increased as biodegradation in these pure and mixed cultures progressed. While this method was robust enough to confirm aerobic biodegradation of 1,4-dioxane, it was unable to statistically distinguish metabolic from cometabolic degradation mechanisms.

In addition to carbon CSIA, this study also developed a method for stable hydrogen isotope analysis of 1,4-dioxane. It was applied to establish the isotope signatures of commercial 1,4-dioxanes, and to determine the kinetic isotope fractionation associated with degradation in various environments. It was shown that biodegradation by *P. dioxanivorans* CB1190 and by industrial activated sludge could enrich both ^{2}H and ^{13}C in residual 1,4-dioxane. Mixed microbial cultures were found to have similar enrichment factors for carbon and hydrogen when compared with those established for the pure cultures, suggesting that removal of 1,4-dioxane in industrial activated sludge was certainly due to biodegradation. In contrast, abiotic aeration and sonolytical degradation only enriched ^{2}H in 1,4-dioxane. These results revealed that combined carbon and hydrogen isotope analyses of 1,4-dioxane allow differentiation of biological processes from abiotic mechanisms, and thus are valuable for assessing natural attenuation and engineered bioremediation of 1,4-dioxane.

The present study extends from previous knowledge on biodegradation of 1,4-dioxane by using stable isotopic and molecular biological tools to better predict biodegradation, as well as differentiate biodegradation from other degradation processes. This research quantified the effects of selected transition metals and organic ligands on 1,4-dioxane biodegradation, evaluated the usefulness of functional gene biomarkers to indicate the biodegradation potential in complex environments, developed methods for hydrogen and carbon isotope analysis of 1,4-dioxane, established enrichment factors associated with biotic and abiotic degradation of 1,4-dioxane, and applied CSIA methods for validating biodegradation in environmental samples.

7.2. Suggestions for Future Research and Field Application. Successful bioremediation requires a multidisciplinary approach involving expertise in hydrology, biogeochemistry, microbial ecology, and process engineering for better understanding of the physicochemical, biological, and geological factors which may influence microbial degradation. Successful bioremediation depends upon provision of biodegradative microbes, or appropriate electron donors and/or electron acceptors required to stimulate indigenous microbes in polluted environments. Environmental factors including pH, soil structure, nutrients, bioavailability of organic pollutants, dissolved oxygen content, temperatures, and various co-contaminants may influence rates and product profile of bioremediation. Thus, it is very important for environmental scientists and engineers to understand the effects of these environmental factors to better predict biodegradation of toxic compounds, including 1,4-dioxane.

A comprehensive investigation into the potential synergistic or inhibitory effects of co-contaminants on 1,4-dioxane biodegradation is needed. The present study and limited previous research (*88*) demonstrated that co-contaminants (including TCA and its abiotic breakdown product 1,1-dichloroethene (DCE), and transition metals Cu(II), Cd(II) and Ni(II)) as well as

organic ligands (including tannic acid, alginic acid and L-cysteine) can influence biodegradation of 1,4-dioxane. Other transition metals (chromium, arsenic, manganese, cobalt, iron), volatile organic compounds (other chlorinated ethenes and ethanes, carbon tetrachloride, methyl ethyl ketone, methyl isobutyl ketone, acetone, methanol and xylene isomers), solvent stabilizers (1,3-dioxolane, 1,2-butylene oxide, propylene oxide, trimethylpentene (diisobutylene), nitromethane, isopropyl alcohol, pyridine, triethylamine, epichlorohydrin), ethoxylated surfactants, polyethylene glycols (triethylene glycol, ethers of propylene glycol, propylene glycol stearate, propylene glycol oleate, propylene glycol cocoate), phthalates, herbicides and pesticides may be found as co-contaminants of 1,4-dioxane in groundwater or industrial wastewater. Further study on the effects of these compounds will assist in designing systems for effectively bioremediating waste streams and sites simultaneously contaminated by 1,4-dioxane and other chemicals.

To evaluate the feasibility of bioremediation to treat 1,4-dioxane at a contaminated site or wastewater treatment plant, environmental engineers should first conduct treatability studies using the samples collected from that specific site or reactor. In addition to monitoring 1,4-dioxane, it is recommended to determine concentrations of transition metals and organic compounds, since their individual and interactive effects may influence biodegradation of 1,4-dioxane. The analyses of these parameters will also provide inputs for the prediction of the bioavailability of transition metals using computer models such as PHREEQC 3.0. Note that PHREEQC 3.0 can be used to determine speciation of heavy metals in a defined environmental chemistry. Moreover, it is possible to apply CSIA and molecular biological tools to validate biodegradation of 1,4-dioxane in microcosms. Microcosm studies and applications of monitoring tools altogether are thus valuable for validating biodegradation at bench, pilot, and

full scale, and facilitate the selection of appropriate bioremediation strategy for site-specific treatment of 1,4-dioxane.

Development of molecular biological tools specifically for 1,4-dioxane allows applications in monitoring, assessing, and validating natural and enhanced bioremediation. Previous scientific research applied tools such as enzyme activity probing, PCR-DGGE, functional gene arrays, etc., for confirming 1,4-dioxane biodegradation (*16, 18, 81, 138, 139, 144*). However, the availability of tools specific for 1,4-dioxane and 1,4-dioxane-degrading bacteria were limited.

Fluorescent *in situ* hybridization (FISH) is an assay that can be applied for detecting microorganisms capable of biodegrading certain compounds. In FISH, DNA or RNA probes tagged with fluorescence dyes are used to target sequences within the cells. Hybridization of the probes with the targets allows the emission of fluorescent light that can be observed through microscope (such as epiflorescent microscope) or sorted by flow cytometry. FISH can be used for site characterization and monitoring which can be used in conjunction with qPCR or other molecular biological tools for better assessment. Unfortunately, FISH is still not available for 1,4-dioxane-degrading microorganisms. Development of a FISH method that targets functional genes relevant to 1,4-dioxane biodegradation such as DXMO, PrMO, or PHE, preferably in a field-deployable platform will provide another useful tool for environmental engineers to confirm biodegradation and identify microorganisms that can degrade the compound.

It was shown that 1,4-dioxane could be effectively removed from complex environments through bioaugmentation with *P. dioxanivorans* CB1190 (reference (*19*) and in this study). To date, bioaugmentation with bacteria capable of growing on 1,4-dioxane has not been implemented in the field. To apply this technology for 1,4-dioxane treatment in subsurface aquifers, the efficacy

of bioaugmentation by pure cultures or consortia should be evaluated. Previous research revealed that *in situ* culture distribution and steady state cell concentration of desired bacteria are critical factors that can impact the effectiveness of field-scale bioaugmentation (*247-249*). Since *P. dioxanivorans* CB1190 is best-studied 1,4-dioxane-metabolizing microbe, its viabilty in the subsurface following bioaugmentation should be examined.

While CSIA is valuable to characterize biodegradation, current CSIA methods still have some limitations for use in field applications. CSIA methods developed in the present research possess high method detection limits (21 mg L^{-1} and 100 mg L^{-1} for analysis of stable carbon and hydrogen isotopic analysis, respectively). Since the remediation goals for 1,4-dioxane by the United States Environmental Protection Agency range from 0.94 to 6.1 μg L^{-1} (*238*), the current method will require a considerably larger sample volume and subsequent concentration. Further development of the sample concentration method as well as optimization of analytical methods for stable isotope analysis is necessary to apply this technique for μg L^{-1} range aqueous samples of 1,4-dioxane. In addition, further insights may be obtained by incorporating stable oxygen isotopic analysis in the suite of CSIA methods for 1,4-dioxane.

CHAPTER 8

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