EFFECTS OF CO-CONTAMINANTS ON BIODEGRADATION OF

1,4-DIOXANE

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Effects of Co-contaminants on Biodegradation of 1,4-Dioxane. (May 2013)

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1,4-Dioxane is a commonly used industrial solvent stabilizer, a groundwater contaminant, and a probable human carcinogen. Due to its chemical and physical properties, treatment of 1,4-dioxane-contaminated groundwater is not cost effective. Two well-studied oxygenase-expressing bacteria *Mycobacterium vaccae* JOB5 (referred as JOB5 hereafter) and *Rhodococcus jostii* RHA1 (referred as RHA1 hereafter) have been shown to individually degrade both 1,4-dioxane and common co-contaminants, e.g. trichloroethylene (TCE) and trichloropropane (TCP). However, little study has been devoted to the biodegradation of both 1,4-dioxane and co-contaminants. To determine the effects of co-contaminants on 1,4-dioxane biodegradation, strains JOB5 and RHA1 were used to degrade 1,4-dioxane and mixtures of 1,4-dioxane and TCE or 1,4-dioxane and TCP. Propane- and 1-butanol-induced JOB5 and RHA1 were able express oxygenases to degrade both 1,4-dioxane, TCE, and TCP. Complete degradation of 1,4-dioxane/TCE mixture was only observed in propane-induced strain JOB5. Product toxicity caused incomplete degradation of 1,4-dioxane by 1-butanol-induced JOB5. Furthermore, competitive inhibition was observed between 1,4-dioxane and TCE in propane- and 1-butanol-

induced JOB5 and RHA1. The findings of this study provide a major basis for developing an

effective *in-situ* remediation method for 1,4-dioxane-contaminated ground water.

DEDICATION

This thesis is dedicated to my beloved fiancé, Kelsey Suzanne Roberts.

ACKNOWLEDGMENTS

NOMENCLATURE

TCP   1,2,3-Trichloropropane

TCE   Trichloroethylene

SF   Slope Factor

PEL   Permissible exposure limits

R2A   Reasoner's 2A

AMS   Ammonium Mineral Salts

PMA   Propidium monoazide

PCR   Polymerase Chain Reaction

CHAPTER I

INTRODUCTION

Introduction

1,4-Dioxane is a commonly used stabilizer for chlorinated solvents such as vinyl chloride (VC), dichloroethene (DCE), trichloroethene (TCE), and 1,2,3-trichloropropane (TCP). 1,4-Dioxane is a probable human carcinogen and a common subsurface contaminant as a result of improper disposals of industrial waste or accidental solvent spills. Due to its chemical and physical properties, it is difficult to attenuate 1,4-dioxane by volatilization or sorption. Several aerobic bacteria can degrade 1,4-dioxane, suggesting *in-situ* bioremediation of 1,4-dioxane is a promising treatment option. Some of the known 1,4-dioxane-degraders can also degrade its co-contaminants. However, the impacts of 1,4-dioxane's co-contaminants upon its degradation are unknown.

Objective and hypotheses

The **objective** of this study is to ascertain whether the removal of 1,4-dioxane is effective in the presence of individual and mixtures of chlorinated solvents. As these contaminants are degraded by the same enzymes, the presence of co-contaminants might cause an inhibitory effect on 1,4-dioxane biodegradation. Thus, I hypothesize that the presence of co-contaminants will competitively inhibit the biodegradation of 1,4-dioxane (**Hypothesis 1**). Degradation of chlorinated solvents can also generate product toxicity, which has the potential to decrease the viability of the degradative bacterial strains. My second hypothesis is that the product toxicity

generated from the biodegradation of co-contaminants is profound and might subsequently decrease the viability of 1,4-dioxane-degrading cultures (**Hypothesis 2**).

Two well-studied oxygenase-expressing bacteria *Mycobacterium vaccae* JOB5 (referred as JOB5 hereafter) and *Rhodococcus jostii* RHA1 (referred as RHA1 hereafter) have been shown to individually degrade both 1,4-dioxane and common co-contaminants, e.g. trichloroethylene (TCE) and trichloropropane (TCP). In this study, these two strains were used to test the hypotheses.

Thesis overview

My results indicated that propane- and 1-butanol-induced JOB5 and RHA1 were able to degrade 1,4-dioxane, TCP, and TCE. Complete degradation of 1,4-dioxane/TCE or TCP mixture was only observed in the samples containing propane-induced strain JOB5. Competitive inhibition was observed between 1,4-dioxane and TCE or TCP with strain JOB5 showing a 85% decrease in degradation between pure 1,4-dioxane and mixture samples. Further, product toxicity was observed with both TCE and TCP and caused incomplete degradation of 1,4-dioxane. Degradation with TCP caused the greatest decrease in viable cell count by as much as 38%. Strain JOB5 induced with propane, strain showing the highest degradative potential, experienced significant losses when degrading either TCE or TCP. The effects of both competitive inhibition and product toxicity must therefore be considered when developing methods for *in-situ* 1,4-dioxane-contaminated groundwater remediation.

CHAPTER II

LITERATURE REVIEW

Chemical and physical properties of 1,4-dioxane

1,4-Dioxane, shown below in **Error! Reference source not found.**, is primarily used as a stabilizer used in conjunction with 1,2,3-trichloropropane (TCP, Figure 2) and other chlorinated solvents such as trichloroethylene (TCE, Figure 3). 1,4-Dioxane is a flammable, colorless liquid, with a faint pleasant odor. It is miscible in water and has a boiling point of 101.1ºC. 1,4-Dioxane has a very low octanol-water partition coefficient (log K_{ow}) of -0.27; this is indicative of it being highly mobile in groundwater. It also has a Henry's law constant of 4.80 x 10^{-6} atm m^3/mol, suggesting that soil gas measurement techniques will not be useful for tracking it. The low Henry's law constant also means that any presence in surface water or groundwater will not volatilize heavily and the majority will remain in the body of the water. It has a very low organic carbon partition coefficient (log K_{oc}) of 1.23 which indicates that it will not be readily absorbed by soil or sediment (1).
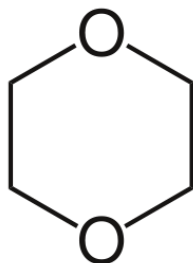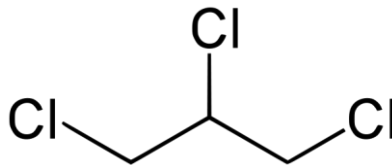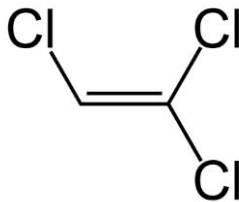
Figure 1: 1,4-Dioxane

