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Degradation of cyclic ethers by microorganisms isolated from contaminated groundwater

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Degradation of cyclic ethers by microorganisms isolated from contaminated groundwater

By:
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Abstract

Groundwater contamination is an extremely important topic in environmental science. Ex-situ removal of contaminants is costly and largely ineffective on contaminants that have high vapor pressures and readily mix with water. Bioremediation or bio-augmentation are attractive alternative ways to remediate a site, as they may require less work and be more cost-effective.

Groundwater samples from a superfund site contaminated with chlorinated compounds and other volatile organic compounds. Samples were enriched to select for organisms capable of degrading cyclic ethers, specifically tetrahydrofuran and 1,4-dioxane. The isolates were tested for their degradation capacity and to determine if they were impacted by the presence of aliphatic chlorinated compounds.

Consortia of organisms were isolated and grow readily on rich media as well as in high concentrations of tetrahydrofuran and 1,4-dioxane (616 mM THF, 586 mM 1,4-dioxane). They were also shown to be able to grow readily in the presence of, and directly on, tetrachloroethylene (0.2 mM).

Previous research done at the source of the organisms has shown the presence of functional and phylogenetic genes that may co-metabolize tetrahydrofuran and 1,4-dioxane. This research has confirmed the previous hypothesis that degradation was occurring by microorganisms on site. 16sRNA analysis was completed on the isolates, and the majority of organisms have not been previously seen in degradation of these compounds. Commonly known degraders were not found in the samples, suggesting other degradation pathways are being used.

Introduction

Groundwater contamination from anthropogenic sources can severely impact not only the individuals who are in contact with the water, but also the ecosystems that are fed by water from the hyporheic zone, which connects the contaminated groundwater to the surface water (Danielopol et al. 2003). However, the impacts of many contaminants in groundwater, and ways to remove them, have not been adequately studied. In particular, 1,4-dioxane and tetrahydrofuran (THF), two cyclic ethers, readily move in groundwater, and are considered by the Environmental Protection Agency to be, respectively, "likely" or "probably" carcinogenic to humans (Department of Health and Human Services Agency for Toxic Substances and Disease Registry 2012; U.S. Environmental Protection Agency Office of Research and Development 2011). Each of these substances also pose non-carcinogenic risks to humans, and they are therefore limited in drinking water to concentrations not exceeding 0.46 µg/L (1,4-dioxane) and 4.6 µg/L (THF) (Isaacson, Mohr, and Field 2006). While studies on biodegradation of these compounds have been initiated, there are still a great deal of unknowns. The objective of this research is to isolate the microorganisms that are capable of degrading 1,4-dioxane and THF in groundwater from a specific site which appears to show naturally occurring degradation, and to determine if this information can be applied to other contaminated sites.

Background

The impacts of many contaminants in groundwater, and cost-effective ways to remove them, are evolving areas of study. In particular, man-made chemicals have become a source of concern. Multiple classes of anthropogenic chemicals are not readily biodegradable, which can cause significant problems in polluted areas. Many of these chemicals that are toxic and/or possible carcinogens are also not biologically active enough for biodegradation (Danielopol et al. 2003). Means of remediation have included *in-situ* and *ex-situ* processes, but these are costly methods. Over the past thirty years, advances have been made in the field of bioremediation for certain types of chemicals, but the possibilities have not yet been developed for others.

Cyclic ethers such as tetrahydrofuran (THF) and dioxanes have emerged as "contaminants of concern" at numerous contaminated sites, as analytical techniques have been developed capable of quantifying them in groundwater at low concentrations. 1,4-dioxane in

particular was widely used as a stabilizer in chlorinated solvents, so it is likely present at sites impacted by solvents (Santa Clara Valley Water District 2001). These ether compounds have low vapor pressure, sorb poorly to organic carbon, and are highly water soluble, so they can easily move in soil and groundwater (Isaacson, Mohr, and Field 2006). It was not until the work completed by Bernhardt and Diekmann (1991) that there was evidence of degradation by organisms.

Continued work has expanded knowledge of microorganisms and consortiums of microorganisms that are capable of degrading THF and dioxanes, especially 1,4-dioxane. Organisms have been isolated from a variety of sources, including industrial sludge (Parales et al. 1994), activated sludge from pharmaceutical factories (Chen et al. 2010), uncontaminated soil (Nakamiya et al. 2005), as well as soil that was contaminated by a groundwater plume (Sun, Ko, and Ramsay 2011). Isolated organisms tend to be within the *Pseudonocardia* or *Rhodococcus* genera, but *Flavobacterium* and even a filamentous fungi have also been found (Kampfer et al. 2006; Sun, Ko, and Ramsay 2011; Mahendra and Alvarez-Cohen 2005; Nakamiya et al. 2005).

However, applying these organisms as in-situ bioremediation via bio-augmentation- the process of adding high concentrations of specific organisms to enhance the rate of contaminant degradation- has not been shown in literature (Abdulsalam et al. 2011). One of the biggest problems surrounding the use of these organisms is their rate of degradation. In previous work, the rate of degradation has varied widely from 28.5 mg THF/h (g dry weight) to 203.9 mg THF/h (g dry weight) (Chen et al. 2010). Prolonged growth on only nutrient rich agar may negatively impact their degradation capacity, even if only temporarily (Chen et al. 2010). This can inhibit the ability to utilize these organisms for bioremediation, as it makes culturing large populations difficult. There have also been observations that chlorinated solvents inhibit the degradation of these compounds (Mahendra, Grostern, and Alvarez-Cohen 2013). Chlorinated solvents are often found as a co-pollutant in places contaminated with cyclic ethers, which suggests that the efficiency of bioremediation would be limited in these cases. Isolating an organism capable of degrading recalcitrant ethers that would not be inhibited by co-contaminants, and which could be grown on standard media would be a valuable option for in-situ bioremediation.

Pseudonocardia dioxanivorans strain CB1190 is one of the most studied dioxane degraders. First isolated in 1994, at least six additional publications have been released on the

identification, genomic sequencing, biodegradation kinetics and metabolic degradation pathways, as well as the impact of chlorinated solvents on its growth.

P. dioxanivorans CB1190 is a gram-positive actinomycete that is capable of aerobically degrading 1,4-dioxane (Mahendra and Alvarez-Cohen 2005). It is capable of completely mineralizing 1,4-dioxane and can co-metabolically degrade with THF, methane, propane, toluene or ethanol (Mahendra, Grostern, and Alvarez-Cohen 2013). It grows slowly on all media-including richer ones such as LB, TSA and R2A (Mahendra and Alvarez-Cohen 2005). Degradation is catalyzed by monooxygenases that are sensitive to common co-contaminants of 1,4-dioxane such as 1,1,1-trichloroethane (TCA) or 1,1-dichloroethene (DCE) (Mahendra, Grostern, and Alvarez-Cohen 2013).

P. dioxanivorans CB1190 was unable to degrade TCA or DCE as either the sole substrate or co-metabolically (Sales et al. 2011). Degradation of 1,4-dioxane was suppressed by the presence of chlorinated volatile organic compounds (cVOCs), but it was reversible once *P. dioxanivorans* CB1190 was returned to solely 1,4-dixoane (Mahendra, Grostern, and Alvarez-Cohen 2013). *P. dioxanivorans* CB1190 had an inhibition effect with low concentrations of cVOCs, meaning that for it to have the potential for bioremediation, the sites chosen would require concurrent dioxane and chlorinated solvent degradation (Mahendra, Grostern, and Alvarez-Cohen 2013). The inhibition is not due to the destruction of the cell membrane by the chlorinated compounds, nor by the formation of toxic by-products, but by the cVOCs binding to the enzymes and inactivating them (Zhang, Gedalanga, and Mahendra 2016). 1,1-dichloroethene and cis-1,2-dichloroethene were found to be the most inhibitory, with concentrations of less than 5 mg L^{-1} and 50 mg L^{-1} respectively caused causing stress and inhibition of degradation (Zhang, Gedalanga, and Mahendra 2016).

Site Background

This research will utilize data obtained from the Solvents Recovery Service of New England, Inc. (SRSNE) Superfund site in Southington, CT (Figure 1-1) (Arcadis, 2015). As detailed in the 1998 Remedial Investigation (RI) and the 2005 Feasibility Study (FS), between 1957 and 1991, SRSNE processed over 41 million gallons of waste solvents, fuels, paints, and similar liquid materials. A portion of these materials entered the soil due to placement of distillation sludge in two unlined lagoons on site, occasional overflow of materials from these lagoons to ditches adjacent to the site, and incidental spills and leaks (Arcadis, 2015). Resulting

releases produced a complex, multi-component, non-aqueous phase liquid (NAPL) source zone in the overburden and bedrock, along with associated aqueous phase plumes (Arcadis, 2015).

Figure 1-1. 'Aerial Photograph-1980' from 2015 Conceptual Site Model Update (Arcadis, 2015).

Dense NAPL (DNAPL) and, to a lesser extent, light NAPL (LNAPL) have been encountered at borings and monitoring wells at the site (Arcadis, 2015). DNAPL and LNAPL samples collected from monitoring wells have contained predominantly aromatic and chlorinated hydrocarbons, but also alcohols, furans, ketones and polychlorinated biphenyls. The associated volatile organic compound (VOC) plume in groundwater extends generally southward along the river valley, and is controlled by a hydraulic containment system that includes overburden and bedrock groundwater extraction wells (Figure 1-2).

Figure 1-2. 'Estimated NAPL-Zone Boundary in Bedrock' from 2015 Conceptual Site Model Update (Arcadis, 2015).

The site has been subject to extensive remedial efforts, including in-situ thermal treatment of the overburden NAPL zone, capping, "hot spot" soil excavation, and on-going hydraulic containment ("pump and treat") of both overburden and bedrock groundwater (U.S. Environmental Protection Agency 2015). While the majority of the focus has been on the thermal treatment area, where the contamination was greatest, groundwater sampling across the contaminated plume has been taking place for many years. Site studies to date have demonstrated extensive on-going biodegradation of aromatic and chlorinated compounds, as demonstrated by the presence of breakdown products and specific organisms known to completely degrade trichloroethene (TCE) (primarily *Dehalococcoides*). The groundwater plume covers a large area, but within a distance of approximately 700 feet, the concentration of THF drops about three orders of magnitude in the groundwater plume, dependent upon the depth interval sampled (deep overburden vs. shallow bedrock). In deep overburden, the concentration changes from 1,960 times the federal drinking water standards or CT groundwater standards-collectively called the "action level"- (4.6 µg/L) to 4.88 times the action level (US EPA 2015, Figure 1-3). The concentration of 1,4-dixoane drops from 1,200 times the action level (20 ug/L) to 126 times the action level (US EPA 2015, Figure 1-3).As there is no active remediation going on in this area, and neither THF nor 1,4-dioxane sorb to organic carbon, the most plausible explanation of this phenomenon is that biodegradation is taking place.

In 2014, 12 wells across SRSNE were sampled using BioTraps™. Bio-Trap™ samplers are passive sampling tools that are inserted into a groundwater well which are colonized by microbes (Julie Seuker, ARCADIS, 2014). The microbes can then be extracted and analyzed. The analyses applied to the extracted Deoxyribonucleic acid (DNA) from the BioTrap™ samplers looked for phylogenetic and functional genes of interest (Julie Seuker, ARCADIS, and Monica Heintz, ARCADIS, 2015). This data can be used to estimate the total bacteria in the wells, but it does not include Archaea and any other organisms that do not grow readily on the substrates provided (Julie Seuker, ARCADIS, 2014). Quantitative polymerase chain reaction (qPCR) analysis was completed to look for analyses phylogenetic and functional genes known to be characteristic of two degradation mechanisms for chlorinated VOCs (cVOCs); the aerobic co-metabolism is the more pertinent of the two (Julie Seuker, ARCADIS, 2014).

Further genetic testing was done in 2016 with 16 wells, which included all of the wells from the 2014 sampling. These qPCR tests were done with 1,4-dioxane biodegradation potential in mind (Julie Seuker, ARCADIS, and Monica Heintz, ARCADIS, 2015).

The goal of this study is to isolate, culture and identify the microorganism(s) and to evaluate their degradation capacity for both THF and 1,4-dioxane. The objective of this research is to determine what microorganisms are capable of degrading 1,4-dioxane and THF in groundwater from a specific site, the Solvents Recovery Service of New England, Inc. (SRSNE), which appears to show naturally occurring bioremediation.

Methods

Sampling Locations

Groundwater samples within the plume have shown that there is a wide range of VOCs present, including chlorinated ethanes and ethenes, ketones, aromatics (benzene, toluene, ethylbenzene and xylenes (BTEX)), methylene chloride, styrene, THF, and 1,4-dioxane (ARCADIS 2015). Previous research has shown that some of these compounds may inhibit the capacity of organisms to degrade cyclic ethers (Mahendra, Grostern, and Alvarez-Cohen 2013). One goal of this research is to identify if there are organisms present at the site that are capable of degrading cyclic ethers in the presence of chlorinated compounds.

Sample well locations were selected based upon several criteria including the location within the groundwater plume, the decrease in concentration over time at the location, and dissolved oxygen concentration in the water. While anaerobic degradation is an important pathway, it is significantly more difficult to study in a laboratory setting; therefore, aerobic conditions were considered. Bio-Trap™ samplers were not used, as they are a purchased item, and adding a step of culturing from that media was beyond the scope of this research. Therefore, simple low-flow sampling techniques were to be used.

The groundwater plumes of 1,4-dioxane and THF are well defined at SRSNE from the numerous sampling events over time and high spacial density of monitoring wells that range from the shallow overburden to deep bedrock (Figures 1-3, 1-4). Wells that were chosen were from both the overburden and bedrock, to determine if there were different organisms in the depths and if the sample locations themselves were different.

CPZ-6A

CPZ-6A is a middle overburden/deep overburden well with the screen between 9.13 feet below ground surface (ft. bgs) and 24.13 ft. bgs (Figure 2-1). It has been sampled since 1996, and has shown decreasing concentrations in 1,4-dioxane and THF, as well as other contaminants including trichloroethene. During the 2014 groundwater sampling event, the dissolved oxygen (DO) concentration was 1.29 mg/L (ARCADIS 2015).

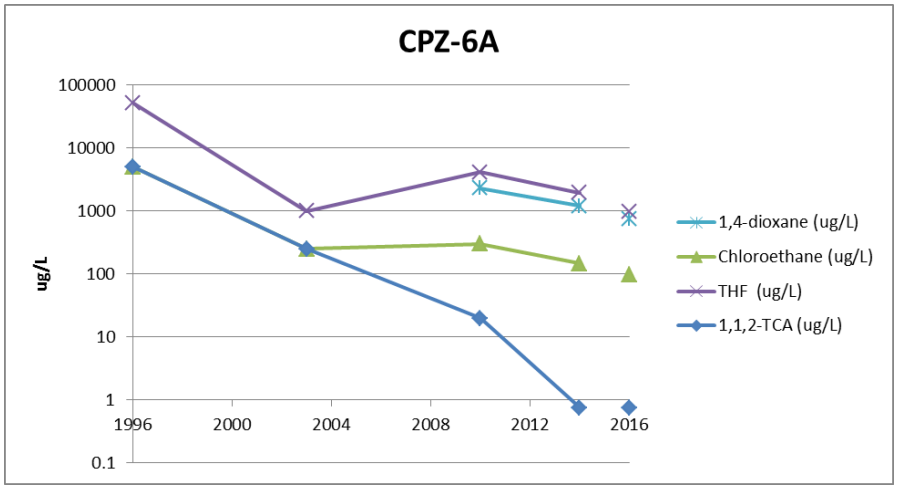

Figure 2-1. Concentration of various contaminants in well CPZ-6A over time.

MW-907M

MW-907M is a middle overburden well with the screen between 28.1 ft. bgs and 38.1 ft. bgs. It was recently developed in 2010 (Figure 2-2). The concentrations seen of THF and 1,4-dioxane are higher in 2014 than seen in the other two wells. During the 2014 groundwater sampling event, the DO was 0.93 mg/L (ARCADIS 2015).

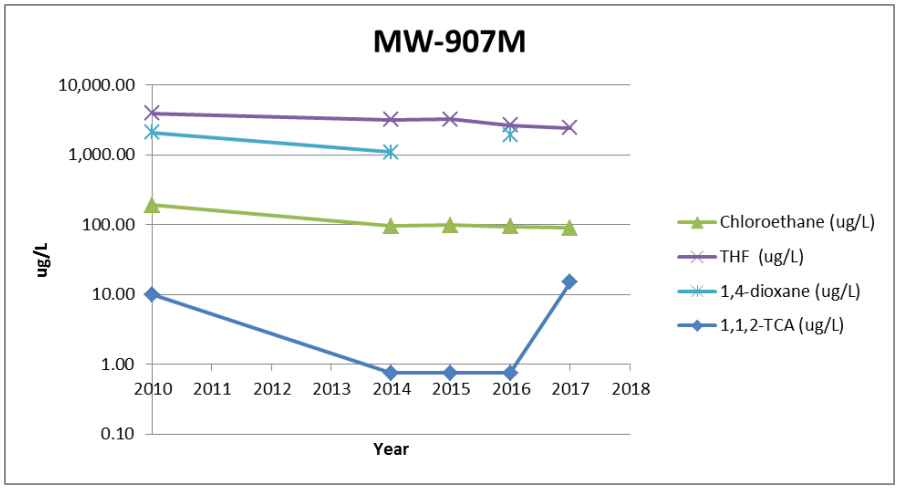

Figure 2-2. Concentration of various contaminants in well MW-907M over time.

P101-A

P101-A is a shallow bedrock well with the screen between 66 ft. bgs and 96 ft. bgs. It has been sampled since 1996, but was not sampled in 2003 (Figure 2-3). Therefore, although there are four data points, it is not as a complete dataset as CPZ-6A. During the 2014 groundwater sampling event, the DO was 5.0 mg/L (ARCADIS 2015).

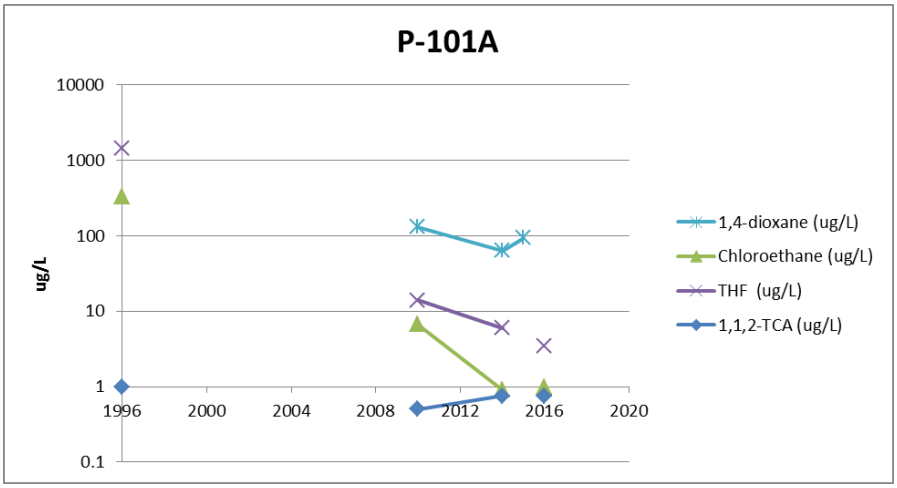

Figure 2-3. Concentration of various contaminants in well P-101A over time.

