

TOWARDS IMPROVED BIOREACTOR DESIGN FOR REMOTE FIELD SCALE
HYDROCARBON BIOAUGMENTATION

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Abstract

Hydrocarbon contamination has a negative impact on the environment and can persist in the environment for long periods of time without some form of treatment. Microbial bioremediation utilizing bacteria is an increasingly applied method for treating hydrocarbon contamination. It is a more environmentally friendly option, generally cheaper, and provides a complete breakdown of the contamination into water and carbon dioxide. With its growing popularity, research on the topic has also increased. However, there is limited research on producing the bacteria in the field in a cost-effective manner. To address this, a series of experiments meant to evaluate media types and growth conditions for maximum growth in field bioreactors were evaluated. Investigation of various sources of field-related contamination were also evaluated to determine which source or sources of contamination have a detrimental effect on the target bacterial consortium so that these contamination sources can be addressed. The first chapter provided a background on the subject and the issues faced with bacterial bioremediation. The second chapter investigated the type of nutrients and growth conditions that provide optimal growth while factoring in cost. It was found that Bushnell Haas media supplemented with dextrose yielded high bacterial numbers while still being cost-effective. Chapter 3 investigated the bacterial community in two separate environments. The first was conducted in sterile laboratory conditions, where standard laboratory procedures were used to culture the consortium. The second environment was more representative of the field bioreactor, utilizing an air pump for aeration, larger vessels and the same media types evaluated in Chapter 2. From the results in Chapter 3, we were able to build on the findings from Chapter 2 and determine that Bushnell Haas not only produced sufficient cell density but also yielded a bacterial community of the target genera *Bacillus* spp. and *Pseudomonas* spp. Chapter 4 evaluated three main sources of contamination in the field bioreactors, including air for aeration, water, and exposure to the environment. From these experiments, it was evident that unsterilized water had the most significant impact on the community. Chapter 5 then evaluated the bacterial community in a field bioreactor used for treating contaminated soil. The

results of this chapter revealed that the community consisted of a majority of contaminating bacteria. Based on the findings of Chapter 4, suggestions are made on which sources of contamination should be addressed to increase the proportion of target genera. Each of these chapters contains a conclusion on the findings of each respective chapter. The final chapter then provided a conclusion to all the chapters, bringing together the findings from the four previous chapters. In addition to bringing the chapters together, future directions of this research are suggested.

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CHAPTER 1 - INTRODUCTION

LITERATURE REVIEW

Hydrocarbons are ubiquitous in the environment; these compounds can originate from various sources, including the natural degradation of organic matter and incomplete combustion. Hydrocarbons can be divided into three main classes: aliphatic hydrocarbons, aromatic hydrocarbons and heteroatomic hydrocarbons. Aliphatic hydrocarbons can be saturated or unsaturated and have a chain structure. Aromatic hydrocarbons contain at least one benzene ring (monocyclic aromatic hydrocarbons) and are planar, which means there is at least one ring structure in the hydrocarbon and all the atoms are in the same two-dimensional plane. Hydrocarbons such as benzene, toluene, ethylbenzene, and xylene, commonly referred to as BTEX are all aromatic hydrocarbons (Varjani 2017; Pandolfo et al. 2023). These molecules are considered light weight and are volatile, meaning they often gas off into the atmosphere. These hydrocarbons are typically easier to degrade, and in older contaminated sites, more of these hydrocarbons will have evaporated off leaving behind the heavier weight hydrocarbons. Aromatic hydrocarbons may also consist of multiple benzene rings (polycyclic aromatic hydrocarbons) and can be categorized into low-molecular-weight and high-molecular-weight compounds. The low molecular weight aromatic hydrocarbons usually consist of 2 to 3 aromatic rings, and the high molecular weight aromatic hydrocarbons consist of 4 or more rings. Heteroatomic hydrocarbons are hydrocarbon structures that include an atom other than carbon, such as nitrogen or oxygen as seen in Figure 1.1.

Additionally, hydrocarbons are categorized into fractions 1 to 4. These fractions are classified by the number of carbons in the molecule. Fraction one consists of hydrocarbons with 6 to 10 carbon atoms. The hydrocarbons in fraction one are considered volatile. Due to their low molecular weight, they easily evaporate into the atmosphere. Fraction two is considered semi-volatile and consists of hydrocarbons

that contain 10 to 16 carbon atoms. Fraction three consists of hydrocarbons with 16 to 34 carbon atoms and is considered non-volatile, and lastly, fraction four contains hydrocarbons with 35 or more carbon atoms and is the least volatile (Varjani 2017; Pandolfo et al. 2023).