Figure 2: 1,2,3-Trichloropropane

Figure 3: Trichloroethlyene

Toxicity and regulation

1,4-Dioxane does not bioaccumulate in fish or food webs. It is known to cause vertigo, drowsiness, headache, anorexia and irritation of the eyes, nose, throat, and lungs in humans after short-term exposure (1). Chronic exposure has been linked to dermatitis, eczema, drying and cracking of skin, and liver and kidney damage. The current reproductive effects for 1,4-dioxane are unknown but it is assumed to be weakly genotoxic with a developmental study on rats indicating that the developing fetus may be a target of toxicity.

Although inhalation is the most common and concerning exposure route for 1,4-dioxane, it can be absorbed through inhalation, dermal contact, and ingestion. The United States Evinronmental Protection Agency (EPA) has classified 1,4-dioxane as "likely to be carcinogenic to humans" through all exposure routes . The EPA uses slope factor (SF), typically measured in kilogram days per milligram, to measure the relative toxicity of a carcinogenic compound. Slope factor is

a measure of the dose and response for a carcinogenic compound. A given dose is multiplied by the slope factor to determine the risk of development of cancer for a given pathway. 1,4-Dioxane has a slope factor of 0.011 kg/d mg when ingested orally. Within the United States, Colorado has established water cleanup standards at 3.2 µg/L. The Occupational Safety and Health Administration (OSHA) airborne permissible exposure limits (PEL) is 360 mg/m^3 (1). Sweden has established an airborne PEL of 90 mg/m^3 (14).

Current treatment technologies for 1,4-dioxane

The physical and chemical properties of 1,4-dioxane make *in-situ* removal of 1,4-dioxane from contaminated sites very difficult. 1,4-Dioxane cannot be removed with liquid-phase granulated activated carbon through adsorption. While advanced oxidation techniques involving hydrogen peroxide and ultraviolet light (UV) or ozone have been shown to effectively remove 1,4-dioxane, these techniques are often prohibitively expensive. Distillation has been proven to destroy it; however the relatively high boiling point renders this treatment uneconomical in most applications. These methods often require *ex-situ* treatment for any groundwater contamination. Sei et al. (12) have shown that while the potential for 1,4-dioxane biodegrdation exists within the natural environment, it is not ubiquitous and is often ineffective. Phytoremediation has been used as a means of treating 1,4-dioxane-contamined groundwater in shall (6). Chlorination be effectively remove 1,4-dioxane, but the chlorination byproducts are between 12 and 1,000 times more toxic than the 1,4-dioxane itself. Given that 1,4-dioxane is biodegradable, bioremediation of 1,4-dixoane can be an economical treatment method. As 1,4-dioxane is a solvent stabilizer for TCE and TCP, groundwater is commonly contaminated with mixtures of 1,4-dioxane and these

solvents. The presence of chlorinated solvents might affect the efficiency of biotreatment for 1,4-dioxane.

Co-metabolic Biodegradation of 1,4-dioxane.

Multiple oxygenase-expressing bacteria are known to degrade 1,4-dioxane (3, 7, 10). Recent findings in the Dr. Chu laboratory have shown that two aerobic strains, *Mycobacterium vaccae* JOB5 (hereafter referred as strain JOB5) and *Rhodococus jostii* RHA1 (hereafter referred as strain RHA1), can be easily cultured in complex nutrient media and their degradative enzymes can be easily induced for 1,4-dioxane biodegradation. Previous research has focused principally on isolating 1,4-dioxane degraders and the enzyme kinetics associated with this degradation, but has not considered the effects of co-contaminant degradation on the degradation of 1,4-dioxane.

These two strains biodegrade 1,4-dioxane via a co-metabolic reaction – a non-growth-linked degradation processes.   Bacteria use their existing enzymes that are expressed to degrade their growth substrate to degrade target contaminants without gaining any benefits (i.e., energy or building blocks).   Strains JOB5 and RHA1 can express various oxygenases depending on their growth substrates. For example, when incubating with propane or 1-butanol, strains JOB5 and RHA1 can produce propane monooxygenase or butane monooxygenase, respectively, to degrade a range of chlorinated solvents, including common co-contaminants of 1,4-dioxane such as TCP or TCE   As common co-contaminants are degraded by the same enzymes as 1,4-dioxane, the presence of co-contaminants might cause an inhibitory effect on 1,4-dioxane biodegradation. In competitive inhibition, both inhibitors (co-contaminants in this case) and substrate (i.e., 1,4-

dioxane as the target compound) compete for the same binding site of the enzyme to prevent the substrate to form the enzyme-substrate that is necessary for degradation.

Product toxicity may or may not occur during co-metabolic degradation. The potential adverse effect of product toxicity is to decrease the viability of degradative cultures, limiting the overall capability of biodegradation. As chlorinated solvents and 1,4-dioxane are degraded co-metabolically, product toxic might occur during the degradation and to damage or even inactivate the 1,4-dioxane degrading strains. Thus, this study examined the occurrence and extent of product toxicity during degradation of 1,4-dioxane and its co-contaminants .

CHAPTER III

MATERIALS AND METHODS

Strains and culture conditions

Strains JOB5 was kindly provided by Dr. Robert Steffan, Shaw Environmental Inc.