Groundwater Sampling

Overnight sampling was used to sample the three wells- CPZ-6A, P101-A, and MW-907M. HydraSleeve™ groundwater samplers were used to collect the samples. The HydraSleeve™ is a valved bag which once placed in the screened interval of the well will allow for water to move into the sampler (GeoInsight 2016). There is minimal disturbance of the well water, which means only the desired point in the well is sampled. A premeasured length of suspension line can be attached to the outside of the well, with the attachment spring clip on the other side (GeoInsight 2016).

Media Preparation

Bushnell-Haas broth (HiMedia M350) was the preferred growth media. Preparation of the Bushnell-Haas was done by suspending 3.27 grams of powder into 1.0 liter of distilled water. The mixture was stirred and its pH was adjusted to be between 7.20 and 7.24 standard units. The mixture was dispensed into bottles and sterilized by autoclaving at 15 lbs. pressure at 121^{0}C for 20 minutes. The bottles were stored at room temperature.

R2A agar (BD Difco 218261) was the preferred solid media for isolation. Preparation of the R2A agar was done by suspending 18.2 g of R2A powder to 1.0 liter of deionized (DI) water. The mixture was stirred and dispensed into a flask to be sterilized by autoclaving at 15 lbs. pressure at 121^0C for 20 minutes. Once cooled, Petri plates were poured and allowed to cool and solidify. Once solid, the plates were bagged and stored at room temperature.

Carbon Source Preparation

Initial carbon source was made by dispensing 1.0 mL of pure carbon source (either THF or 1,4-dioxane) to 100 mL of de-ionized (DI) water. The container was tightly closed and shaken lightly. Additional concentrations were made by adding 5.0 mL of pure carbon source to 100 mL distilled water and 50 mL of pure carbon source to 100 mL DI water. The diluted solutions were kept in the flammables cabinet unless being used in an experiment. Prior to use the container was shaken lightly to confirm solution. The dilutions were as follows (concentrations and molar equivalents):

Initial (1 ml/100 mL):

- THF: 12 micro molar (mM)
- 1, 4-dioxane: 12 mM

Low (5 mL/100 mL):

- THF: 62 mM
- 1, 4-dioxane: 59 mM

Moderate (50 mL/100 mL)

- THF: 616 mM
- 1, 4-dioxane: 586 mM

For additional experiments, trichloroethylene (TCE) and tetrachloroethylene (PCE or PERC) were diluted using the same technique. Both were diluted to 0.05 mM, and tetrachloroethylene was also diluted to a higher concentration of 0.2 mM.

Sample Site Diversity

Initial diversity was assayed using R2A agar plates. In 9 mL of 0.1% peptone broth, 1 mL of groundwater was aseptically added to the test tube. The test tube was closed, lightly shaken, and then 1mL of the inoculated 0.1% peptone broth was added to the next test tube. This process

was repeated until each groundwater sample had three sets of diluted 0.1% peptone broth. The sample was serially diluted to 10^{-5}. The process was repeated for the other groundwater samples.

R2A agar plates were used for culture isolation and enumeration. From each dilution tube 0.1 mL was plated to R2A agar. Colonies were counted daily for 7 days and different types were recorded to see differences between dilutions and wells. Shannon Index will be used to determine differences in original samples and diversity. Shannon Index was calculated using the equation [1]:

[1] $$H' = \sum_{i=1}^{R} p_i \ln p_i$$

Where p_i is the proportion of total organisms belonging to type i (Shannon 1948).

For microbial populations, Bio-Trap™ samplers were deployed in 2014 and again in 2016. The data obtained from the samplers uses cells per bead as the unit of measurement, as the BioTrap™ contains Bio-Sep© beads, which are an inert structural material (Nomex®) covered with powdered activated carbon (Seuker, Julie, ARCADIS, and Monica Heintz, ARCADIS 2016). These beads are 2 to 4 millimeters in diameter and are the surface the microbes colonize on (Seuker, Julie, ARCADIS, and Monica Heintz, ARCADIS 2016).

qPCR data collected from the site in 2014 and 2016 were analyzed to infer results regarding sample site diversity in a limited scope. The genetic testing looked for specific genes that show the potential for biodegradation of certain carbon substrates, or for specific phylogenetic genes which would indicate the presence of specific species of interest (Seuker, Julie, ARCADIS 2014). Two of the three wells were sampled in 2014, but all three were analyzed for specific functional genes in 2016 (Seuker, Julie, ARCADIS 2014 and Seuker, Julie, and Monica Heintz, ARCADIS 2016). In 2016 CPZ-6A was sampled in two places- middle overburden (MOB) and middle/deep overburden (MOB/DOB).

Enrichment Technique

Initial Enrichment

40 mL of Bushnell-Haas broth was added to nine 125 ml flasks. Each location had three flasks: one for 1,4-dioxane alone, one for THF alone, and one for both carbon sources. 6 mL of groundwater sample from the wells was added to each of the respective flasks. For each well

group (i.e. the three flasks with that well's water in it), one flask had 1mL of 12 mM THF, another had 1mL of 12 mM 1,4-dioxane and the third had 1mL each of 12 mM THF and 12 mM 1,4-dioxane. All flasks were incubated in a shaking incubator (120 rpm) at room temperature for seven days. After a week of growth, another 1 mL of carbon source(s) was added to the flask.

At two and a half weeks, cultures that did not grow were removed from the experiment. The remaining cultures were: CPZ 6A (THF), P101A (THF + 1,4-dioxane), MW-907M (THF), MW-907M (1,4-dioxane), and MW-907M (THF+1,4-dioxane).

Culturing Enriched Organisms

Samples that grew in the initial enrichment were selected further by a secondary enrichment, at two different concentrations. In a 125 mL flask, 40 mL of Bushnell-Haas broth and 10 mL of sample from the initial enrichment were combined along with the higher concentration of carbon source. The 6 successful enrichments were doubled to twelve- to account for the two different concentrations of carbon source to be used [62 or 616 mM THF; 58 or 586 mM 1,4-dioxane].

Growth of Isolates from Selected Carbon Source

9.6 mL of Bushnell-Haas broth was added to 3 125 mL flasks. The flasks were inoculated with 0.2 mL of culture and 0.2 mL substrate (THF [616 mM], 1,4-dioxane [586 mM] or mix). The samples were incubated at room temperature in a shaker for 10 days.

The optical density (OD) was taken on days 0, 2, 4, 6, 8 and 10 at 600 nanometers (nm). The OD of a bacterial culture is a way to measure the growth of the culture- the cells generally do not absorb the light, but prevent it from hitting the photoelectric cell (Widdel 2010).

Growth of Isolates with Varying Environmental Parameters

Effect of Aeration

The effect of aeration in growth of various isolates will be tested by adjusting the amount of media found in each culture. 0.2 mL of culture and 0.2mL of carbon source [616 mM THF; 586 mM 1,4-dioxane] were added to 125 mL flasks. The volume of Bushnell-Haas broth was varied to be 10 mL, 20 mL, 40 mL or 80 mL. Cultures were incubated at room temperature, shaking. Growth was determined at 600 nm by sampling each culture at days 0, 2, 4, 6, 8 and 10.

Effect of Carbon Concentration

The effect of carbon concentration on microbial growth was tested by varying the amount of carbon source found in each culture. Using the results from the *Effects of Aeration* experiment, the ideal volume of Bushnell-Haas broth, 10 mL, was added to 125 mL flasks along with 0.2 mL of culture. The volume of carbon provided was 0.2 mL, 0.1 mL, 0.01 mL or 0.005 mL of [616mM THF; 586mM 1,4-dioxane], with 0.2 mL acting as the control. Cultures were incubated at room temperature, shaking. Growth was determined at 600 nm by sampling each culture at days 0, 2, 4, 6, 8 and 10.

Growth after Prolonged Storage on Rich Media

Isolates were tested to determine if they would retain their degradation capacity after being grown on rich media (R2A agar). Samples were swabbed onto the R2A and grown for two weeks before the cultures were returned to Bushnell-Haas media. 10 mL of Bushnell-Haas broth, 0.2 mL of culture and 0.2 mL of carbon [616 mM THF; 586 mM 1,4-dioxane] were added to 125 mL flasks. Cultures were incubated at room temperature, shaking. Growth was determined at 600 nm by sampling each culture at days 0, 2, 4, 6, 8 and 10.

Effect of Aliphatic Chlorinated Compounds

As previous research has shown that organisms capable of degrading 1,4-dioxane and THF are inhibited by the presence of chlorinated compounds, diluted trichloroethylene (TCE) and tetrachloroethylene (PERC or PCE) were added to the hypothesized "ideal" conditions which would be derived from the earlier experiments (10 mL Bushnell Haas, 0.2 mL carbon [616 mM THF; 586 mM 1,4-dioxane]). Isolates were grown in Bushnell-Haas with TCE [0.05 mM] or PERC [0.05 mM]. The control was unaltered Bushnell-Haas with the choice carbon source.

Growth on Aliphatic Chlorinated Compounds

After the results of the previous experiment, samples were grown on PERC [0.2 mM] as the sole carbon source. 10 mL of Bushnell Haas was combined with 0.2 mL of culture and 0.2 mL of diluted PERC. A control of the isolate growing with choice carbon source (THF, 1,4-dioxane or mix [616 mM THF; 586 mM 1,4-dioxane] was used to determine if there was a difference in growth between the two. Samples were incubated at room temperature in a shaker for 10 days. Growth was determined at 600 nm by sampling each culture at days 0, 2, 4, 6, 8 and 10.

Gram Staining

Sterile swabs were used to collect samples of the isolates to grow on R2A plates. Samples of isolates were fixed onto glass slides. A primary stain (crystal violet) was added to the slide for one minute, and then washed gently with DI H_2O for 5 seconds to remove excess stain. Iodine was then added to the slide and left for one minute, then rinsed with the decolorizer (ethanol or acetone) for 3 seconds before being rinsed with DI H_2O. The secondary stain, safranin, was added to the sample for one minute, and then washed with DI H_2O for 5 seconds.

Growth of Isolates from Selected Carbon Source- Carbon Dioxide Evolution

48mL of Bushnell-Haas broth was added to each biometer flask, 1.0 mL of culture and 1.0 mL of substrate (THF, 1, 4-dioxane [616 mM THF; 586 mM 1,4-dioxane] or mix OR 0.2 mM PERC). The ascarite tower was replaced, and 10 mL of potassium hydroxide (KOH) was added to the sidearm. The large stopper was replaced and the smaller stopper was placed on top of the syringe. The samples were incubated at room temperature, not shaking (Figure 2-4). The KOH was removed and titrated on days 2, 4, 6, 8 and 10.

On days 2, 4, 6, 8 and 10 stopcock to the ascarite tower was opened and the stopper was removed. Using a syringe, the KOH was removed from the sidearm and transferred to a separate flask and sealed with a rubber stopper. The KOH in the sidearm was replaced using 10 mL of fresh 0.2N KOH, and the stopcock was closed and the rubber stoppers replaced. 1 mL of saturated barium chloride and 0.1 mL of phenolphthalein were added to the recovered KOH samples and to a control fresh 10mL of KOH. The KOH solutions were titrated with 0.05N HCl until the solution turned colorless. CO_2 [mg] was determined by the equation [2] (Cerqueira et al. 2011):

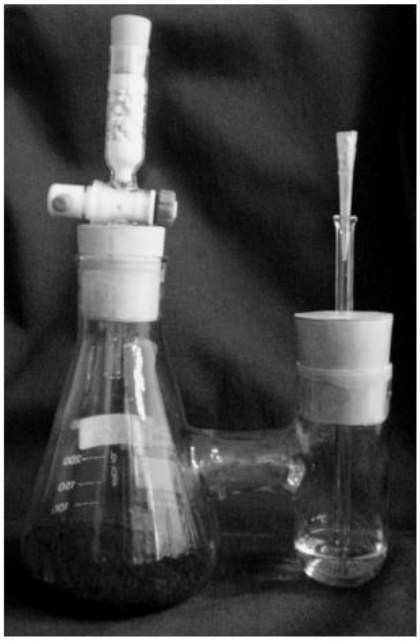

Figure 2-4. Setup of a biometer flask used for CO_2 evolution determination (Gonçalves and Franchetti 2015).

[2] $$CO_2\ evolved\ (mg) = (V_C - V_E)\left(mol\ {}^{CO_2}/_{2}\right)(molarity\ HCl)(CF)$$

where $Correction\ Factor\ (CF) = {}^{molarity\ HCl}/_{molarity\ KOH}$

V_C = volume to titrate control KOH

V_E = volume to titrate experimental KOH

Growth Analysis

During each growth experiment, the turbidity of the samples were read at 600 nm on a UV/VIS spectrophotometer. 0.3 mL of sample was diluted with 1.0 mL of DI H_2O. The absorbance was the multiplied by a constant to account for dilution.

To produce a single value that could be used for statistical analysis, the specific growth rate (μ) [3] in $days^{-1}$ and generation times (g) [4] in days were calculated. These constants were calculated using the exponential phase of growth. Two values taken from each sample point were used- the time measured (t) and the OD reading from that time point. OD_2 and t_2 are the higher absorbance measured and the later date than OD_1 and t_1 (Widdel 2010).

[3] $$\mu = \frac{\ln OD_2 - \ln OD_1}{t_2 - t_1}$$

[4] $$g = \frac{\ln 2}{\mu}$$

The constants were used in ANOVA 2-way analyses. When results showed statistically significant results, Tukey HSD and Dunnett Method were used to find the specific points of difference. Dunnett Method was used when a control was in the experiment.

For the first chlorinated solvent experiment, an average control was produced for each sample. This was created by averaging the controls from the previous experiments. This was done because with the amount of samples being analyzed, it was not time-economically feasible to split the experiment into two groups, and do five experiments per culture. An average control would also be more statistically useful than single data points. The average control was compared to the prior control data to determine if it was statistically different, and it was shown

to not be, and therefore valid to use in the analyses. This average control was also used in the analysis of whether the cultures lost degradation capacity when grown on rich media.

Results

Sample Site Diversity

The results from this experiment are limited. The Shannon Index only covers organisms that were able to grow quickly on aerobic R2A plates. There may be many more organisms that were not seen during the experiments. Any conclusions based off of this data are not conclusive to the entire population. Not all samples had enough growth to be used in the Shannon Index analysis. R2A plates needed to have between 10 and 250 colonies (Figures 2-5 through 2-8). Of the 36 plates, only 7 had the right quantity of colonies to be useable in the analysis (3 from CPZ-6A, 3 from P101-A and 1 from MW907-M).

Initial Enrichment

Initial enrichment was done over an extended timeline, to allow for any potential growth of even a small population of organisms. The initial concentration of THF was 12 mM and 1,4-dioxane was 12 mM. Growth was determined via visual appearance of flocs in the Bushnell Haas media. Of the nine initial flasks, three did not have growth and were removed from the experiment. The six remaining flasks were then doubled to test higher concentrations- 62 and 616 mM of THF; 59 and 586 mM of 1,4-dioxane. Samples that did not show any growth via visual inspection were removed from the experiment (Figures 2-9 and 2-10).

Initial Degradation

Initial degradation tests were done and experiments were done in triplicate for sufficient statistical analysis. Samples were grown with 1mL of THF or 1,4-dioxane or an equal mix of the two (Table S-1; Figures 3-1, 3-2, 3-3). Of the four THF experimental samples MW-907M low (62 mM), high (616 mM) and CPZ-6A low (62 mM) and high (616 mM), MW-907M low had the highest specific growth rate (μ). However, during the growing process CPZ-6A high began growing fungal colonies in the Bushnell Haas and was kept in the experiments because of this abnormality.

Of the four THF + 1,4-dioxane experimental samples, MW-907M low (616 mM THF + 586 mM 1,4-dioxane), high (616 mM THF + 586 mM 1,4-dioxane) and P101-A low (616 mM THF + 586 mM 1,4-dioxane) and high (616 mM THF + 586 mM 1,4-dioxane), there was no statistical difference between the means. However, both of the P101-A samples did not grow as readily as the MW-907M samples, and were removed from the rest of the experiments.

As there were only two 1,4-dioxane experimental samples, MW-907M low (59 mM) and high (586 mM), the lack of statistical difference between the means is difficult to interpret. However, it is not surprising that there was no difference, as the two experimental samples were from the same starting location (Table 1-1 and 1-2).

The samples from this point forth will be labeled:

- CPZ-6A (isolated on 616 mM THF)
- MW-907M A (isolated on 62 mM THF)
- MW-907M B (isolated on 58 mM 1,4-D)
- MW-907M C (isolated on 616 mM THF + 586 mM 1,4-D)

Table 1-1. Two-way ANOVA analysis comparing growth rates of initial isolates (p-value $\leq$ 0.05).

Grouping	P-value	F-value	Total df
THF Samples	0.019	6.04	11
THF + 1,4-D Samples	0.798	0.34	11
1,4-D Samples	0.093	4.81	5

Table 1-2. Tukey Pairwise Comparisons for THF Samples. Means that do not share a letter are significantly different.

Factor	N	Mean	Grouping	
MW-970M THF A	3	0.2283	A	
MW-970M THF B	3	0.13342	A	B
CPZ-6A THF A	3	0.1011		B
CPZ-6A THF B	3	0.099		B

Aeration

There was no statistical difference in the growth based on the volume of Bushnell Haas the isolates were grown in (Table 2). Mean μvalues for each of the experimental cultures did not differ significantly, and this was also seen in the turbidity growth analysis (Table S-2; Figures 4-1 through 4-4). Therefore for all subsequent experiments samples were grown in 10mL of Bushnell Haas media.

Table 2. Two-way ANOVA analysis comparing growth rates of isolates based on the volume of Bushnell-Haas in the culture (p-value ≤ 0.05).

Isolate	P-value	F-value	Total df
CPZ-6A	0.749	0.41	11
MW-970M A	0.731	0.44	11
MW-970M B	0.196	1.98	11
MW-970M C	0.824	0.3	11

Effect of Carbon Concentration

There was no statistical difference in the growth of isolates based on the reduced amount of carbon provided (Table 3). Mean μ values for each of the experimental cultures did not differ significantly, and this was also seen in the turbidity growth analysis (Table S-3; Figures 5-1, 5-2, 5-3, 5-4). Therefore for all subsequent experiments samples were grown with the highest amount of carbon.

Table 3. Two-way ANOVA analysis comparing growth rates of isolates based on the volume of carbon provided (p-value ≤ 0.05)

Isolate	P-value	F-value	Total df
CPZ-6A	0.637	0.59	11
MW-970M A	0.39	1.14	11
MW-970M B	0.812	0.32	11
MW-970M C	0.69	0.5	11

Growth after Prolonged Storage on Rich Media

After being grown on R2A agar for two weeks, there was no statistical difference in growth between the cultures returned to Bushnell-Haas and the control (Tables 4 and S-4; Figures 6-1 through 6-4).