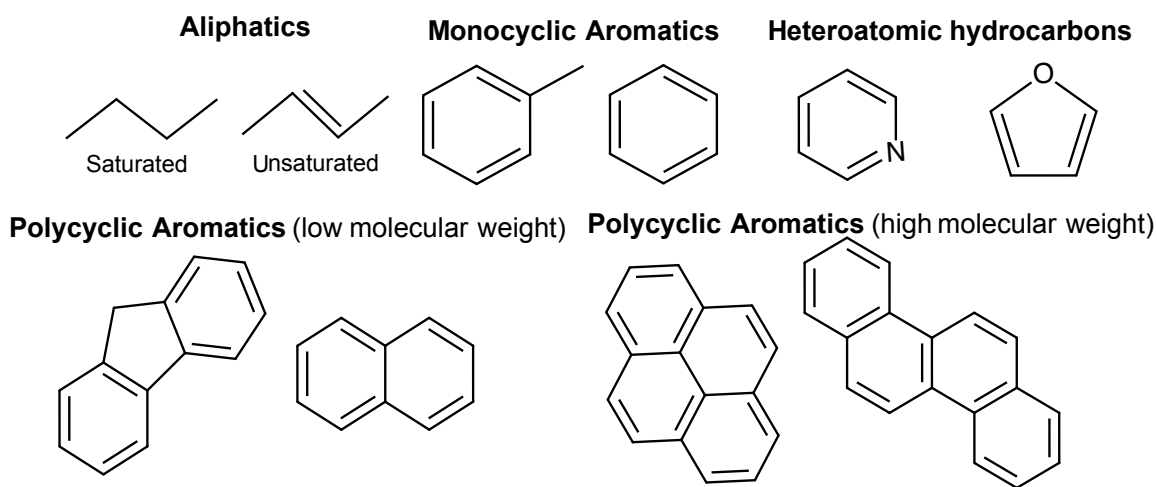


Figure 1.1. Structure of aliphatic hydrocarbons, monocyclic aromatic hydrocarbons, heteroatomic hydrocarbons and polycyclic aromatic hydrocarbons of both low and high molecular weight

Hydrocarbon contamination, such as diesel or crude oil, has a significant impact on the plants, animals and people living near contaminated areas (Ehis-Eriakha et al. 2020; Varjani and Upasani 2019). The constituents of hydrocarbon contamination, such as benzene, ethyl benzyne, toluene, xylene and polyaromatic hydrocarbons (PAHs), can all have serious health impacts. These pollutants are mutagenic, carcinogenic, teratogenic and immunotoxic (Varjani et al. 2017; Abdel-Shafy and Mansour 2016). Without human intervention, these spills can remain in the environment for decades with minimal reduction in the amount of contamination present. Additionally, the longer these contaminants remain in the ground, the more challenging it becomes to remove the contamination successfully. (Varjani et al. 2017). Some of these locations are treated to deal with the contamination. However,

many of the methods used come with their own list of undesirable side effects or byproducts. For example, a commonly used method for dealing with hydrocarbon contamination involves excavating the contaminated soil and hauling it to a secondary location, where it is periodically turned over to allow contaminants to evaporate until it meets landfill requirements for contamination levels (Mekkiyah et al. 2023). The soil is then transported to a landfill, where it will remain indefinitely (Lemming et al. 2010). This method of remediation does not treat the soil; it simply moves the contamination somewhere else. This method also removes that soil from its original environment and introduces new soil to the area. Other traditional methods of hydrocarbon remediation include thermal desorption, which involves burning off the contamination, potentially releasing harmful toxins into the air and leaving the soil devoid of nutrients (Lemming et al. 2010). This results in the soil being inhospitable to many of the fauna and flora that would have originally inhabited the area. As a cleaner, greener, and often more cost-effective alternative, bioremediation has been gaining popularity as a remediation method. Bioremediation can be categorized into several main categories. There is biostimulation, a process that involves adding limiting nutrients to contaminated soil, allowing microbes already present in the soil to break down hydrocarbons more efficiently (Chettri et al. 2021). There is bioaugmentation, a process that involves adding microbes known to have hydrocarbon-degrading capabilities to a contaminated site. There is also biopiles, which is an ex-situ treatment where the soil is excavated and placed in piles. These piles can be treated with various methods, including fertilizer, bacteria, compost or a combination of all three (Azubuike et al. 2016). There are additional methods of bioremediation, all with their own advantages and disadvantages; however, for the purpose of this research, the focus will be on bioaugmentation.