(Lawrenceville, NJ). Strain RHA1 was kindly provided by Dr. Bill Mohn, University of British

Columbia, Canada. Strains of RHA1 and JOB5 were cultivated in 50 mL of Reasoner's 2A

(R2A) broth medium in a 30°C incubator for approximately 48 hrs until $OD_{600} = 0.8 \sim 1.5$. Cells

were harvested by centrifugation at 10,000 g for 5 min and then washed and resuspended in

Ammonium Mineral Salts (AMS) medium to $OD_{600} = 0.5 \sim 1.0$. Resuspended cultures were

incubated with either 1-butanol (10 mg/L) or propane (40% headspace v/v) for 24 hrs to induce

butane- and propane-monooxygenases, respectively. Cells were then harvested by centrifugation

at 10,000 g for 5 min and then washed with and resuspended in AMS medium to $OD_{600} = 0.5 \sim 1.0$

for experimental use.

Biodegradation tests

Biodegradation of 1,4 dioxane was performed in a series of 40 mL glass bottles containing

resting cells of either strains JOB5 or RHA1 and 20 mg/L of 1,4-dioxane. The initial cell

concentration was measured as optical density using a spectrophotometer at A_{600} and as volatile

suspended solids (VSS).   The bottles were divided into three sample categories: 1) Resting cells

and 20 mg/L of 1,4-dioxane; 2) Resting cells and 5 mg/L of co-contaminant (TCE or TCP); and

3) Resting cells, 20 mg/L of 1,4-dioxane, and 5 mg/L of TCE or TCP. Killed controls (KC) for

each sample category were prepared by adding 50μL of concentrated sulfuric acid to inhibit

biological reactions prior to the addition of either solvent or 1,4-dioxane. The bottles were then

incubated while mixing at 30°C for 72hrs to allow for complete degradation based upon previous

degradation tests in the Dr. Chu Laboratory. After 72 hrs, samples were removed from incubator

and liquid and gas phase samples were taken to determine 1,4-dioxane and TCP or TCE

concentration.

Chemical analysis

For samples containing TCE, 200 µL of headspace was extracted and injected into a Agilent

Technologies 6890N gas chromatography/ flame ionization detection system to determine TCE

concentration. The injector, oven, and detector temperatures were set at 225 °C, 60 °C, and 250

°C, respectively. The TCE peak occurred at a retention time of 6.2 min. Standard curves were

generated using headspace samples with known concentrations. The detection limit was 0.5

mg/L. The data was collected and analyzed using Agilent ChemStation software. The

concentration of TCE in KC control samples was compared with the concentration of TCE in

active cell samples to determine the relative percent degradation.

For samples containing TCP, 175 µL of headspace was extracted and injected into a Agilent

Technologies 6890N gas chromatography/ flame ionization detection system to determine TCP

concentration. The injector, oven, and detector temperatures were set at 225 °C, 100 °C, and 250

°C, respectively. The TCP peak occurred at a retention time of 6.2 min. Standard curves were

generated using headspace samples with known concentrations. The detection limit was 0.5

mg/L. The data was collected and analyzed using Agilent ChemStation software. The

concentration of TCP in KC controls was used to determine the abiotic loss. The amounts of

TCP degraded were determined by comparing the concentrations of TCP in controls to those in the samples.

The 1,4-dioxane in liquid samples was extracted using dichloromethane in a ratio of 1 mL of dichloromethane per 1 mL of liquid sample in a method adapted from Draper et al. 2000 (5). The samples were then vortex mixed and incubated for 16-24 hr in a 30°C incubator. 1 mL of the extracted liquid was extracted and injected into a Agilent Technologies 6890N gas chromatography/ flame ionization detection system to determine 1,4-dioxane concentration. The injector, oven, and detector temperatures were set at 150 °C, 60 °C, and 250 °C, respectively. The 1,4-dioxane peak occurred at a retention time of 9.5 min. Standard curves were generated using liquid samples with known concentrations. The detection limit was 1 mg/L. The data was collected and analyzed using Agilent ChemStation software. The concentrations of 1,4-dioxane in KC controls were compared with the concentrations of 1,4-dioxane in active cell samples using a developed standard curve to determine the relative percent degradation.

Live/Dead cell differentiation

Cells were treated with propidium monoazide (PMA[TM]) dye acquired from Biotium, Inc. after degrading 1,4-dioxane or 1,4-dioxane/TCE mixture for 3 days. Cell membrane-impermeable PMA modifies only the DNA of dead cells with destroyed cell membranes and has been successfully used to quantify viable bacterial cells (8, 9, 11, 13). 2.5 µL of PMA dye was added to 1 mL sample of suspended cells. PMA samples were then gentle shaken for 10 min while covered to limit light exposure. Lastly, samples were then placed on an ice block under a halogen lamp and shaken for 15 min according to instructions supplied by Biotium, Inc. After

PMA treatment, DNA was extracted and used for real-time polymerase chain reaction (PCR) analysis. PMA modified DNA cannot be amplified by the PCR reactions, thus only DNA from live cells can be PCR amplified. . PCR results were compared between cells with no exposure to contaminants and live cell samples to determine percent active cells remaining after biodegradation.

CHAPTER IV

RESULTS

This study presents the first known in-depth analysis of the effects of presence of co-contaminants on the bioremediation of 1,4-dioxane. The results of this study indicate that the presence of co-contaminants do inhibit 1,4-dioxane degradation by as much as 85%. The degradation of co-contaminants was also found to reduce the concentration of viable cells in degradation samples. Of the two bacterial strains observed, JOB5 was shown to better degrade 1,4-dioxane in the presence of inhibiting co-contaminants. JOB5 was capable of fully degrading the 1,4-dioxane in both pure 1,4-dioxane samples and 1,4-dioxane mixtures. Similarly, JOB5 was shown to be more resilient to the effects of toxicity of degradation byproducts. However, JOB5 experience a more pronounced difference in remaining active cells between 1,4-dioxane and co-contaminants, potentially making it more susceptible to high co-contaminant concentration.