Table 4. Two-way ANOVA analysis comparing growth rates of isolates that had been stored on rich media for a prolonged period of time (p-value ≤ 0.05).

Grouping	P-value	F-value	Total df
CPZ-6A	0.495	0.56	5
MW-970M A	0.485	0.59	5
MW-970M B	0.089	5.02	5
MW-970M C	0.568	0.39	5

Effect of Aliphatic Chlorinated Compounds

One of the four isolates was significantly impacted by the presence of the chlorinated compounds (Table S-5; Figures 7-1 through 7-4). CPZ-6A (THF) (p-value = 0.012) was further analyzed to determine what was the source of the differences (Table 5-1).

CPZ-6A: The Dunnett Method showed that the control and PERC samples were similar; while the two TCE samples were significantly different from the control level mean (Table 5-2). When further analyzed- separating the PERC and TCE samples into two ANOVA analyses- CPZ-6A (THF) was significantly impacted by the presence of TCE (p-value = 0.015) in both low (0.005mL) and high concentrations (0.2mL) and less impacted by the presence of PERC (p-value = 0.094) (Table 5-3).

Table 5-1. Two-way ANOVA analysis comparing growth rates of isolates based on the volume of aliphatic chlorinated compounds added to the culture (p-value ≤ 0.05).

Isolate	P-value	F-value	Total df
CPZ-6A	0.012	5.62	14
MW-970M A	0.648	0.64	14
MW-970M B	0.076	2.94	14
MW-970M C	0.755	0.47	14

Table 5-2. Tukey Pairwise Comparisons for CPZ-6A to determine which aliphatic chlorinated compound was impacting the culture. Means that do not share a letter are significantly different.

Isolate	N	Mean	Grouping	
0.1232 mM THF + $1.00x10^{-5}$ mM PERC	3	0.1914	A	
0.1232 mM THF + $2.5x10^{-7}$ mM PERC	3	0.1355	A	B
0.1232 mM THF + $1.00x10^{-5}$ mM TCE	3	0.1068	A	B
0.1232 mM THF + $2.5x10^{-7}$ mM TCE	3	0.0733		B
Control (0.1232 mM THF)	3	0.05104		B

Table 5-3. Further inspection of the impact of the presence of aliphatic chlorinated compounds on isolate CPZ-6A (p-value ≤ 0.05).

ANOVA Analysis	p-Value	F-value	Total df
0.1232 mM THF + $1.00x10^{-5}$ mM PERC, 0.1232 mM THF + $2.5x10^{-7}$ mM PERC vs. Control	0.094	3.59	8
0.1232 mM THF + $1.00x10^{-5}$ mM TCE, 0.1232 mM THF + $2.5x10^{-7}$ mM TCE vs. Control	0.015	9.07	8

Growth on Aliphatic Chlorinated Compounds

Due to the results from the previous experiment, subsequent experiments with PERC were conducted. Isolates were grown in Bushnell-Haas with PERC [0.2 mM] as the sole carbon source, with a combination of both the standard cyclic ether and PERC, and a control of just the standard cyclic ether (Table S-6; Figures 8-1, 8-2, 8-3, and 8-4). Most isolates were not impacted by the presence and were capable of degrading the PERC [0.2 mM].

Of the four isolates, one had somewhat statistically significant results. MW907-M B had an impact from being grown on just PERC (p-value = 0.07) (Table 6-1). The mean specific growth rate for the samples grown with just PERC was greater than the mean specific growth rates of both the control and the combination of 1,4-dioxane and PERC (Table 6-2).

Table 6-1. Two-way ANOVA analysis comparing growth rates of isolates when grown with solely PERC as the carbon source, or when combined with choice carbon source (p-value $\leq$ 0.05).

Isolate	P-value	F-value	Total df
CPZ-6A	0.127	2.97	8
MW-970M A	0.586	0.58	8
MW-970M B	0.772	0.27	8
MW-970M C	0.07	4.27	8

Table 6-2. Details from ANOVA analysis for MW-970M B. The mean for the sample that grew just on $4.00x10^{-5}$ mM PERC was higher than the mean for the combined or the control.

Factor	N	Mean	StDev	95% CI
Control	3	0.0782	0.0365	(0.0035, 0.1530)
0.0116 mM 1,4-D + $4.00x10^{-5}$ mM PERC	3	0.0721	0.0408	(-0.0026, 0.1469)
$4.0x10^{-5}$ mM PERC	3	0.1844	0.0735	(0.1097, 0.2592)

Gram Staining

All samples were shown to be Gram negative cocci. There was little difference in the appearance of the different samples between the wells.

CO_2 Evolution

Two series of experiments for CO_2 evolution were completed: one with the standard cyclic ethers as the sole carbon source, and one with PERC as the sole carbon source. It was

important to determine if there was a difference in degradation capacity with the two substrates; if there was no difference it could mean that different metabolic pathways could be the source of the degradation.

Standard carbon substrate (Table 7-1): On average, CPZ-6A evolved 11.5% ± 2.9% of the carbon available into CO_2. MW-907M A evolved 14.9% ± 3.8% of the carbon available into CO_2. MW-907M B evolved 9.9% ± 0.4% of the carbon available into CO_2. MW-907M C evolved 16.9 ± 1.5% of the carbon available into CO_2. It is unknown what percentage of the THF or 1,4-dioxane was degraded, and if they were degraded completely or partially.

PERC (Table 7-2): On average, CPZ-6A evolved 71.2% ± 17.1% of the carbon available into CO_2. MW-907M A evolved 64.9% ± 9.2% of the carbon available into CO_2. MW-907M B evolved 91% ± 7.1% of the carbon available into CO_2. MW-907M C evolved 84.4% ± 17.1% of the carbon available into CO_2. Again, it is unknown what percentage of the PERC was completely degraded or only partially degraded, and what the metabolic byproducts were.

Table 7-1. Average biodegradation of cyclic ethers by isolates. Total degradation (mg CO_2 evolved) was determined after 10 days.

Isolate	C evolved (mg)	Percent C evolved
CPZ-6A	3.4	11.47% ± 2.86%
MW-970M A	4.42	14.94% ± 3.76%
MW-970M B	5.71	9.89% ± 0.42%
MW-970M C	4.76	16.9% ± 1.52%

Table 7-2. Average biodegradation of PERC by isolates. Total degradation (mg CO_2 evolved) was determined after 10 days.

Isolate	C evolved (mg)	Percent C evolved
CPZ-6A	3.42	71.17% ± 17.14%
MW-970M A	3.12	64.89% ± 9.19%
MW-970M B	4.37	91.03% ± 7.07%
MW-970M C	4.05	84.35% ± 17.08%

Discussion

Initial Enrichment from Contaminated Groundwater

Considering historical research, and the expected problems that arose with previous degraders, the speed of growth and ease of culturing was unexpected. Samples grew readily on both media and isolation via substrate selection occurred with relative ease. The final samples mainly came from MW907-M, as the isolates from P101-A had less growth based on turbidity and CPZ-6A was unable to grow on some of the substrates.

Sample Site Diversity- Overall Diversity

. CPZ-6A had $2.67x10^6$ cells/bead of total eubacteria, while MW-907M had $1.58\ x10^6$ cells/bead total eubacteria (Table 9-1) (Seuker, Julie, ARCADIS 2014). CPZ-6A had higher concentrations of both methanogens ($2.0\ x10^3$ cells/bead) and sulfate reducing bacteria ($8.71x10^5$ cells/bead) than MW-907M ($5.87x10^2$ cells/bead and $3.89x10^2$ cells/bead respectively (Seuker, Julie, ARCADIS 2014). CPZ-6A had high concentrations of *Desulfitobacterium spp*. ($2.5x10^2$ cells/bead), an organism capable of degrading PCE and TCE (Seuker, Julie, ARCADIS 2014). MW-907M had relatively low numbers of all cVOC degraders and low numbers of aerobic co-metabolic enzymes present (Seuker, Julie, ARCADIS 2014). *Dehalococcoides spp.*, the most well-known cVOC degrader, was found in low concentrations in both wells ($2.5x10^3$ cells/bead in CPZ-6A and $3.72x10^2$ cells/bead in MW-907M) (Seuker, Julie, ARCADIS 2014). These data may suggest that the organisms in this environment are likely not known cVOC degraders or that this degradation path is not their main source of energy.

Sample Site Diversity- qPCR Results

Multiple research articles have looked at the genetic information of 1,4-dioxane and THF degraders. A variety of pathways, both direct metabolism and co-metabolism, have been found and a multitude of functional genes have been determined to play a role in degradation. The main co-metabolic pathways for 1,4-dioxane use methane (MMO), propane (PrMO or PPO), phenol (PHE), THF (THFMO) or toluene (RDEG, RMO, T2MO, T4MO, T*p*MO) monoxygenases (Gedalanga et al. 2014; Mahendra and Alvarez-Cohen 2006). Dioxane Monooxygenase (DXMO) is the main monooxygenase known for dioxane degradation, and is used as the taxonomic identifier for *Pseudonocardia dioxanivorans* CB1190 and other known dioxane degraders (Gedalanga et al. 2014). Alcohol (DDH) and Aldehyde Dehydrogenase (ALDH) are known to be

a part of the degradation process of THF for *Pseudonocardia tetrahydrofuranoxydans* strain K1 (Gedalanga et al. 2014).

In 2014, functional and phylogenetic tests were run primarily to determine what cVOC degraders were found in the two wells. One functional gene is relevant regarding the chlorinated tests done- Toluene Dioxygenase (TOD). TOD is capable of co-metabolism of TCE (Microbial Insights 2015). CPZ-6A had $2.47x10^5$ cells/bead with TOD, while MW-907M had $2.75x10^3$ cells/bead (Microbial Insights 2015) (Table 9-2).

The 2016 functional gene analysis provided diverse results. The biggest surprise was that all three wells showed very low concentrations of DXMO, ALDH and PPO (Seuker, Julie, ARCADIS, and Monica Heintz, ARCADIS 2016) (Table 9-2). All three wells had high quantities of PHE (Seuker, Julie, ARCADIS, and Monica Heintz, ARCADIS 2016). All three wells also showed high quantities of soluble MMO (SMMO) and low quantities of PPO (Seuker, Julie, ARCADIS, and Monica Heintz, ARCADIS 2016).

While known functional genes DXMO and ALDH were not found on site, there is known degradation occurring. This means that there is likely either an unknown directly degrading monooxygenase or co-metabolic degradation of these cyclic ethers.

Environmental Parameters- Aeration and Carbon Concentration

The lack of impact on the growth of the isolates based on the volume of Bushnell-Haas media was not surprising, considering the low DO found in the original environment. From the volumes and concentrations tested, the organisms were not limited nor harmed by the concentration of cyclic ether being provided. This was an initial concern, as the majority of experiments were being conducted with concentrations higher than the standard 50 mg/L CB1190 has been grown on (Mahendra and Alvarez-Cohen 2006).

Growth after Prolonged Storage on Rich Media

There was no impact on the growth of the isolates after being stored on R2A agar. This is different from *P. dioxanivorans* CB1190, which grows poorly on all rich media. The ease of culturing these organisms makes them a much more appealing organism for future biodegradation research.

Impact of Aliphatic Chlorinated Compounds

The biggest surprise of these series of experiments was that the majority of samples were not negatively impacted by the presence of chlorinated compounds; in fact many were able to grow on just PERC. One sample (CPZ-6A THF) was negatively impacted by the presence of TCE, but was not negatively impacted by PERC and was able to grow on PERC.

Growth on Aliphatic Chlorinated Compounds

On average, the majority of isolates were capable of degrading PERC as well as their preferred cyclic ether. This may indicate that the isolated samples are actually a consortium of organisms, or that the singular organism has different metabolic pathways.

CO_2 Evolution

On average, samples evolved more of the PERC into CO_2 than the preferred cyclic ether. This may be due to the significantly smaller concentration of PERC over cyclic ether, or there may have been a previously dormant organism in the culture which was able to degrade the PERC. Regardless, the capacity for degradation of chlorinated compounds may contribute to why we may have seen degradation in the groundwater on site. If the organisms degrading 1,4-dioxane and THF can also degrade chlorinated compounds, then it would lack one of the biggest problems *P. dioxanivorans* CB1190 has- its degradation capacity is reduced in the presence of less chlorinated compounds (TCA and DCE) (Mahendra, Grostern, and Alvarez-Cohen 2013).

16s RNA Analysis

Taxonomic analysis of the 16s data was provided by MR DNA. After processing, operational taxonomic units (OTUs) were defined with 97% similarity (Table 10). OTUs are a practical way for individuals working in the ecological community to use modern DNA analyses for taxonomy (Caron et al 2009). Consecutive OTUs do not have a close phylogenetic relationship, and the use of OTUs overall could provide better taxonomy results than just present morphological, physiological, and behavioral data (Caron et al 2009). Final OTUs were taxonomically classified using BLASTn against a databased derived from GreenGenes, RDPII and NCBI ("Data Processing (basic Overview)."). Two values were provided- count and percent. Count is the number of sequences in the same, while percent is the relative proportion percentage of the sequences within each sample or taxonomic breakdown (i.e. kingdom) to the designated taxonomic definition ("Data Processing (basic Overview)."). Based on the percent identity, the

OTUs were identified to the specific taxonomic level ("Data Processing (basic Overview).") (Table 16). All known isolates that are capable of degrading THF and 1,4-dixoane were not found in the samples. A variety of sulfur and methane degrading organisms were found, along with a wide variety of others (Tables 11-1 through 11-4 for abridged results).

Of the more than 400 OTUs in the database, six were found to be the most prolific (based on count and percent). All of the isolates have not been previously seen in papers regarding degradation of THF and 1,4-dioxane, meaning that there may be unknown mechanisms or co-metabolism occurring. Two samples- MW-907M B and C- had the same two organisms found (Table 12). The OTUs with the highest counts and percents were:

- *Acidobacterium spp*. (MW THF)
- *Pseudofulvimonas gallinarii* (MW THF)
- *Pseudomonas resinovorans* (MW 1,4-D and MW THF+1,4-D)
- *Bradyrhizobium elkanii* (MW 1,4-D and MW THF+1,4-D)
- *Rhizobium spp.* (CPZ-6A)
- *Exophiala xenobiotica* (CPZ-6A)

Brief information on previous research on isolates will be covered. *Acidobacterium* is a phylum which is found abundantly in soils, but is poorly covered in bacterial cultures (Kielak, Anna M et al. 2016). When properly analyzed the phylum can represent over 50% of the bacterial community found in soil and 20% of the overall microbial community, even in diverse soils (Kielak, Anna M et al. 2016). *Pseudofulvimonas gallinarii* is a new organism with very little information available- the single paper was published in 2010 (Kämpfer et al. 2010). It is a Gram-negative rod shaped bacterium isolated from the air in a duck barn (Kämpfer et al. 2010). Isolates of *Pseudomonas resinovorans* from soil samples have been found to be capable of transforming eugenol into vanillin and other related phenolic compounds (Ashengroph 2011). *Bradyrhizobium elkanii* are an aerobic, motile Gram negative rod which are found as either free-living organisms or plant symbionts in soil (Crovadore 2016). *Rhizobium* are a well-known group of symbiotic nitrogen-fixing bacteria that are found near the roots of plants, and have been found in contaminated soils (Teng et al 2011). One isolate was found to increase the phytoremediation by alfalfa of polycyclic aromatic hydrocarbons (PAHs) (Teng et al 2011). *Exophiala xenobiotica* is black yeast which is often found on human skin, but has been found in

environments rich in hydrocarbons, including oil and gasoline contaminated soils (de Hoog, Zeng and Harrak 2006). *E. xenobiotica* has also been found in car gasoline tanks, but likely have very low degradation rates (Isola et al. 2013). Even with the lack of specifically known enzymes, *E. xenobiotica* was able to degrade toluene fairly well (Isola et al. 2013).

The majority of organisms found were not previously known to be capable of degrading the cyclic ethers and aliphatic chlorinated compounds tested. As more than one organism was found in each sample, it is likely that there is a consortium of organisms co-metabolizing the compounds.

Conclusions

From an environment expected not to be conducive for degradation of cyclic ethers, somehow a significant portion of the THF and 1,4-dioxane found in a portion of the groundwater plume at SRSNE has been removed from the system. As there are not known abiotic means for this having occurred, experiments were completed to determine if there was a biotic source behind the removal.

Organisms were isolated from three groundwater wells, CPZ-6A, MW-907M and P101-A. Of these three, P101-A degraders were slower and had poorer mean growth rates than the other two. Cultures were enriched with high quantities of THF and 1,4-dioxane to promote growth of degrading organisms. Cultures remained in a liquid state unless testing with R2A agar.

Experiments were completed to determine if there was an impact on growth based on aeration, carbon available, and the impacts of prolonged storage on rich media as well as the presence of aliphatic chlorinated compounds. Samples were further tested to determine if they were capable of degrading solely the aliphatic chlorinated compound PERC. The release of CO2 by the organisms was calculated along with the percent degradation. It is unknown if there was complete or only partial degradation of the compounds.

Since 2016, the potential for degradation of THF and 1,4-dioxane has been seen in the functional gene data from the SRSNE Site, but there was no direct evidence that actual biological degradation was occurring beyond decreasing concentrations with time and distance from the source area. With this research, confirmation of biological degradation has been made. While it is unknown whether the degradation is completed by direct or co-metabolic pathways, the enzymes seen in degradation by Pseudonocardia dioxanivorans CB1190 was not found. As multiple organisms were isolated from each isolate, there is support for the theory that complex consortia of organisms are degrading the chemicals. This could explain why there was little to no impact based upon the concentration of carbon source available and the addition of aliphatic chlorinated compounds. However, this could also indicate that unknown dioxane or THF monooxygenases may be the source of the degradation. The majority of organisms isolated have not been found in other research regarding the degradation of THF and 1,4-dioxane.

Emerging contaminants are a significant source of issues in environmental science. Improving bioremediation on all chemicals, especially ones that were first believed to be recalcitrant, may help us increase the amount of groundwater that would be safe for consumption or usage. A star degrader similar to Dehalococcoides spp. has not yet been identified for the degradation of cyclic ethers, as ones previously isolated have issues that would make them not cost-effective. The potential isolation and identification of multiple organisms that are easy to culture and degrade at very high concentrations of THF and 1,4-dioxane could indicate that in the future, bioremediation of these compounds could be an effective reality.

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Tables

Table 9-1. Abridged 2014 Baseline Microbiological Survey. Samples with fewer than 250 cells/bead were under the practical quantitation limit. Gene types are Functional gene (F) and Phylogenetic gene (P) to indicate what the focus of the analysis was on.

Gene Target		**Unit**	**Gene Type**	**CPZ-6A**	**MW-907M**
Dehalococcoides spp.	DHC	cells/ bead	P	2.50E+03	3.72E+02
Dehalobacter spp.	DHBt	cells/ bead	P	<2.5E+02	<2.5E+02
Desulfitobacterium spp.	DSB	cells/ bead	P	3.18E+05	<2.5E+02
Desulfuromonas spp.	DSM	cells /bead	P	<2.5E+02	<2.5E+02
BAV1 Vinyl Chloride Reductase	BVC	cells/ bead	F	<2.5E+02	<2.5E+02
Vinyl Chloride Reductase	VCR	cells/ bead	F	<2.5E+02	<2.5E+02
Dehalogenimonas spp	DHG	cells/ bead	P	<2.5E+02	<2.5E+02
Dehalobacter DCM	DCM	cells/ bead	P	<2.5E+02	<2.5E+02
Chloroform reductase	CFR	cells/ bead	F	<2.5E+02	<2.5E+02
Dehalobium chlorocoercia	DECO	cells/ bead	P	<2.5E+02	<2.5E+02
Trichloroethene Reductase	TCE	cells / bead	F	<2.5E+02	<2.5E+02

Table 9-1 cont**.** Abridged 2014 Baseline Microbiological Survey. Samples with fewer than 250 cells/bead were under the practical quantitation limit. Gene types are Functional gene (F) and Phylogenetic gene (P) to indicate what the focus of the analysis was on.

Gene Target		**Unit**	**Gene Type**	**CPZ-6A**	**MW-907M**
Aerobic Co-metabolism					
Soluble Methane Monooxygenase	SMMO	cells/ bead	F	2.28E+04	5.49E+04
Particulate Methane Monooxygenase	PMMO	cells/ bead	F	1.58E+04	5.29E+02
Toluene Dioxygenase	TOD	cells/ bead	F	2.47E+05	2.75E+03
Phenol Hydroxylase	PHE	cells/ bead	F	2.22E+04	7.45E+03
Toluene Monooxygenase 2	RDEG	cells/ bead	F	7.18E+03	<2.5E+02
Toluene Monooxygenase	RMO	cells/ bead	F	4.09E+04	1.18E+04
Epoxyalkane transferase	EtnE	cells/ bead	F	<2.5E+02	<2.5E+02
Ethene Monooxygenase	EtnC	cells/ bead	F	<2.5E+02	<2.5E+02
Trichlorobenzene Dioxygenase	TCBO	cells/ bead	F	<2.5E+02	<2.5E+02
Other					
Methanogens	MGN	cells/ bead	F	2.00E+03	5.87E+02
Sulfate Reducing Bacteria	APS	cells/ bead	F	8.71E+05	3.89E+02
Total Eubacteria	EBAC	cells/ bead	P	2.67E+06	1.58E+06

Table 9-2. Abridged 2016 Microbial Survey. Microbial Samples with fewer than 250 cells/bead were under the practical quantitation limit. Gene types are Functional gene (F) and Phylogenetic gene (P) to indicate what the focus of the analysis was on.

Gene Target		**Unit**	**Gene Type**	CPZ-6A		MW-907M	P-101A
				MOB	MOB, DOB	MOB	SBR
Dioxane Monooxygenase	DXMO	cells/ bead	F	<2.5E+02	<2.5E+02	<2.5E+02	<2.5E+02
Aldehyde Dehydrogenase	ALDH	cells/ bead	F	<2.5E+02	<2.5E+02	<2.5E+02	<2.5E+02
Propane Monoxygenase	PPO	cells/ bead	F	3.55E+02	6.11E+02	<2.5E+02	<2.5E+02
Soluble Methane Monooxygenase	SMMO	cells/ bead	F	4.93E+02	3.55E+03	8.72E+03	8.70E+03
Phenol Hydroxylase	PHE	cells/ bead	F	2.27E+04	3.93E+04	7.19E+03	1.52E+03
Toluene Monooxygenase 2	RDEG	cells/ bead	F	1.16E+04	2.53E+04	2.56E+03	<2.5E+02
Toluene Monooxygenase	RMO	cells/ bead	F	6.85E+02	1.52E+04	1.70E+04	3.54E+02

Table 10. Identity to reference sequence and designation from MR RNA.

Identity to reference sequence	**Identity Designation**
> 97%	Species
Between 97% and 95%	(unclassified Genus)
Between 95% and 90%	(unclassified Family)
Between 90% and 85%	(unclassified order)
Between 85% and 80%	(unclassified class)
Between 80% and 77%	(unclassified phylum)
< 77%	(unknown)

Table 11-1. MW-907M A abridged results from OTU classifications. List includes some known methanogens, nitrogen-fixing bacteria, and sulfur reducing bacteria.

OTU Classification	Count	% Match
Acidobacterium spp.	47799	24.43%
Pseudofulvimonas gallinarii	30594	15.64%
Mesorhizobium loti	18063	9.23%
Pseudomonas resinovorans	14111	7.21%
Bradyrhizobium elkanii	11445	5.85%
Pseudonocardia sulfidoxydans	4632	2.37%
Methylosinus spp.	2620	1.34%
Nitrobacter spp.	65	0.03%
Methylocystis spp.	50	0.03%
Methylosinus trichosporium	33	0.02%
Methylocystis sp.	12	0.01%

Table 11-2. MW-907M B abridged results from OTU classifications. List includes some known methanogens, nitrogen-fixing bacteria, and sulfur reducing bacteria.

OTU Classification	Count	% Match
Bradyrhizobium elkanii	6118	19.61%
Pseudomonas resinovorans	7112	15.50%
Pseudoxanthomonas yeongjuensis	855	14.45%
Aminobacter anthyllidis	160	12.50%
Acidovorax facilis	11002	6.16%
Pseudonocardia sulfidoxydans	11034	0.22%
Nitrobacter spp.	9	0.11%
Methylosinus spp.	62	0.08%
Methylocystis sp.	1	0.04%
Methylosinus trichosporium	17	0.01%
Methylobacterium thiocyanatum	10	0.01%

Table 11-3. MW-907M C abridged results from OTU classifications. List includes some known methanogens, nitrogen-fixing bacteria, and sulfur reducing bacteria.

OTU Classification	Count	% Match
Pseudomonas resinovorans	44612	22.24%
Bradyrhizobium elkanii	41379	20.63%
Pseudoxanthomonas yeongjuensis	18099	9.02%
Aminobacter anthyllidis	17700	8.82%
Acidovorax facilis	12062	6.01%
Nitrobacter spp.	290	0.14%
Pseudonocardia sulfidoxydans	97	0.05%
Methylosinus spp.	92	0.05%
Methylocystis sp.	57	0.03%
Methylosinus trichosporium	32	0.02%
Methylobacterium thiocyanatum	12	0.01%

Table 11-4. CPZ-6A abridged results from OTU classifications. List includes some known methanogens, nitrogen-fixing bacteria, and sulfur reducing bacteria. Note that *Exophiala xenobiotica* is the relative proportion compared to all other fungus in that sample, not the entire sample.

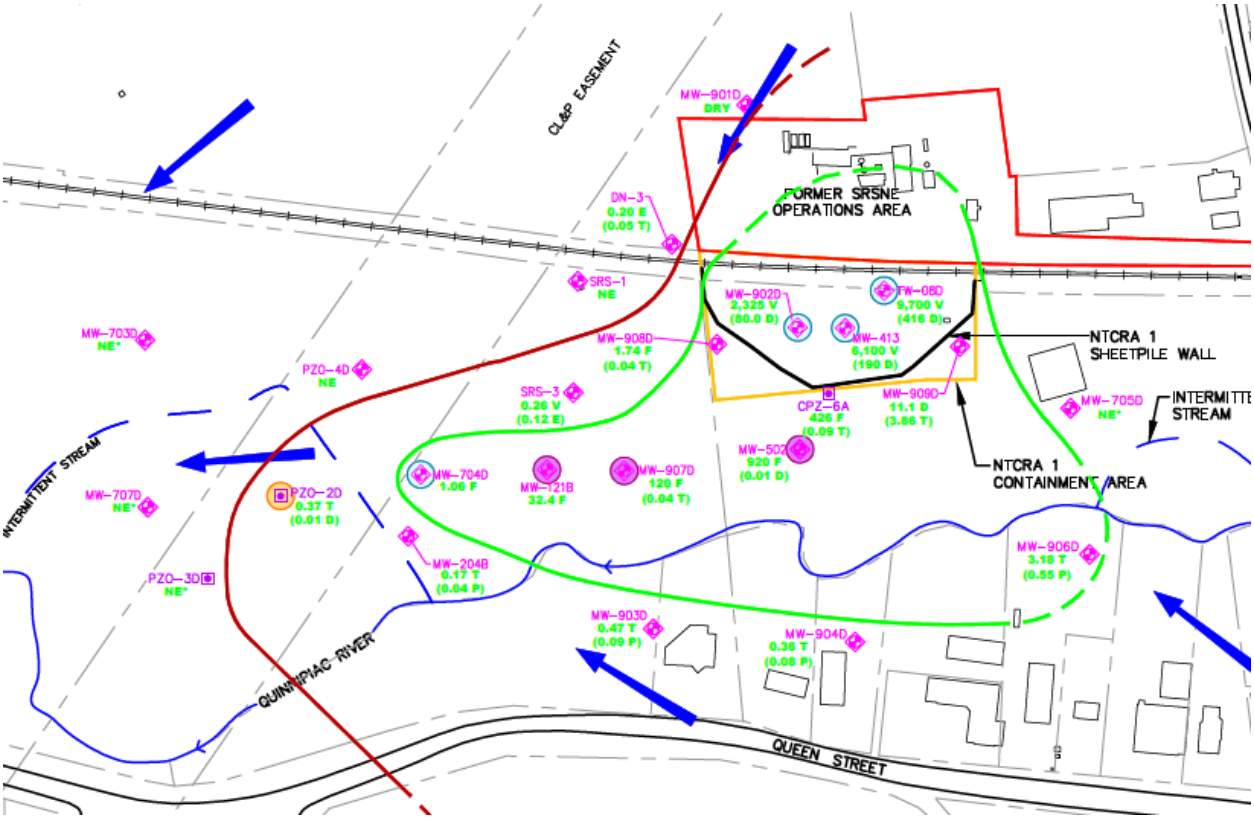
OTU Classification	Count	% Match
Rhizobium spp.	236	18.49%
Pelomonas saccharophila	233	11.88%
Acidovorax delafieldii	173	7.61%
Pseudonocardia sulfidoxydans	389	7.35%
Acidovorax facilis	10817	7.33%
Pseudomonas resinovorans	27196	4.74%
Bradyrhizobium elkanii	34421	4.07%
Desulfovibrio indonesiensis	3	0.55%
Methylosinus spp.	145	0.04%
Methylocystis spp.	7	0.03%
Methylosinus trichosporium	25	0.01%
Exophiala xenobiotica	1092	99.73%

Table 12. Results from 16sRNA analysis- note that *Exophiala xenobiotica* is the relative proportion compared to all other fungus in that sample, not the entire sample.

OTU	Count	Relative Proportion	OTU Classification	Well(s) present
OTU_8	34421	19.61%	*Bradyrhizobium elkanii*	MW 907 1,4D
OTU_1	27196	15.39%	*Pseudomonas resinovorans*	MW 907 1,4D
OTU_8	41379	20.63%	*Bradyrhizobium elkanii*	MW 907M 1,4D+THF
OTU_1	44612	22.11%	*Pseudomonas resinovorans*	MW 907M 1,4D+THF
OTU_34	41700	21.32%	*Acidobacterium spp.*	MW 907M THF
OTU_5	29275	14.97%	*Pseudofulvimonas gallinarii*	MW 907M THF
OTU_6	27772	18.49%	*Rhizobium spp.*	CPZ 6A THF
OTU_80	1092	99.73%	*Exophiala xenobiotica*	CPZ 6A THF

Figures

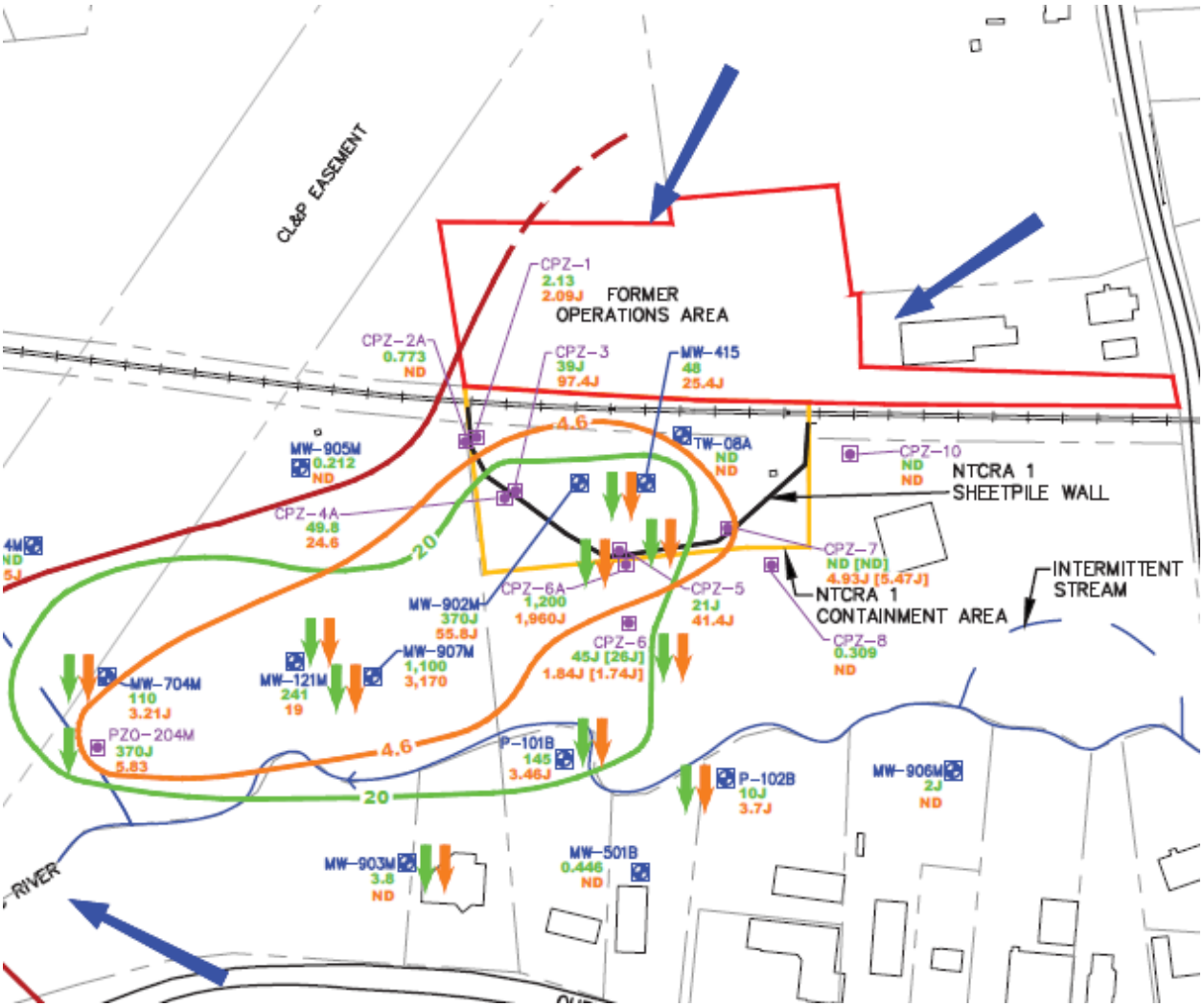
Figure 1-3. 'VOC Exceedance in Deep Overburden' from 2015 Conceptual Site Model Update (Arcadis, 2015). Note the drop in concentration of THF from MW-502 which has a concentration of 4232 µg/L to MW-704D which has a concentration of 4.876µg/L. Concentrations are expressed a value times the regulatory limit.



LEGEND:

- DEEP OVERBURDEN MONITORING WELL
- PIEZOMETER
- ESTIMATED EXTENT OF GROUNDWATER VOC EXCEEDANCES OF MCLs OR CT DEEP CLASS GA GWPCs (2014 SAMPLING RESULTS) (DASHED WHERE INFERRED)
- ESTIMATED NTCRA 2 CAPTURE ZONE BOUNDARY
- GENERALIZED GROUNDWATER FLOW DIRECTION
- MW-502 920 F (0.01 D) — WELL WITH REGULATORY EXCEEDANCE RATIO. NUMBERS >1.0 INDICATE GROUNDWATER REGULATORY LIMIT EXCEEDED. NUMBERS <1.0 INDICATE EXCEEDANCE RATIO FOR COMPOUNDS DETECTED BELOW REGULATORY LIMIT. FIRST NUMBER INDICATES MAXIMUM MULTIPLE OF A DETECTED VOC OVER REGULATORY LIMIT (e.g., 130 INDICATES 130 x LIMIT). LETTER INDICATES COMPOUND WITH INDICATED EXCEEDANCE RATIO (e.g., P = TETRACHLOROETHENE). NUMBERS IN PARENTHESES INDICATE OTHER EXCEEDANCE RATIOS FOR SELECT COMPOUNDS AND WELLS. COMPOUNDS DETECTED IN BLANK(S) ARE NOT INCLUDED IN THIS EVALUATION.
- * NO DETECTIONS ABOVE INTERIM CLEANUP LEVELS (ICLs) AT THIS LOCATION.

Figure 1-4. 'Tetrahydrofuran and 1,4-Dioxane Results: Middle Overburden' from 2015 Conceptual Site Model Update (Arcadis, 2015). Sample locations CPZ-6A and MW-907M are visible, within the capture zone and high concentration contours.



LEGEND:

- MIDDLE OVERBURDEN MONITORING WELL
- PIEZOMETER
- ESTIMATED NTCRA 2 CAPTURE ZONE BOUNDARY
- GENERALIZED GROUNDWATER FLOW DIRECTION
- 1,4—DIOXANE ISOCONCENTRATION CONTOUR (20 ug/L)
- TETRAHYDROFURAN ISOCONCENTRATION CONTOUR (4.6 ug/L)
- DECREASING 1,4—DIOXANE CONCENTRATION TREND INDICATED
- DECREASING TETRAHYDROFURAN CONCENTRATION TREND INDICATED

1,100 1,4—DIOXANE CONCENTRATION (ug/L)
3,170 TETRAHYDROFURAN CONCENTRATION (ug/L)
ug/L MICROGRAMS PER LITER
[ ] DUPLICATE SAMPLE RESULT
J ANALYTE RESULT IS ESTIMATED
NA NOT ANALYZED
ND NOT DETECTED

Figure 2-5. Diversity of microbial population in CPZ-6A. Incubation time is 10 days at room temperature on R2A plates.