1,4-Dioxane degradation

Both propane-induced and 1-butanol-induced JOB5 and RHA1 were able to degrade 1,4-dioxane, as shown in Figure 4. However, only propane-induced JOB5 showed complete degradation of 1,4-dioxane.
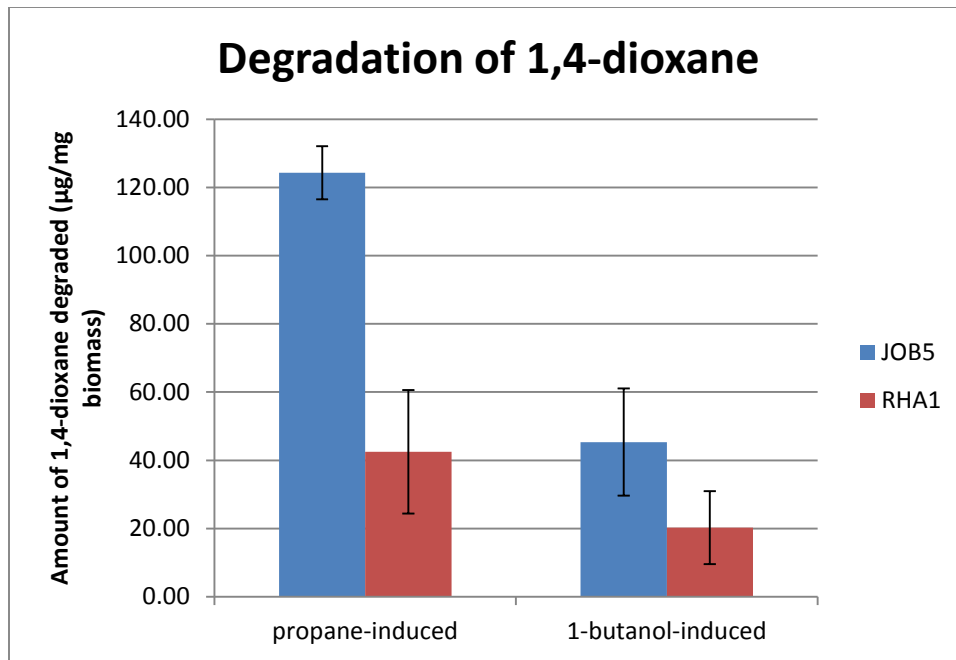
Figure 4: Average degradation of 1,4-dioxane after 3 days

Strain JOB5 performed better than RHA1 in degradation tests, regardless of incubation additive. However, JOB5 displayed a significant reduction in degradation potential when incubated with 1-butanol as opposed to propane, while RHA1 displayed no significant difference in degradation potential when incubated with either propane or 1-butanol.

TCE degradation

Both JOB5 and RHA1were able to degrade TCE when incubated with either 1-butanol or propane as shown in Figure 5.
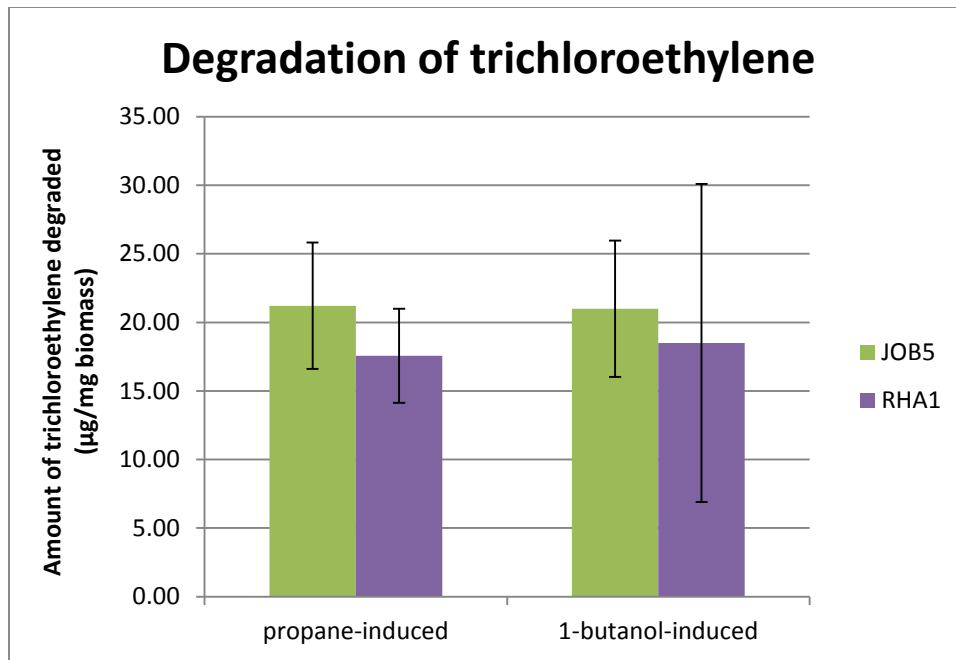
Figure 5: Average degradation of TCE after 3 days

JOB5 and RHA1 displayed comparable degradative potential for TCE. Further, no significant difference in degradation was found between propane- or 1-butanol-unduced JOB5 and RHA1.

TCP degradation

As with TCE, both JOB5 and RHA1were able to degrade TCP when incubated with either 1-butanol or propane as shown in Figure 6.
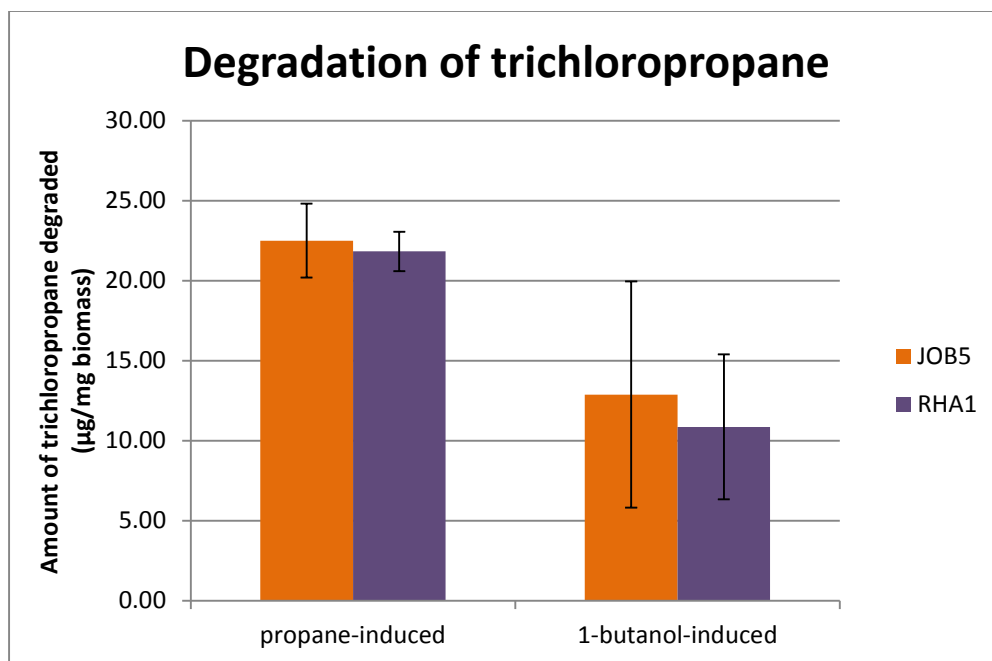
Figure 6: Average degradation of TCP after 3 days