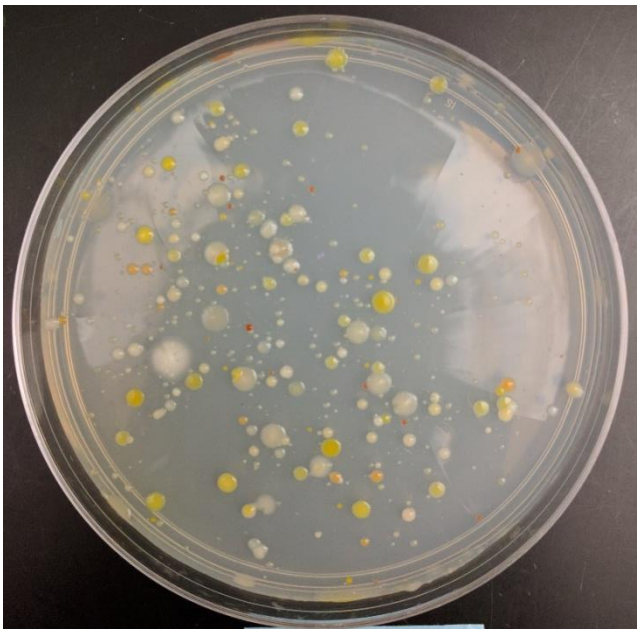


Figure 2-6. Diversity of microbial population in MW-907M. Incubation time is 10 days at room temperature on R2A plates.

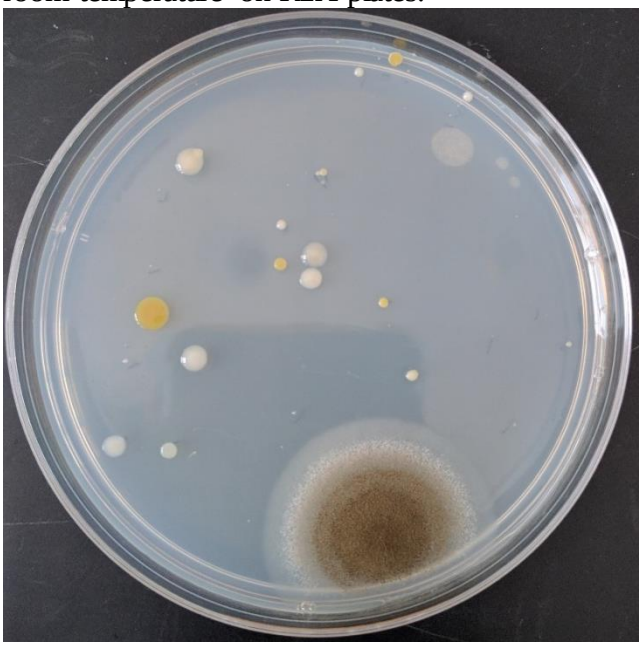


Figure 2-7. Diversity of microbial population in MW-907M. Incubation time is 10 days at room temperature on R2A plates.

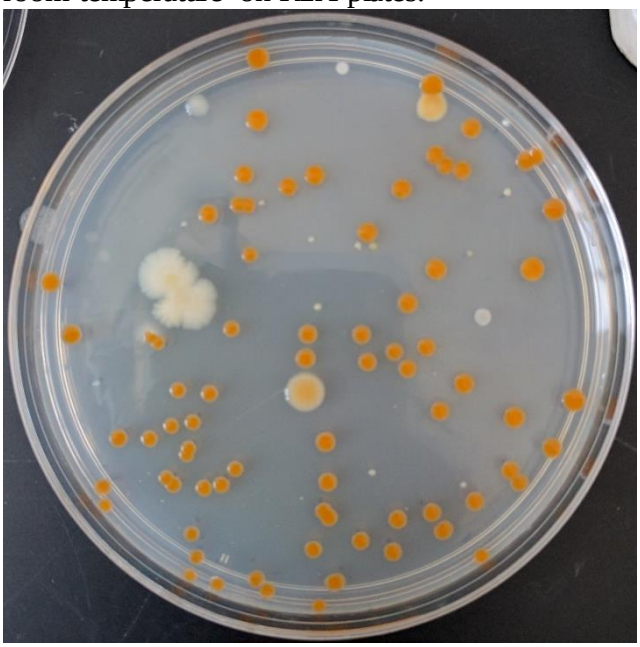


Figure 2-8. Diversity of microbial population in P101-A. Incubation time is 10 days at room temperature on R2A plates.

Figure 2-9. CPZ-6A cultures grown in Bushnell-Haas broth. Cultures were grown with low (left, 62mM) and moderate THF (right, 616mM). Note fungal colony in bottom left of the right flask- fungus has been seen only in this enriched culture

Figure 2-10. MW-907M cultures grown in Bushnell-Haas. Cultures were grown with low (left, 62mM) and moderate THF (right, 616mM).

Figure 3-1. Growth of various samples on THF. Dotted lines indicate samples that were removed from future experiments.

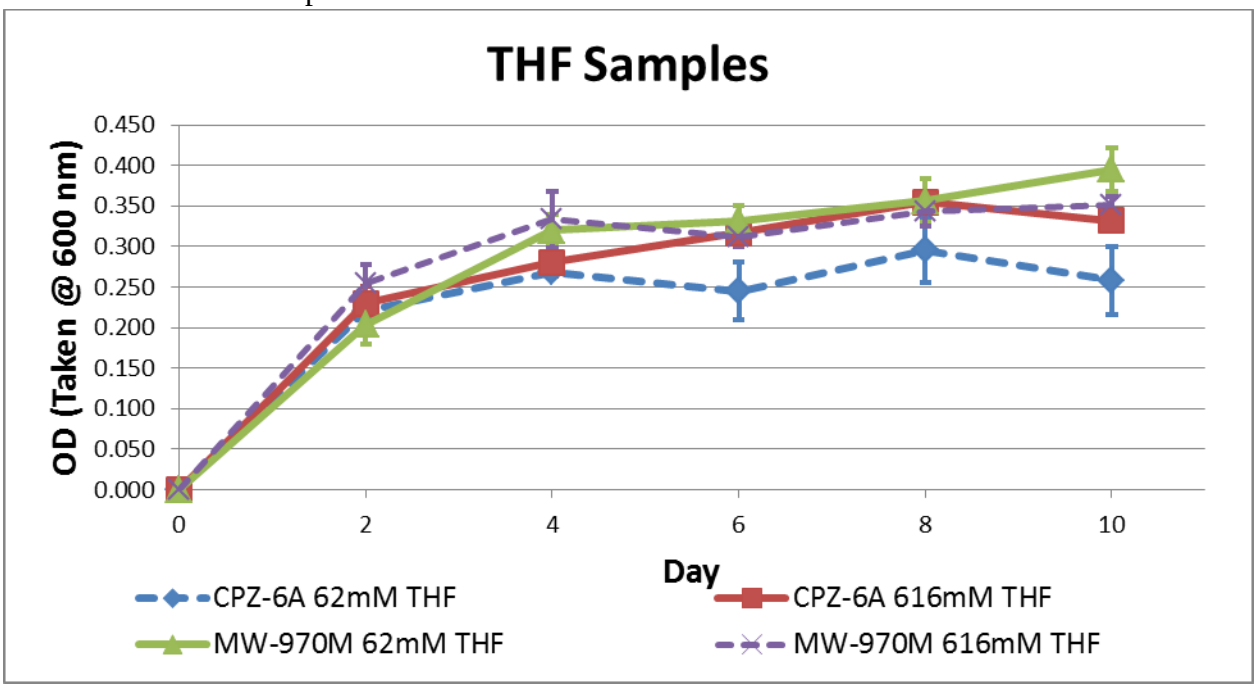


Figure 3-2. Growth of various samples on of 1,4-dioxane. Dotted lines indicate samples that were removed from future experiments.

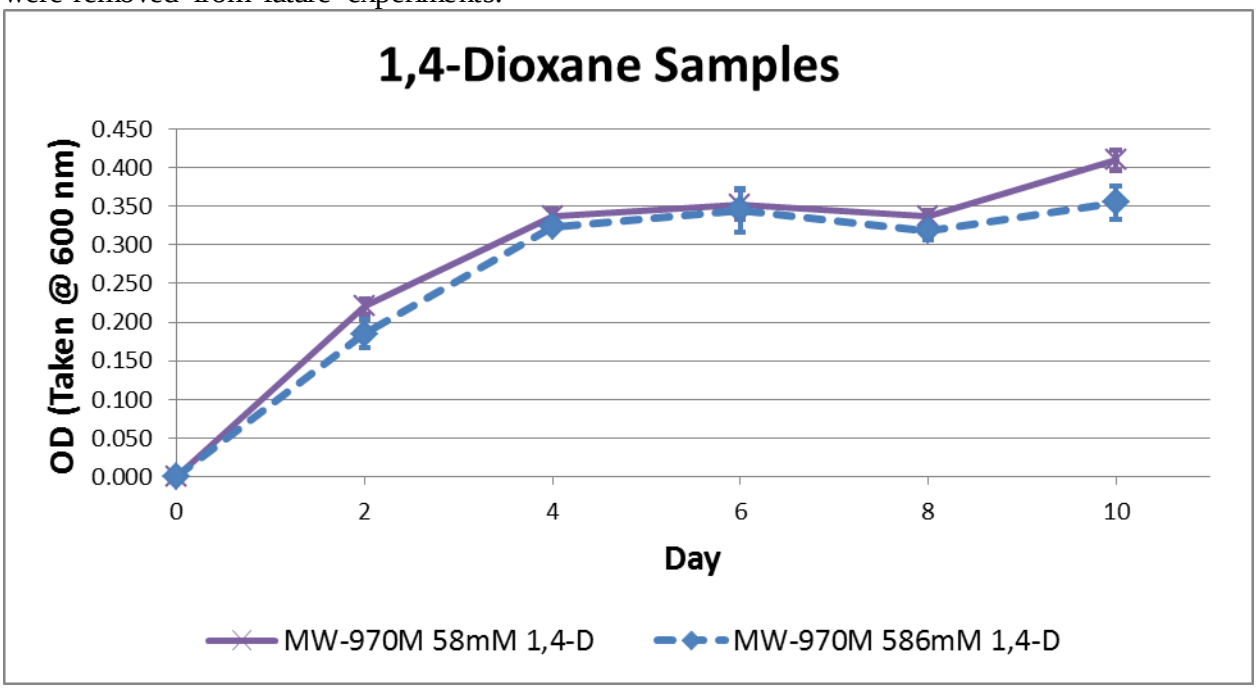


Figure 3-3 Growth of various samples on THF + 1,4-dioxane. Dotted lines indicate samples that were removed from future experiments.

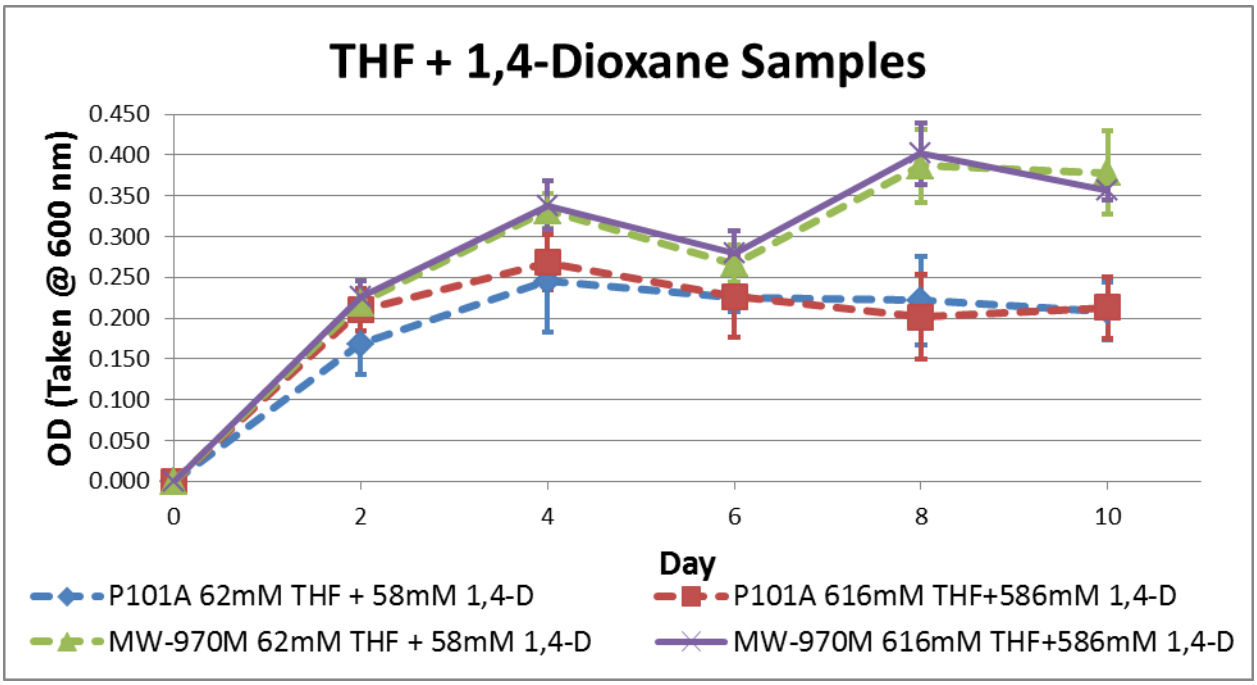


Figure 4-1. Effect of aeration on the growth of sample CPZ-6A.

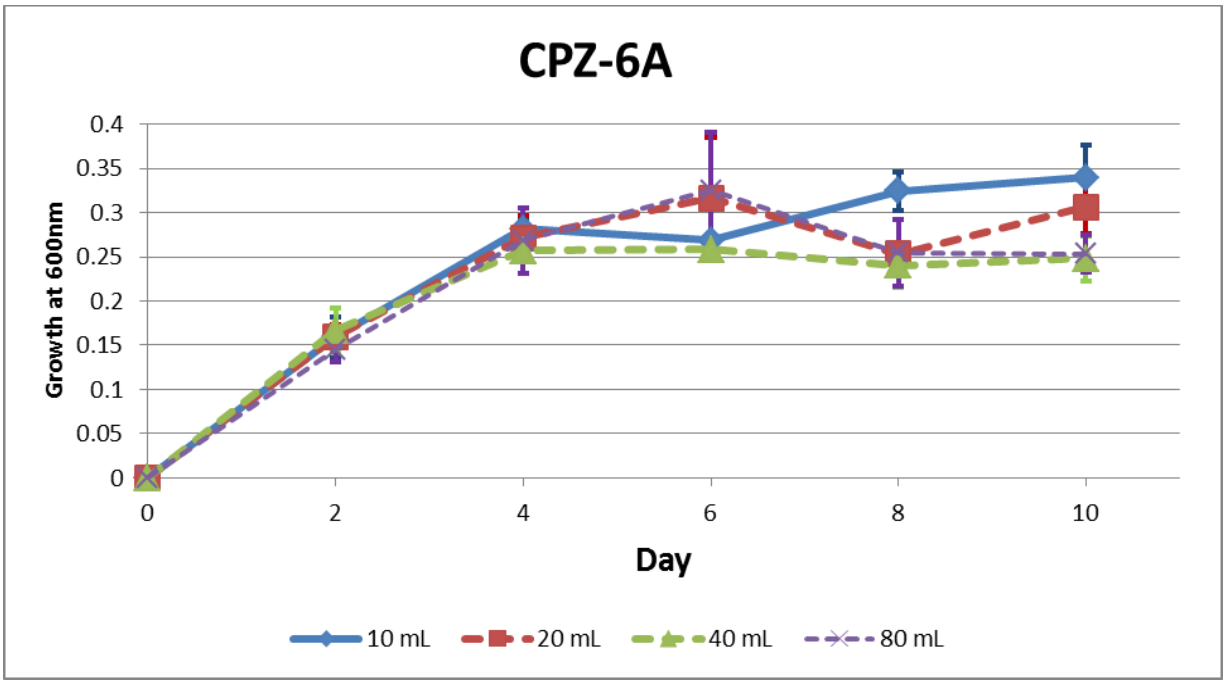


Figure 4-2. Effect of aeration on the growth of sample MW-907M A.

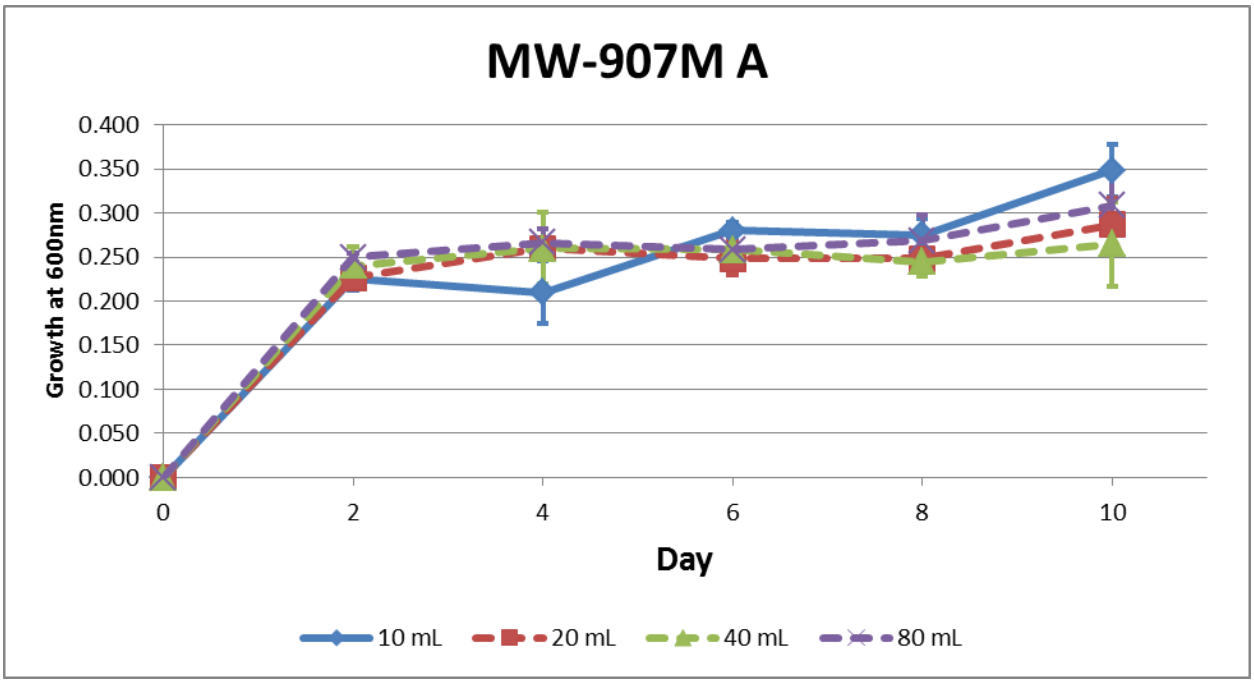


Figure 4-3. Effect of aeration on sample the growth of MW-907M B.