Unlike TCE degradation, propane-induced JOB5 or RHA1were superior to 1-butanol-induced strains. No significant difference in degradative potential between RHA1 and JOB5 was found.

Degradation of mixtures of 1,4-dioxane and co-contaminants

Propane-induced JOB5 was superior to 1-butanol-induced JOB5 in degrading both 1,4-dioxane and TCE in mixture and individually, as shown in Figure 7 Figure 8. Little or no difference in 1,4-dioxane degradation was observed between 1,4-dioxane only and the mixture samples when JOB5 was incubated with propane, and both were capable to fully degrading 1,4-dixane in samples. However, 1-butanol-induced JOB5 degraded 85% less 1,4-dioxane in the mixture than in 1,4-dioxane only.
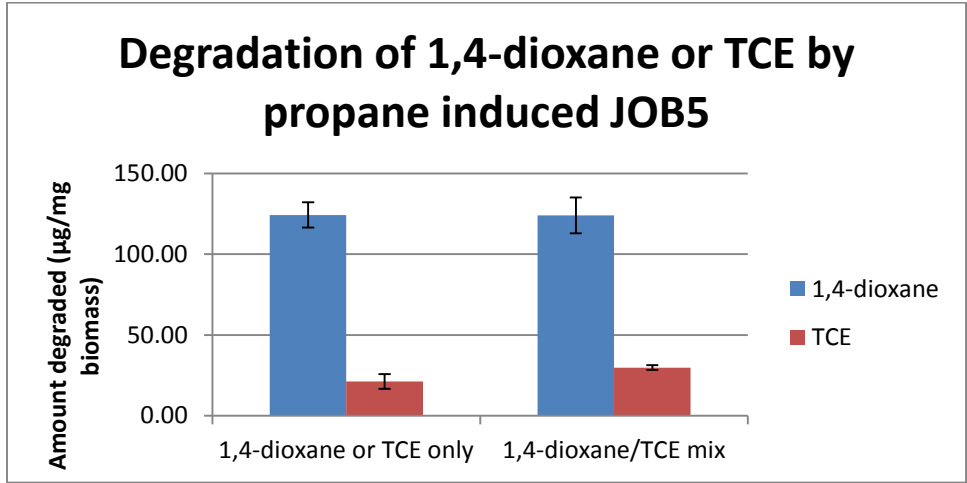
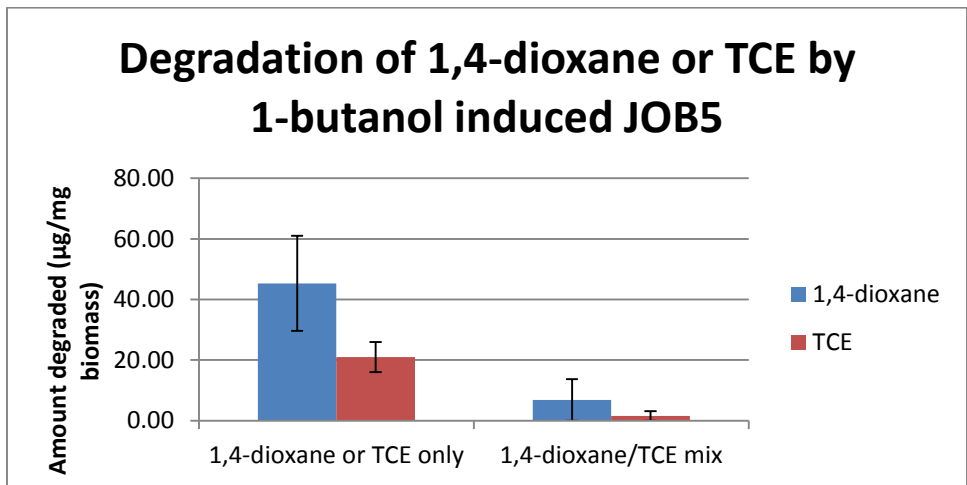**Figure 7: Degradation of TCE mix by propane induced JOB5**

Figure 8: Degradation of TCE mix by 1-butanol induced JOB5

Similarly to JOB, RHA1 demonstrated better 1,4-dioxane degradation when incubated with propane as opposed to 1-butanol as shown in Figure 9 Figure 10. Propane-incubated RHA1 showed somewhat superior degradation of 1,4-dioxane in mixture, however the difference in degradation observed was within experimental error ranges. Unlike JOB5, 1-butanol-induced