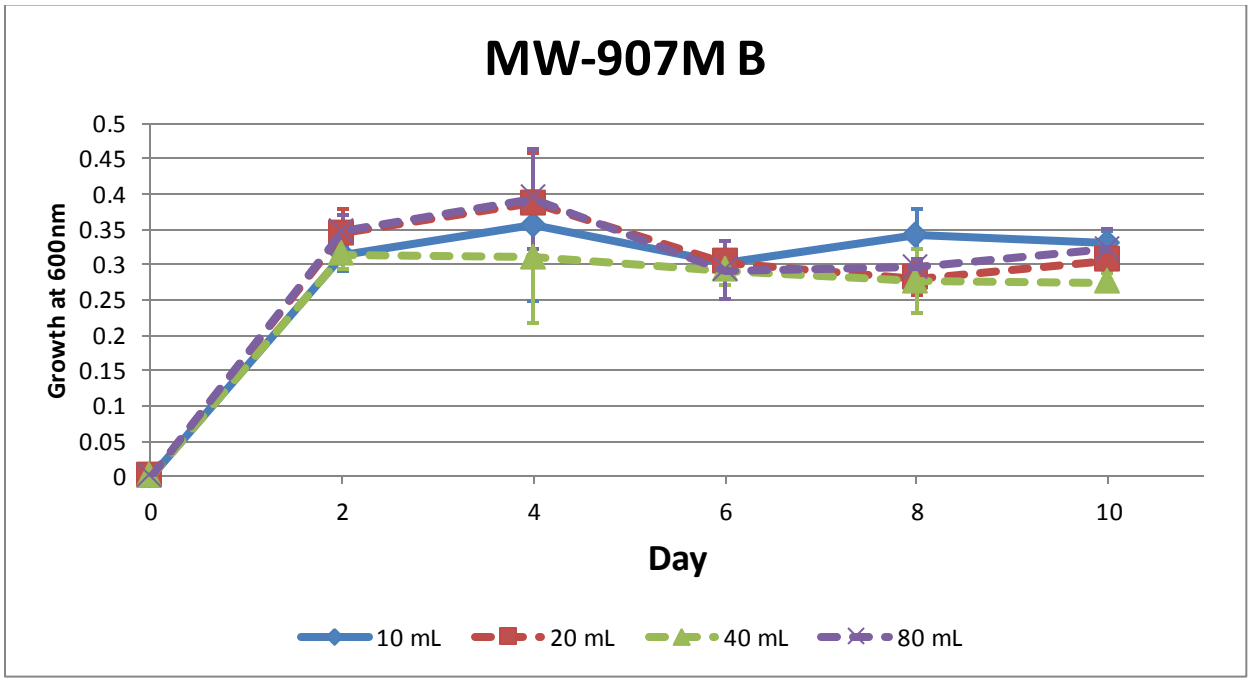


Figure 4-4. Effect of aeration on sample the growth of MW-907M C.

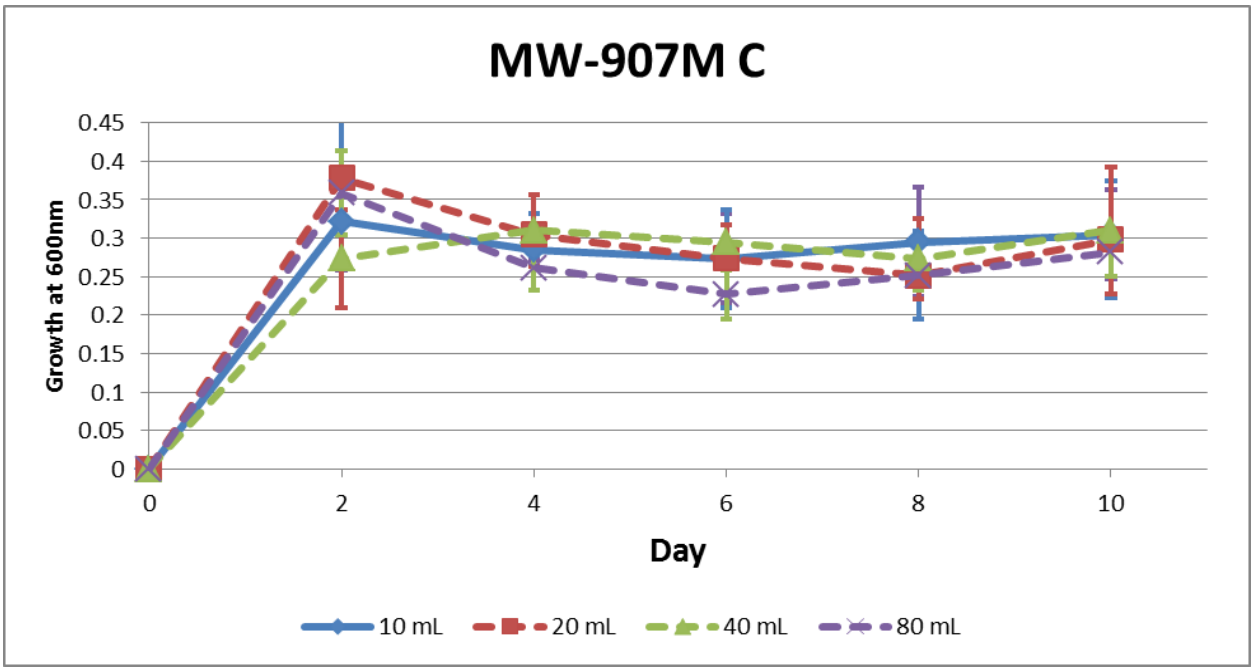


Figure 5-1. Effect of THF concentration on sample CPZ-6A.

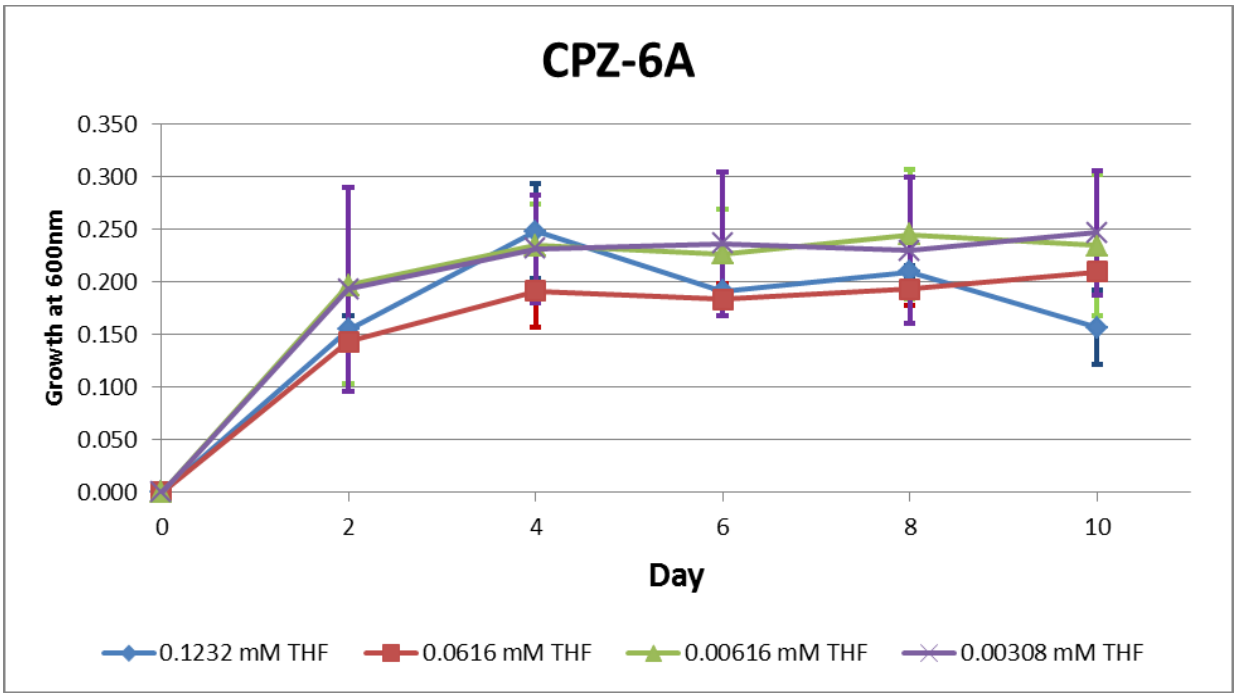


Figure 5-2. Effect of THF concentration on sample MW-907M A.

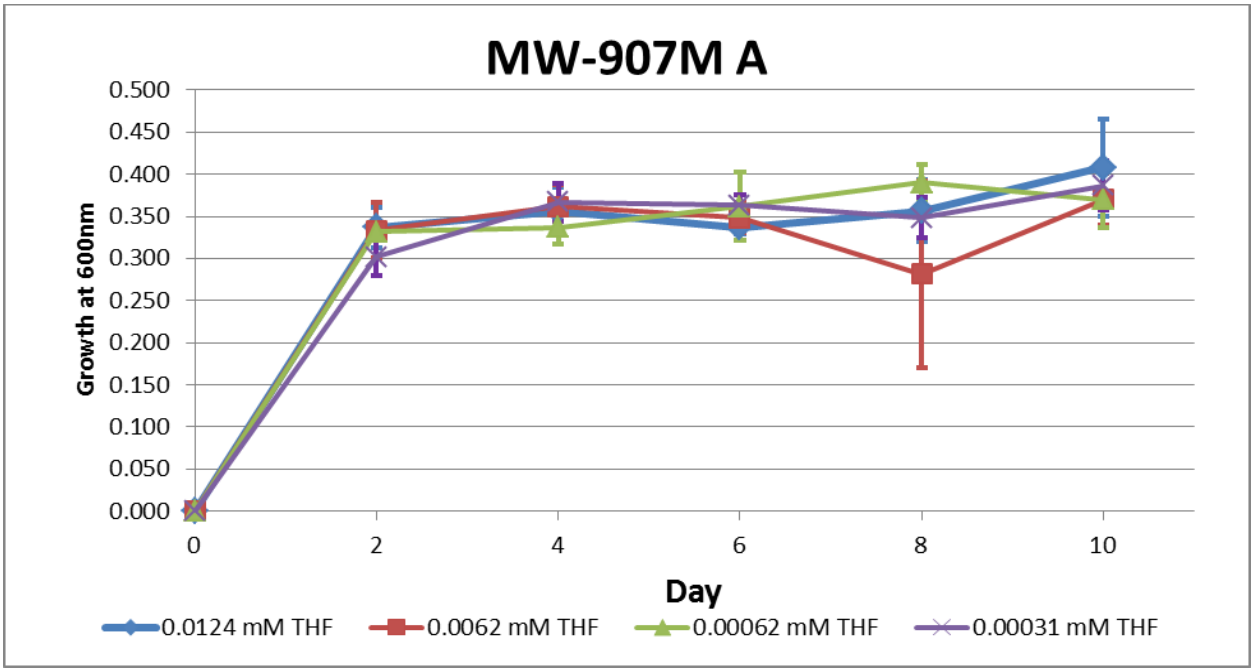


Figure 5-3. Effect of 1,4-dioxane concentration on sample MW-907M B.

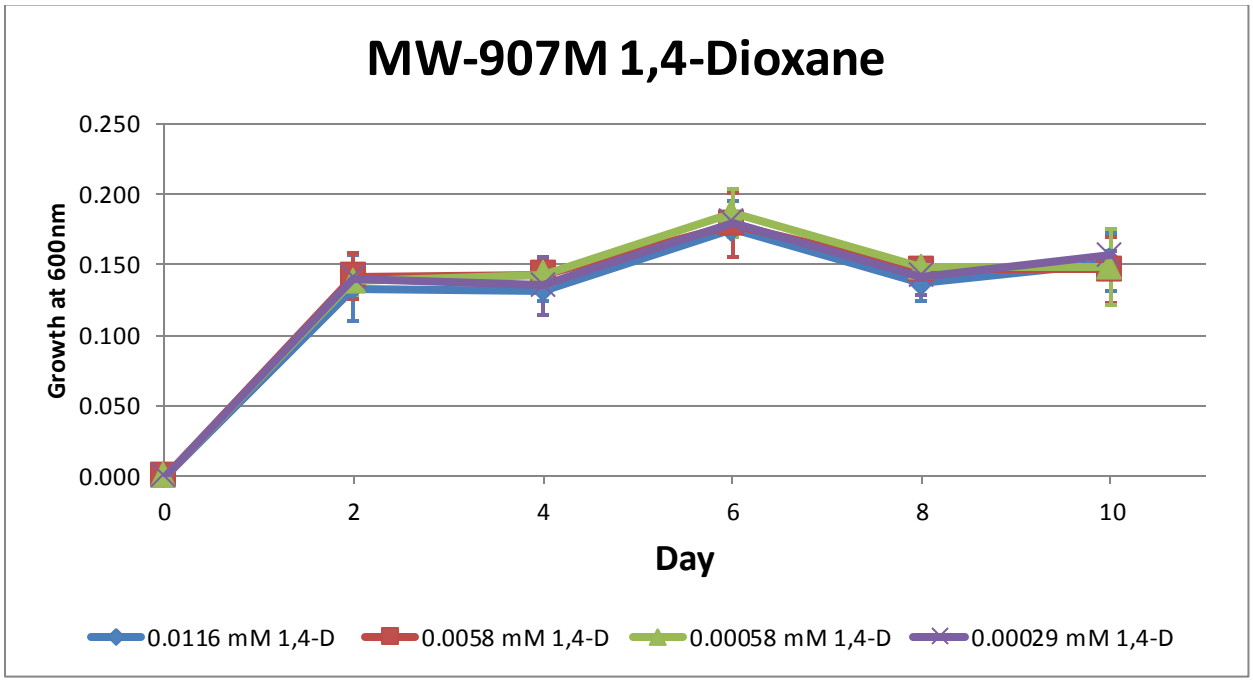


Figure 5-4. Effect of THF + 1,4-dioxane concentration on sample MW-907M C.

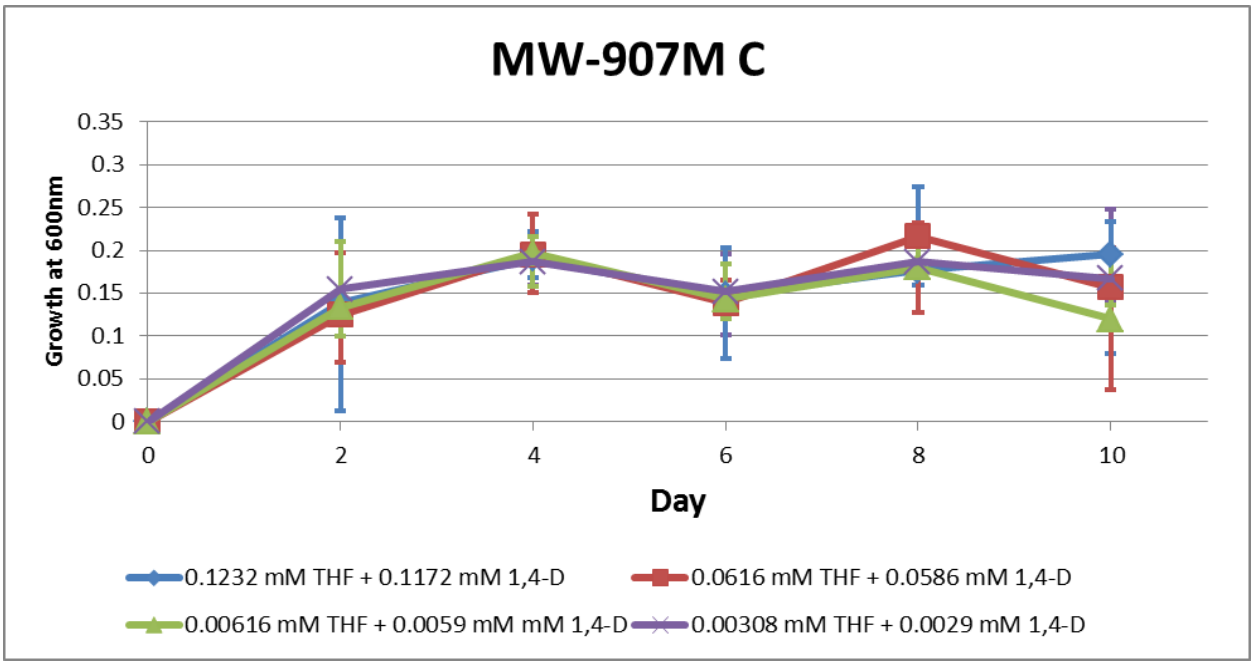


Figure 6-1. Effect of growth after prolonged storage on rich media on sample CPZ-6A.

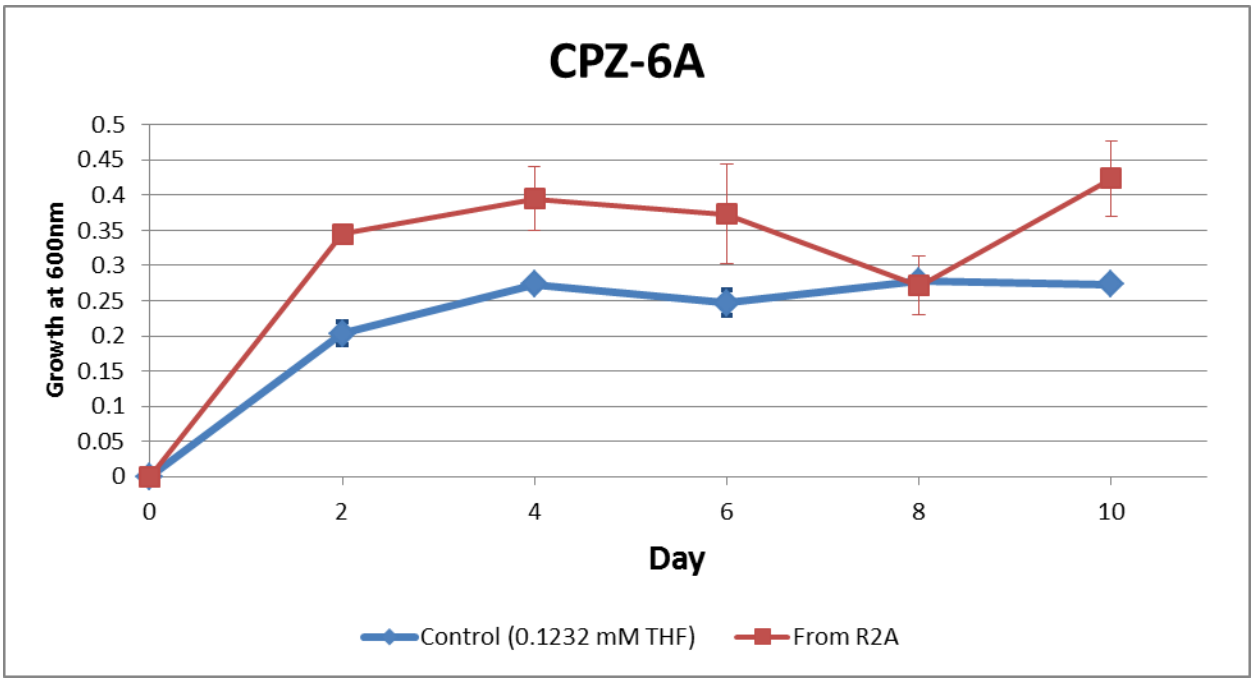


Figure 6-2. Effect of growth after prolonged storage on rich media on sample MW-907M A.