RHA1 did not demonstrate significant degradation between pure 1,4-dioxane and mixture samples.
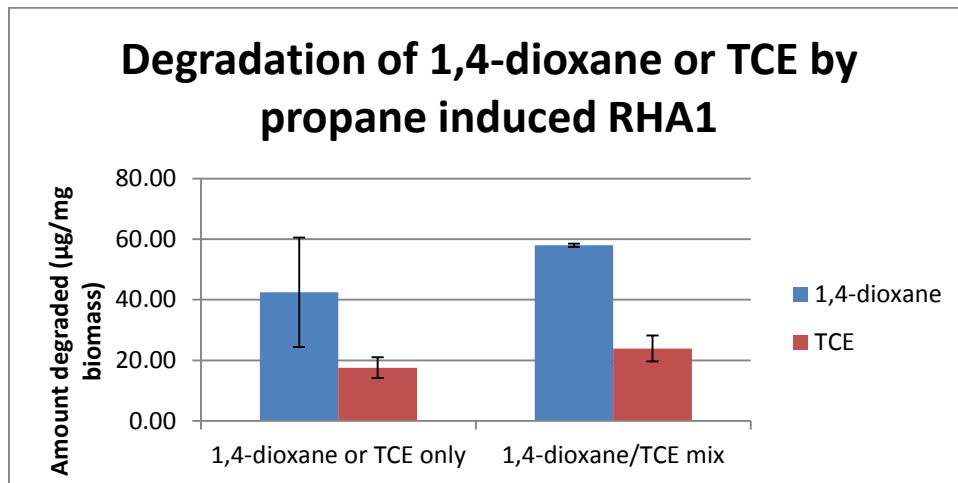

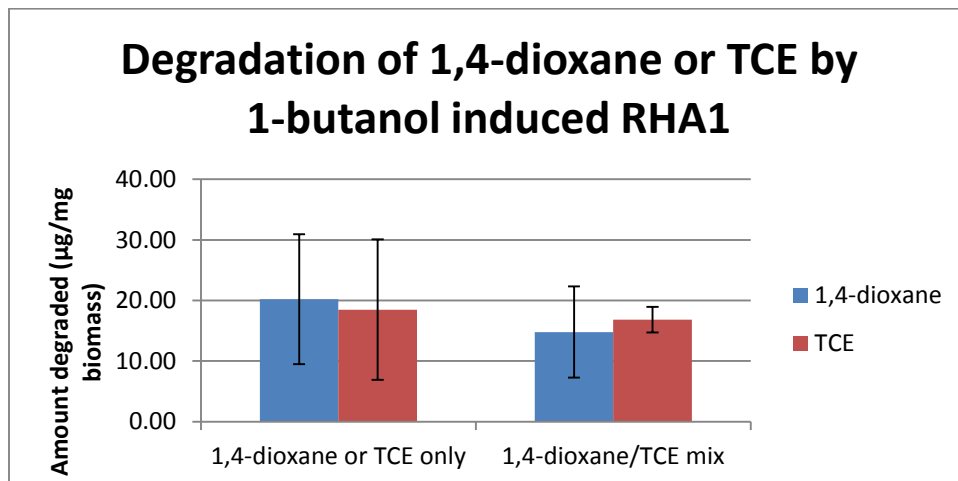**Figure 9: Degradation of TCE mix by propane induced RHA1**

Figure 10: Degradation of TCE mix by 1-butanol induced RHA1

Similar to TCE degradation, propane-induced JOB5 was more effective than 1-butanol-induced in degrading 1,4-dioxane individually and in mixture with TCP as shown in Figure 11Figure 12. Propane-induced JOB5 displayed a reduction degradation of 1,4-dioxane in mixture compared to

pure 1,4-dioxane samples. 1-butanol-induced JOB5 displayed a small improvement in

degradation when 1,4-dioxane was in mixture, however difference in degradative potential fell
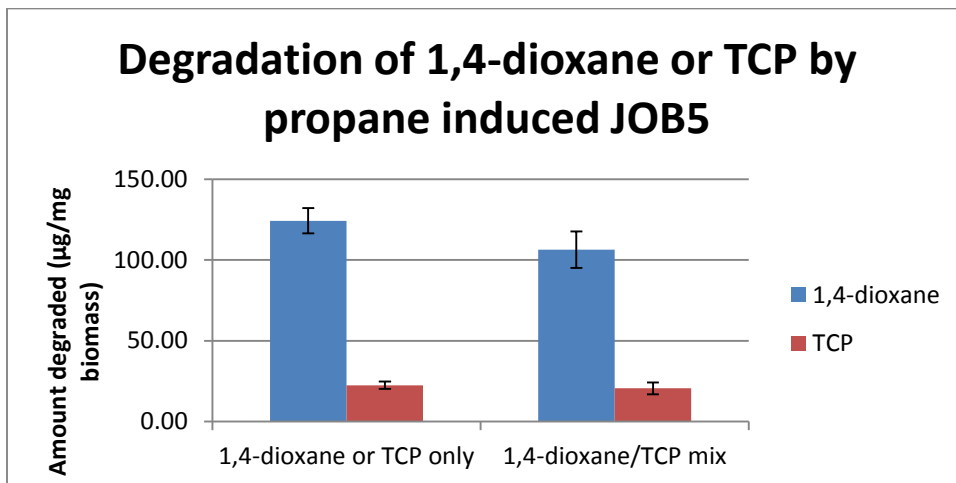
within experimental error ranges.

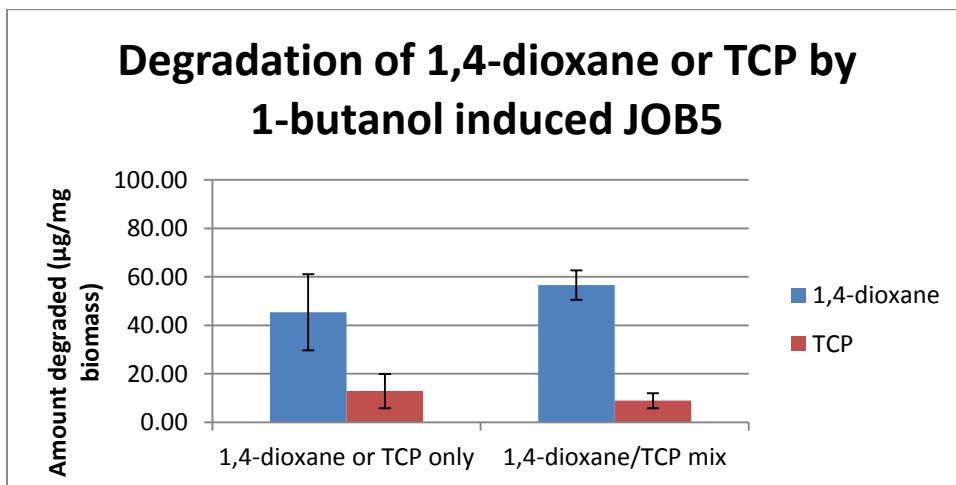**Figure 11: Degradation of TCP mix by propane induced JOB5**

Figure 12: Degradation of TCP mix by 1-butanol induced JOB5

Propane-induced RHA1 displayed superior 1,4-dioxane degradation than 1-butanol-induced

RHA1, shown below in Figure 13 Figure 14. Both propane- and 1-butanol-induced

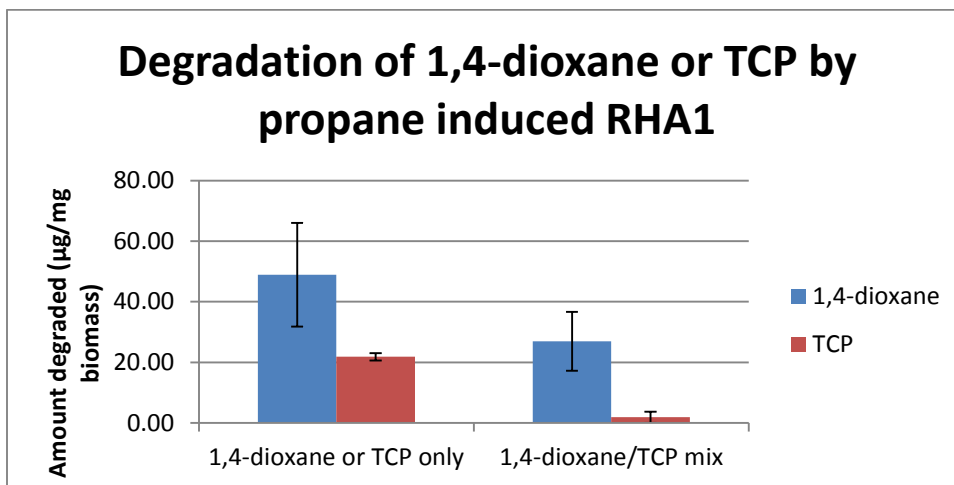RHA1displayed reduction in 1,4-dioxane degradation between pure dioxane and mixture samples.

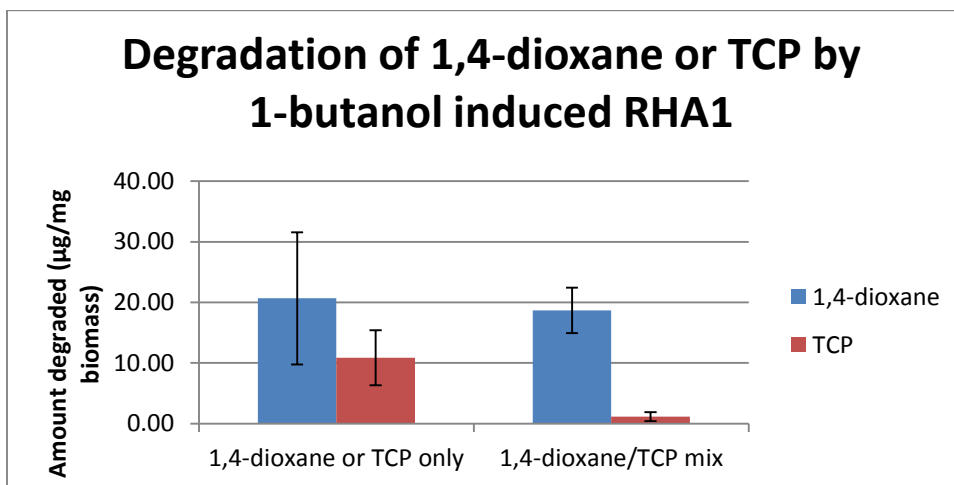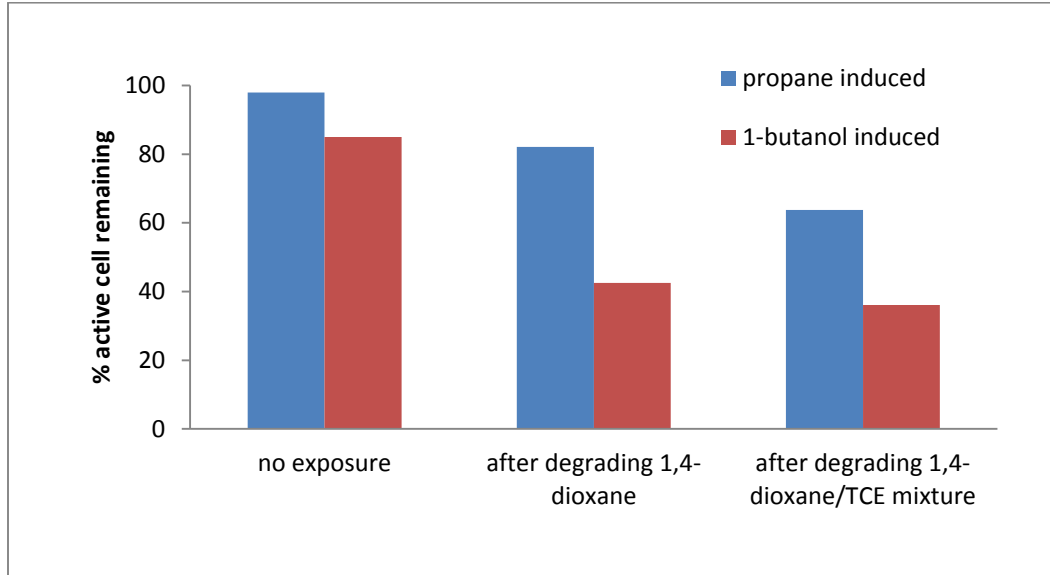**Figure 13: Degradation of TCP mix by propane induced RHA1**