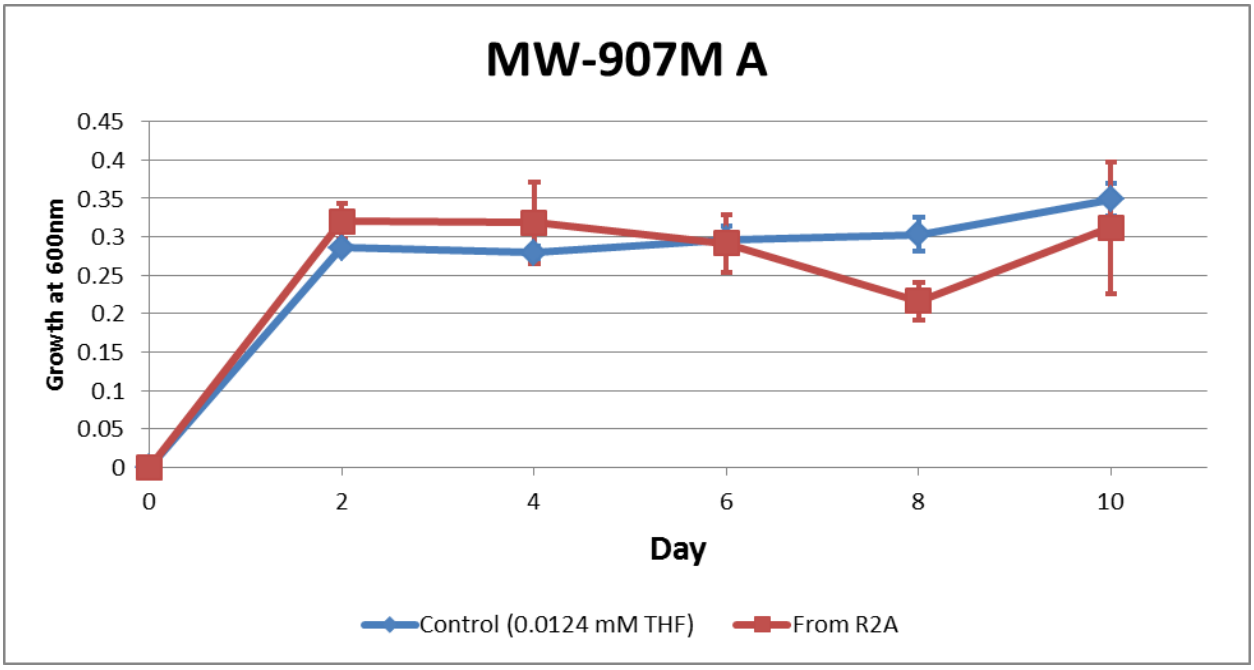


Figure 6-3. Effect of growth after prolonged storage on rich media on sample MW-907M B.

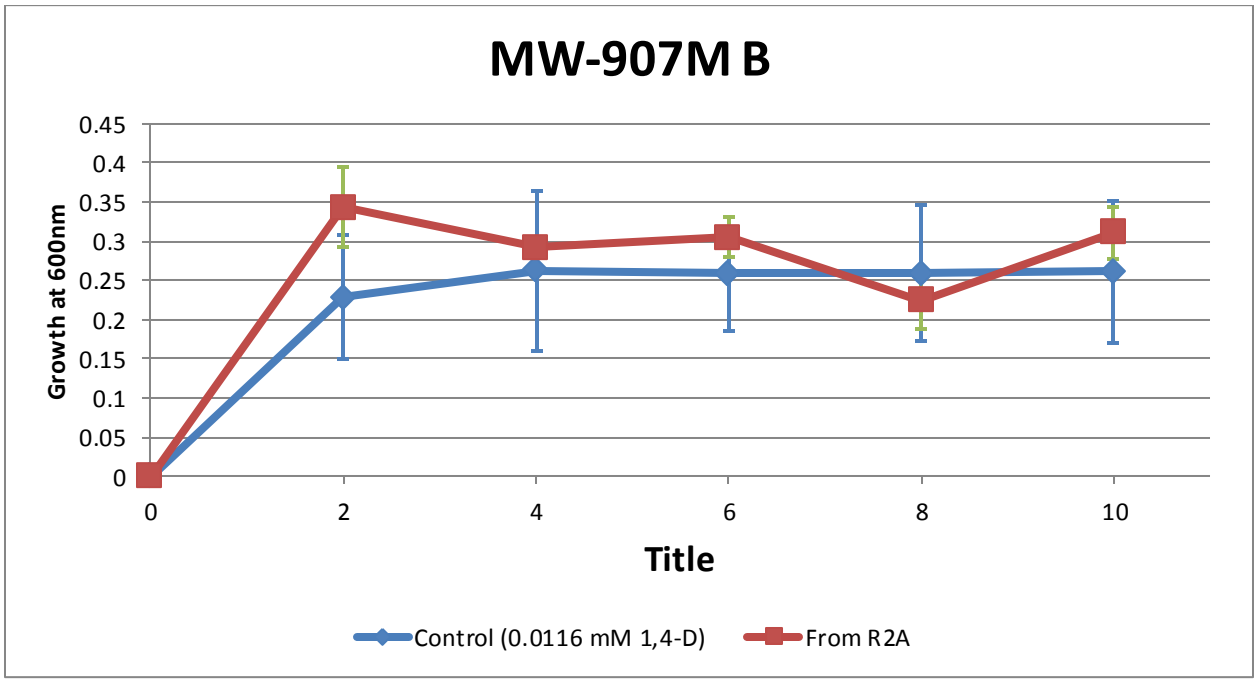


Figure 6-4. Effect of growth after prolonged storage on rich media on sample MW-907M C.

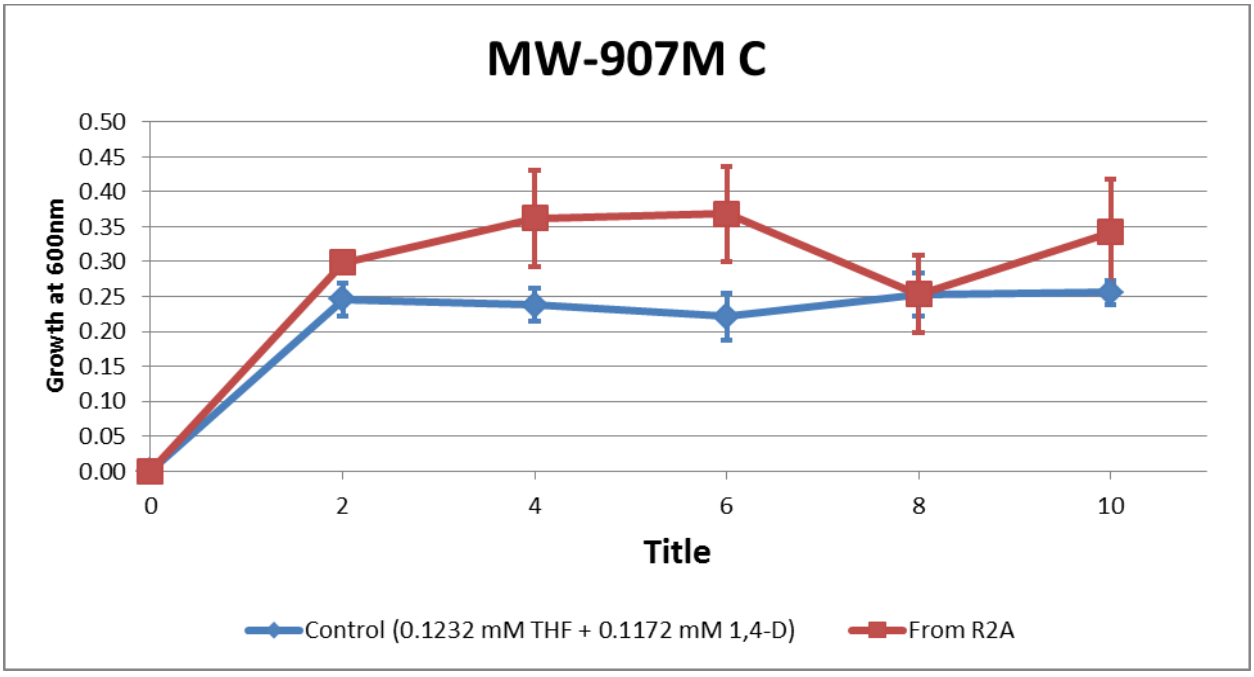


Figure 7-1. Effect of aliphatic chlorinated compounds on the growth of sample CPZ-6A.

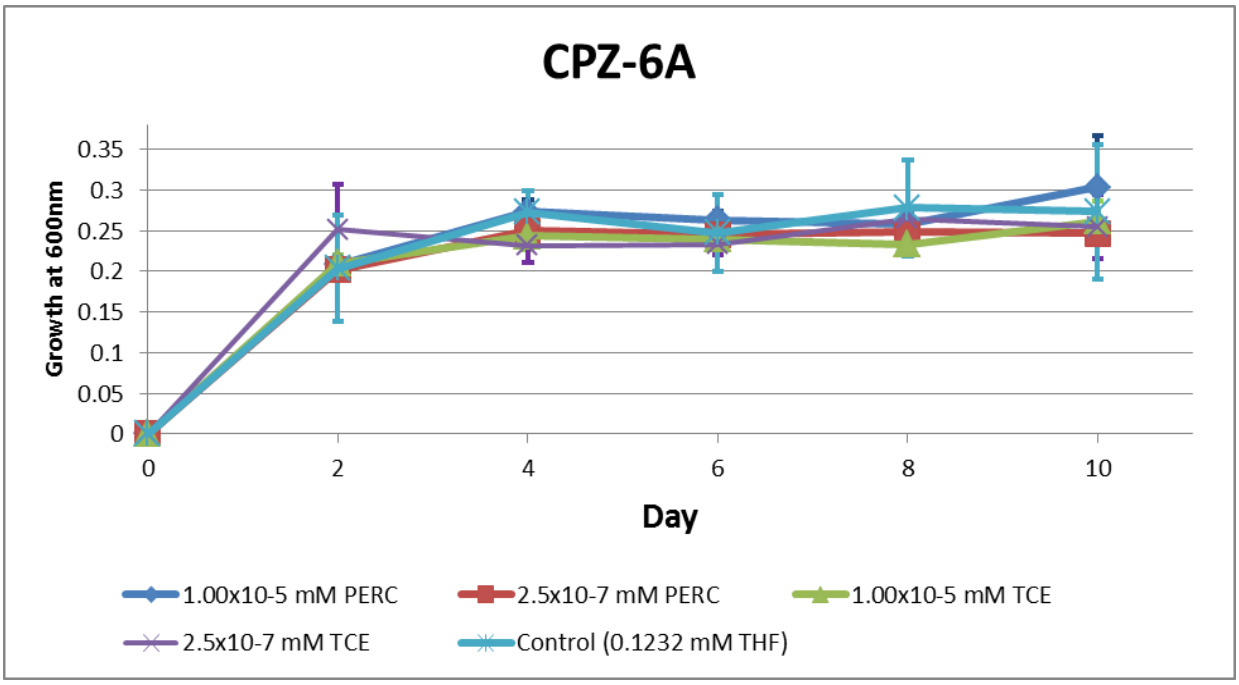


Figure 7-2. Effect of aliphatic chlorinated compounds on the growth of sample MW-907M A.

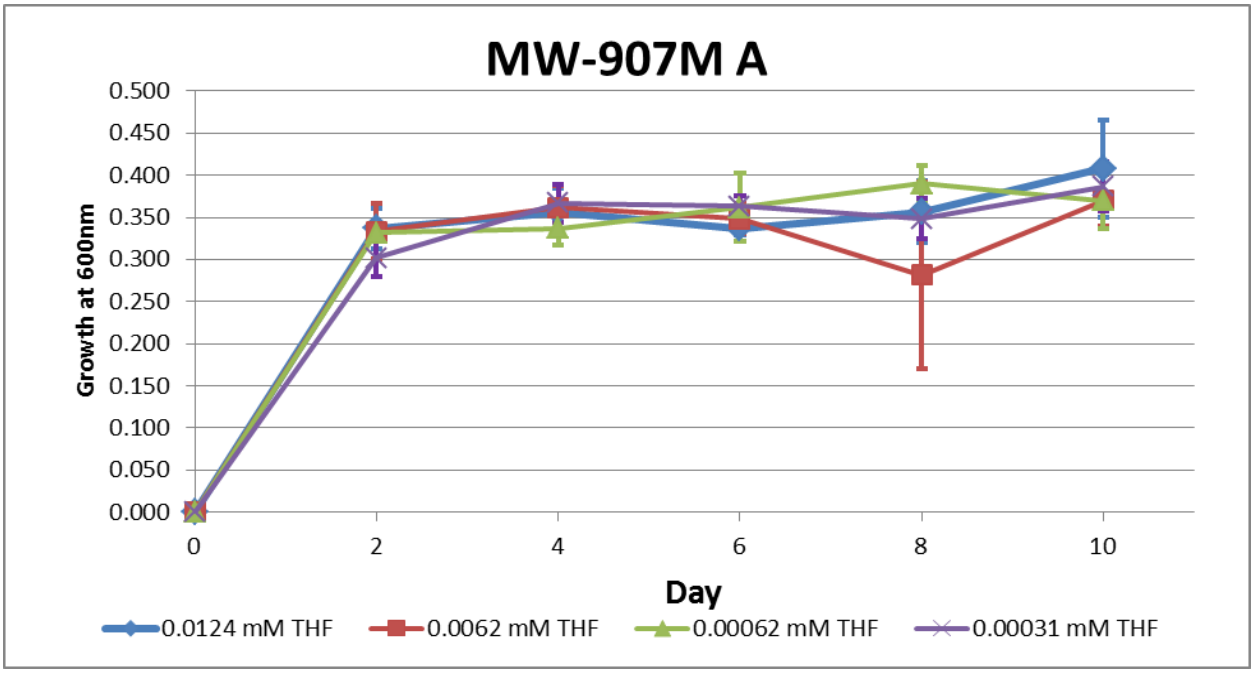


Figure 7-3. Effect of aliphatic chlorinated compounds on the growth of sample MW-907M B.

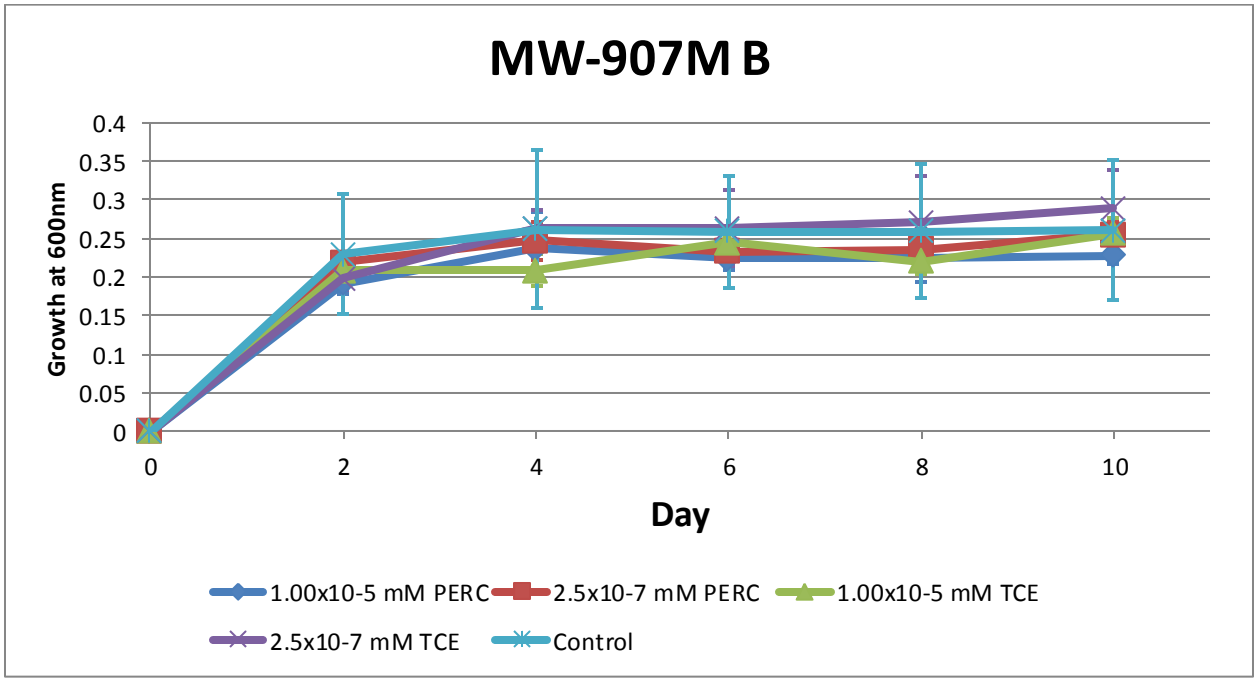


Figure 7-4. Effect of aliphatic chlorinated compounds on the growth of sample MW-907M C.

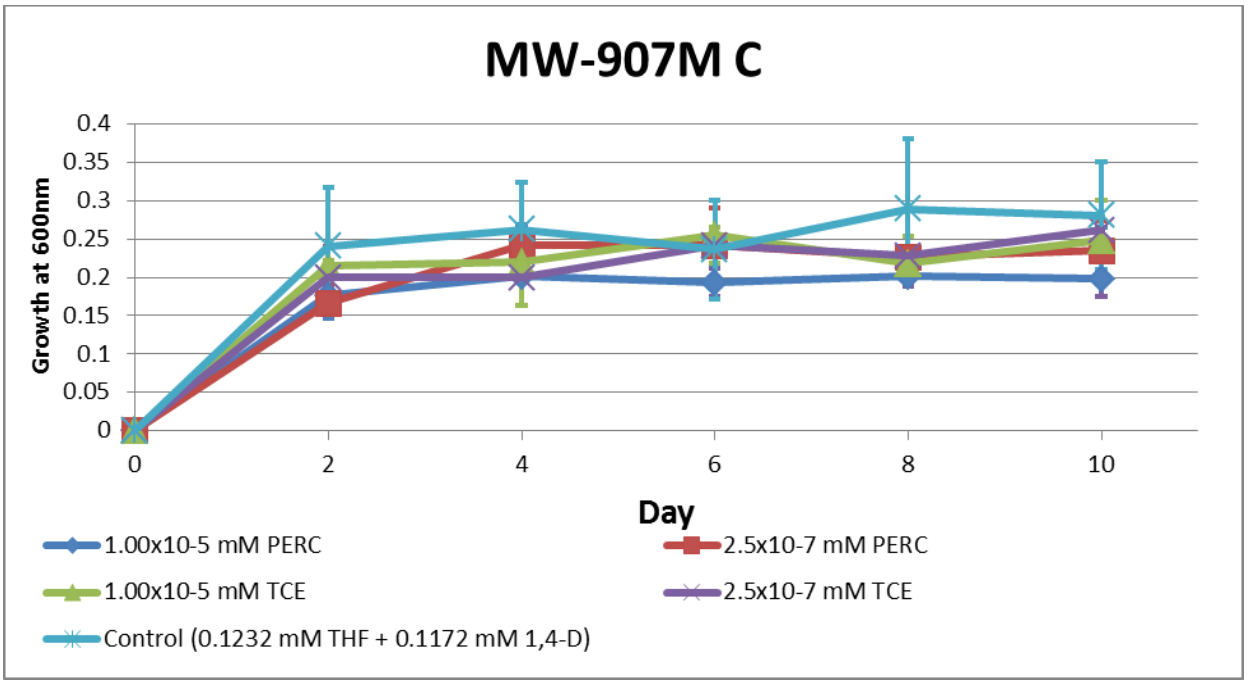


Figure 8-1. Comparison of growth on THF and PERC on sample CPZ-6A.