**Figure 14: Degradation of TCP mix by 1-butanol induced RHA1**

Product toxicity

Active cell concentration was found to have decreased after 72 hrs of degrading both 1,4-dioxane individually and in mixture with co-contaminants, shown in Figure 15.
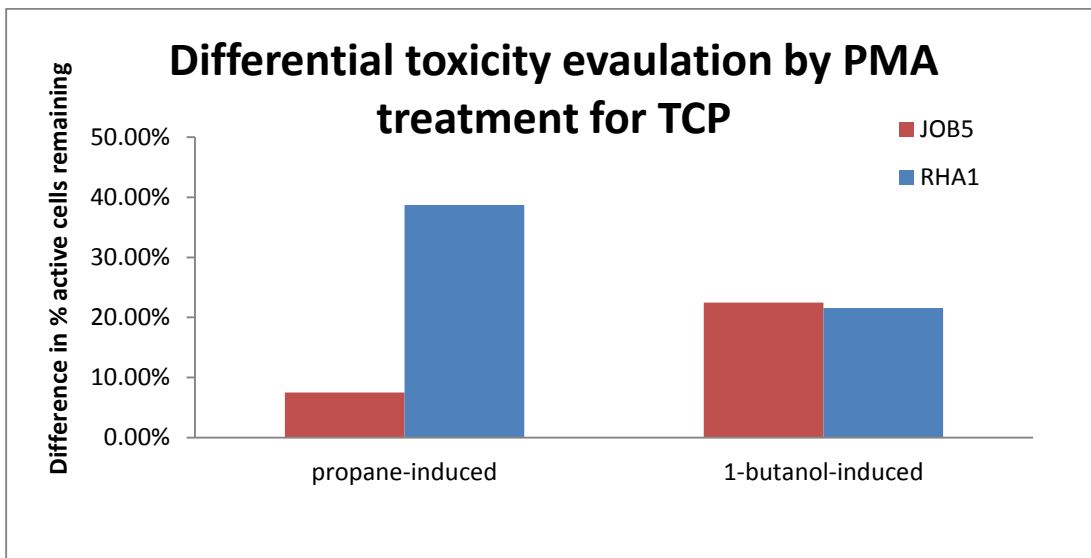
Figure 15: Toxicity due to TCE byproducts during degradation by JOB5

Propane-induced JOB5 was found to have a larger concentration of active cells for every sample than 1-butanol-induced JOB5. However, JOB5 showed a larger difference in the percent active remaining cells between 1,4-dioxane individually and in mixture. Further, in all cases except for propane-induced degradation of TCP, strains incubated with 1,4-dioxane also showed higher larger differences in the percent active remaining cells between 1,4-dioxane individually and in mixture, shown in Figure 16 Figure 17.
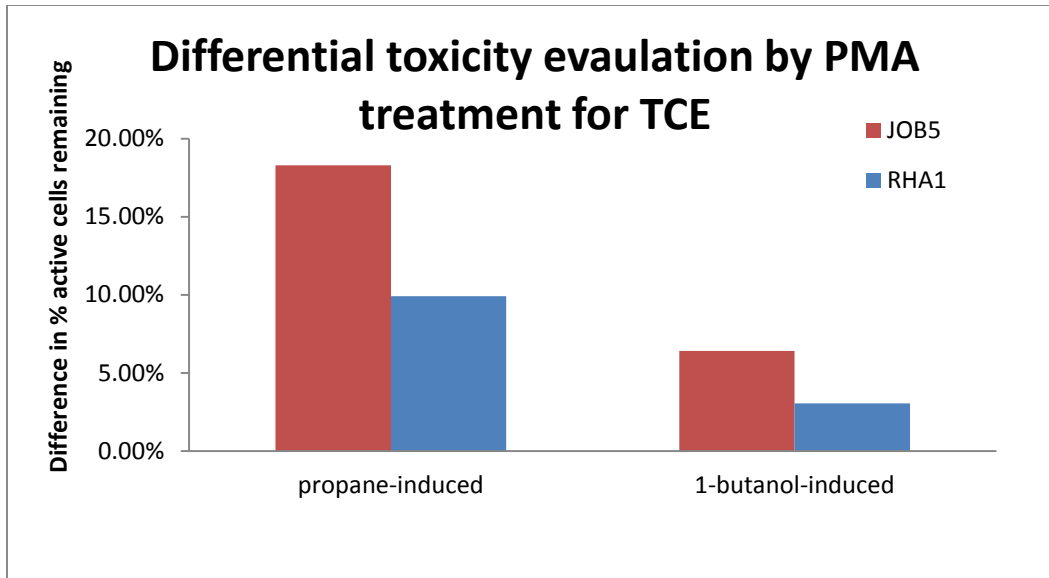
Figure 16: Toxicity due to byproducts during degradation with TCE

Figure 17: Toxicity due to byproducts during degradation with TCP

CHAPTER V

CONCLUSIONS

Competitive inhibition exists when RHA and JOB5 degrade 1,4-dioxane in the presence of co-contaminants TCE and TCP. Further, both RHA1 and JOB5 were better able to degrade 1,4-dioxane when incubated with propane as opposed to 1-butanol.   Incomplete degradation in 1-butanol-induced samples might be the result of product toxicity. Having demonstrated the efficacy of propane-induced JOB5 in degrading 1,4-dioxane in the presence of co-contaminants, further research is necessary to detail the reaction kinetics of 1,4-dioxane degradation as has been done for degradation of pure 1,4-dioxane samples (4, 10). A better understanding of the kinetics behind propane-induced JOB5 is crucial in designing processes for site bioremediation.

The results of this study provide the framework for development of a model *in-situ* treatment method for 1,4-dioxane contaminated groundwater. As pH has been proven to be a key factor in the degradation of isolate 1,4-dioxane, further research needs to be devoted to determining the effects of pH on 1,4-dioxane degradation in the presence of co-contaminants (2). Further environmental condition such as temperature should also be considered. Additional research should be developed to better understand the relationship between product toxicity and JOB5 viability. Varying concentrations of co-contaminants could potentially negate the efficacy of JOB5 in 1,4-dioxane bioremediation.   By determining acceptable co-contaminants thresholds, *in-situ* remediation can be better adjusted to environmental factors unique to each site.

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