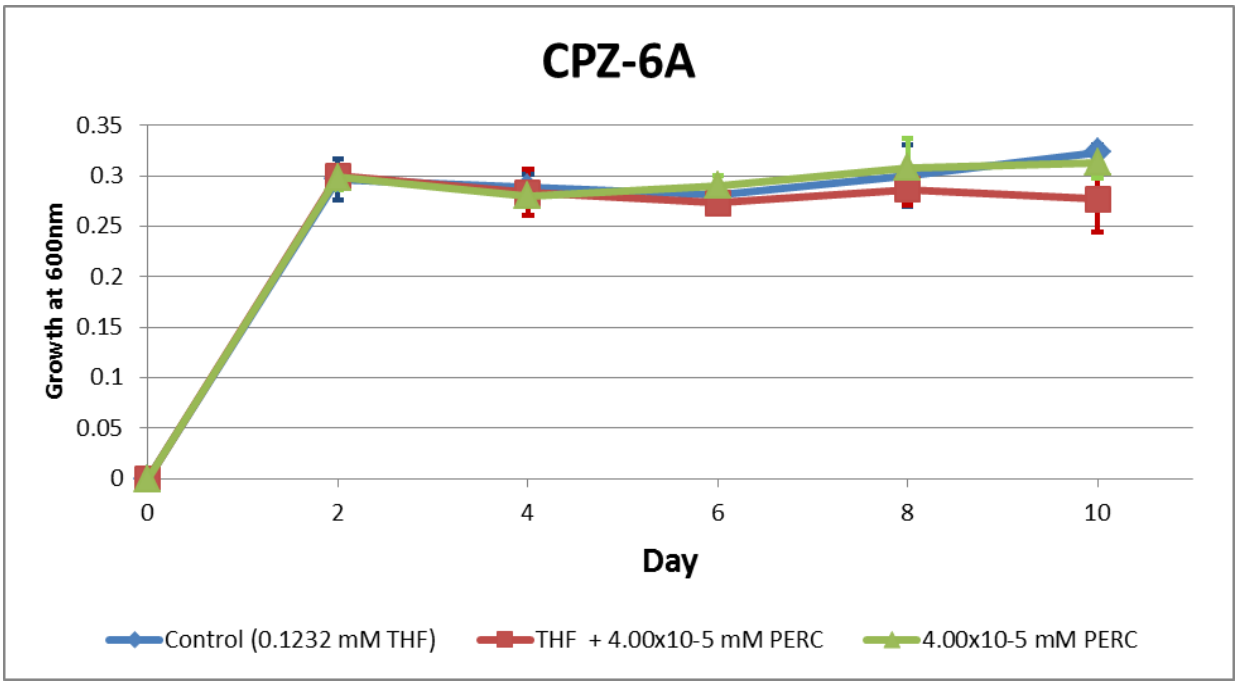


Figure 8-2. Comparison of growth on THF and PERC on sample MW-907M A.

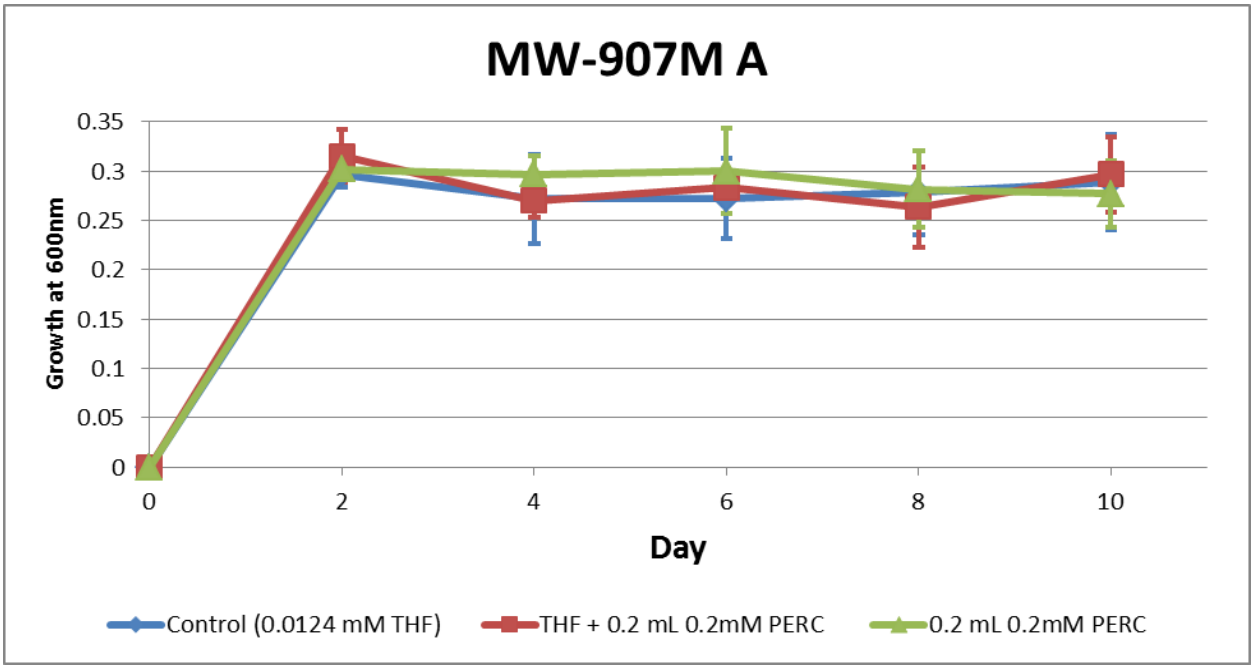


Figure 8-3. Comparison of growth on 1,4-dioxane and PERC on sample MW-907M B.

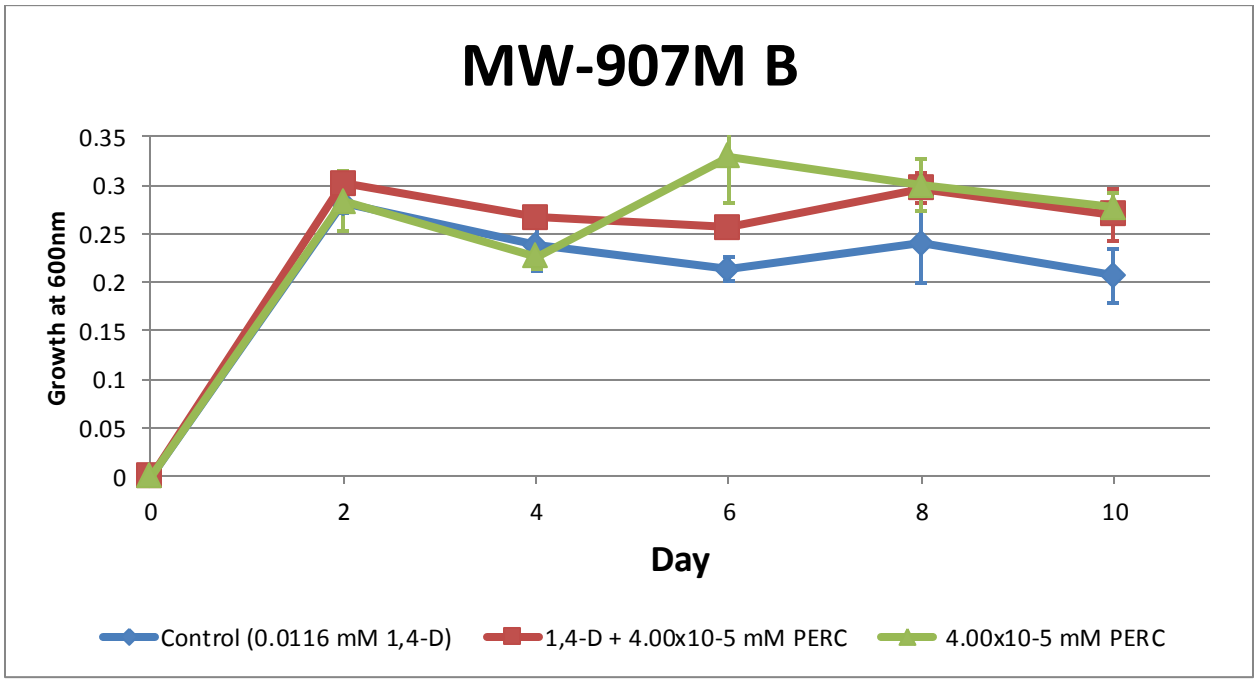
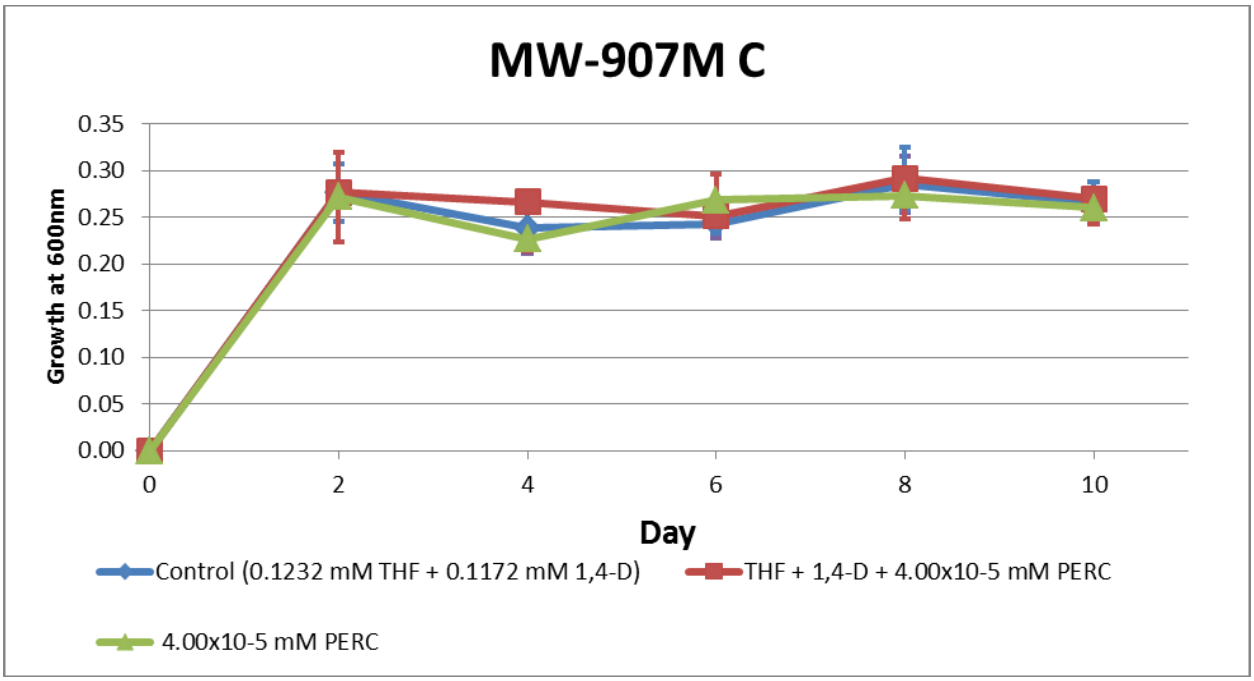


Figure 8-4. Comparison of growth on THF, 1,4-dioxane and PERC on sample MW-907M C.



Appendix

Supplemental Tables

Table S-1. Growth of isolates on selected carbon sources. The growth rate constant (μ) and generation time (g) were determined during the exponential growth phase. The isolates in grey were removed from the experiment.

Isolate	Average μ ($days^{-1}$)	Average g (days)
CPZ-6A 0.0124mM THF	0.10	6.86
CPZ-6A 0.1232mM THF	0.10	7.00
P101A 0.0124mM THF + 0.0116mM 1,4-D	0.22	3.22
P101A 0.1232mM THF+ 0.1172mM 1,4-D	0.12	5.65
MW-970M 0.0116mM 1,4-D	0.21	3.25
MW-970M 0.1172mM 1,4-D	0.28	2.47
MW-970M 0.0124mM THF	0.23	3.04
MW-970M 0.1232mM THF	0.13	5.19
MW-970M 0.0124mM THF + 0.0116mM 1,4-D	0.21	3.32
MW-970M 0.1232mM THF+ 0.1172mM 1,4-D	0.20	3.46

Table S-2. Growth of isolates based on varying volumes of Bushnell-Haas broth. The growth rate constant (μ) and generation time (g) were determined during the exponential growth phase.

Isolate	Average μ ($days^{-1}$)	Average g (days)
CPZ-6A		
10 mL	0.30	2.68
20 mL	0.26	2.62
40 mL	0.22	3.52
80 mL	0.31	2.59
MW-970M A		
10 mL	0.10	26.33
20 mL	0.07	11.56
40 mL	0.07	29.96
80 mL	0.03	35.03
MW-970M B		
10 mL	0.13	8.42
20 mL	0.10	7.62
40 mL	0.09	10.09
80 mL	0.09	9.19
MW-970M C		
10 mL	0.08	10.14
20 mL	0.02	51.28
40 mL	0.10	10.96
80 mL	0.05	16.08

Table S-3. Growth of isolates based on varying volumes of carbon substrate. The growth rate constant (μ) and generation time (g) were determined during the exponential growth phase.

Isolate	Average μ ($days^{-1}$)	Average g (days)
CPZ-6A		
0.1232 mM THF	0.23	3.49
0.0616 mM THF	0.14	9.46
0.00616 mM THF	0.16	5.73
0.00308 mM THF	0.14	7.97
MW-970M A		
0.0124 mM THF	0.05	6.04
0.0062 mM THF	0.07	8.08
0.00062 mM THF	0.05	11.89
0.00031 mM THF	0.1	13.31
MW-970M B		
0.0116 mM 1,4-D	0.12	3.58
0.0058 mM 1,4-D	0.06	17.35
0.00058 mM 1,4-D	0.09	12.3
0.00029 mM 1,4-D	0.13	23.17
MW-970M C		
0.1232 mM THF + 0.1172 mM 1,4-D	0.16	13.52
0.0616 mM THF + 0.0586 mM 1,4-D	0.22	24.45
0.00616 mM THF + 0.0059 mM mM 1,4-D	0.19	11.88
0.00308 mM THF + 0.0029 mM 1,4-D	0.16	4.54

Table S-4. Growth of isolates after prolonged storage on rich media. The growth rate constant (μ) and generation time (g) were determined during the exponential growth phase.

Isolate	Average μ ($days^{-1}$)	Average g (days)
CPZ-6A		
R2A	0.07	21.50
Control	0.15	4.80
MW-970M A		
R2A	0.05	4.80
Control	0.05	21.94
MW-970M B		
R2A	0.09	21.94
Control	0.07	39.46
MW-970M C		
R2A	0.09	39.46
Control	0.07	45.03

Table S-5. Growth of isolates in the presence of aliphatic chlorinated compounds (TCE and PERC). The growth rate constant (μ) and generation time (g) were determined during the exponential growth phase.

Isolate	Average μ ($days^{-1}$)	Average g (days)
CPZ-6A		
0.1232 mM THF + $1.00x10^{-5}$ mM PERC	0.14	5.22
0.1232 mM THF + $2.5x10^{-7}$ mM PERC	0.11	6.17
0.1232 mM THF + $1.00x10^{-5}$ mM TCE	0.07	25.11
0.1232 mM THF + $2.5x10^{-7}$ mM TCE	0.05	14.73
Control (0.1232 mM THF)	0.15	4.8
MW-970M A		
0.0124 mM THF + $1.00x10^{-5}$ mM PERC	0.12	6.04
0.0124 mM THF + $2.5x10^{-7}$ mM PERC	0.09	7.44
0.0124 mM THF + $1.00x10^{-5}$ mM TCE	0.14	6.64
0.0124 mM THF + $2.5x10^{-7}$ mM TCE	0.1	12.76
Control (0.0124 mM THF)	0.05	38.01
MW-970M B		
0.0116 mM 1,4-D + $1.00x10^{-5}$ mM PERC	0.12	9.2
0.0116 mM 1,4-D + $2.5x10^{-7}$ mM PERC	0.08	9.48
0.0116 mM 1,4-D + $1.00x10^{-5}$ mM TCE	0.08	9.21
0.0116 mM 1,4-D + $2.5x10^{-7}$ mM TCE	0.14	4.93
Control (0.0116 mM 1,4-D)	0.07	19.45
MW-970M C		
0.1232 mM THF + 0.1172 mM 1,4-D + $1.00x10^{-5}$ mM PERC	0.07	15.08
0.1232 mM THF + 0.1172 mM 1,4-D + $2.5x10^{-7}$ mM PERC	0.19	5.96
0.1232 mM THF + 0.1172 mM 1,4-D + $1.00x10^{-5}$ mM TCE	0.05	16.85
0.1232 mM THF + 0.1172 mM 1,4-D + $2.5x10^{-7}$ mM TCE	0.09	9.18
Control (0.1232 mM THF + 0.1172 mM 1,4-D)	0.07	10.27

Table S-6. Growth of isolates on PERC versus preferred carbon substrate. The growth rate constant (μ) and generation time (g) were determined during the exponential growth phase..

Isolate	Average μ ($days^{-1}$)	Average g (days)
CPZ-6A		
Control	0.04	18.97
0.1232 mM THF + $4.00x10^{-5}$ mM PERC	0.01	53.82
$4.0x10^{-5}$ mM PERC	0.05	16.26
MW-970M A		
Control	0.04	17.24
0.0124 mM THF + $4.00x10^{-5}$ mM PERC	0.02	56.28
$4.0x10^{-5}$ mM PERC	0.04	22.50
MW-970M B		
Control	0.08	56.46
0.0116 mM 1,4-D + $4.00x10^{-5}$ mM PERC	0.07	27.37
$4.0x10^{-5}$ mM PERC	0.18	14.89
MW-970M C		
Control	0.04	33.95
0.1232 mM THF + 0.1172 mM 1,4-D + $4.00x10^{-5}$ mM PERC	0.06	10.38
$4.0x10^{-5}$ mM PERC	0.07	14.54