Previous research has determined that bacteria can be used to treat hydrocarbon-contaminated soils (Hosokawa et al. 2009). Several genera of bacteria have been utilized in laboratory-scale experiments to degrade hydrocarbons. In this process,

researchers have discovered various mechanisms utilized by bacteria to degrade these hydrocarbons, such as *Bacillus amyloliquefaciens*, which is capable of producing a biosurfactant that enhances the bioavailability of the hydrocarbon to all bacteria present (Brinda et al. 2024). *Pseudomonas* spp. has shown effective degradation of hydrocarbons in many environments, including at cold temperatures. Furthermore, *Pseudomonas putida* exhibits chemotaxis toward pyrene, enabling it to detect contamination and migrate toward it (Rolando et al. 2020). These traits highlight the various adaptations bacteria may use to degrade hydrocarbons and explain why bacterial bioremediation has shown such great potential. Previous research has also revealed correlations between the presence of certain genes and the ability to degrade certain fractions of hydrocarbons (Ehis-Eriakha et al. 2020). The degradation pathway varies depending on the starting hydrocarbon, and the bacteria that start this breakdown process. However, Pandolfo et al (2023) suggests these degradation pathways converge on the Krebs cycle. Their research suggests the initial step of breaking down the hydrocarbons is oxygenation using a monooxygenase enzyme or a dioxygenase enzyme for aromatic hydrocarbons. This oxygenation process requires NADH, which is found inside the bacterial cells, so the hydrocarbon molecule must be transported into the cell. Alkanes often begin being broken down by oxygenation of the sub terminal methyl group, which forms a secondary alcohol, which is then oxidized into a ketone and then an ester. Hydrolysis of the ester leads to a fatty acid and alcohol, which is then oxidized to another fatty acid. This pathway ends up with fatty acids, which can then be used in beta oxidation to produce acetyl-CoA which is then able to enter the Krebs cycle. These pathways get more complex the larger the molecule becomes however it is believed these pathways all work their way down to the Krebs cycle. Certain bacterial species may have different enzymes that allow them to start breaking down different complex hydrocarbons which highlights that to fully degrade hydrocarbons into small, relatively harmless molecules, a diverse range of bacteria with distinct genetic characteristics is required (Pandolfo et al. 2023). A single bacterial species is unlikely to be capable of completely degrading complex hydrocarbons, suggesting

that the solution to full degradation lies in a community of bacteria that work together.

Field-scale bioremediation has been achieved using various techniques, ranging from biostimulation to bioaugmentation. These processes sometimes involve simply adding nutrients or compost to the piles. Others involve adding produced bacteria with known degradation abilities or can also involve introducing genetically engineered microbes. For example, Lukić et al. (2024) treated contaminated groundwater by utilizing a bioreactor setup that recirculated the contaminated groundwater through a bioreactor containing microbes and nutrients before injecting it back into the groundwater to continue treating the entire aquifer. The study by Pelaez et al. (2013) evaluated the use of nutrients, bacteria, and surfactants in laboratory, pilot, and field-scale applications to assess prominent changes in the scale-up process. They found that the addition of nutrients and surfactants, along with aeration and irrigation, resulted in highly efficient degradation of PAHs. In a trial conducted by Mandal et al. (2014), they successfully used bioremediation to treat an effluent pit heavily contaminated with hydrocarbon waste. After the bioremediation treatment, they turned the effluent pit into a pond populated with fish. They evaluated tissue samples from the fish and found no bioaccumulation effect from hydrocarbons, demonstrating the effectiveness of the bioremediation process in removing contamination. This remediation was done using microbes produced at a facility and then transported to the site of the spill.

OUR COLLABORATOR AND BACKGROUND

Delta Remediation is a bioremediation company that has successfully conducted industrial-scale bioremediation for the past decade. Over these 10 years, Delta has developed and continually improved its bioremediation process. Currently, Delta Remediation utilizes its proprietary microbial blend, BioLogix 2XP, which comprises a combination of various *Bacillus* spp. and *Pseudomonas* spp. They culture this blend at the site of the contamination using large tanks that are equipped with

pumps and aeration devices to create a bioreactor. These bioreactors utilize the source of water most readily available, which can be water from a pond or stream, or potable water delivered via a water truck. The bioreactors utilize the physics of a Venturi effect to infuse air from the environment into the bioreactor, maintaining dissolved oxygen levels while also mixing the culture to prevent excessive sedimentation.

Additionally, these tanks are exposed to the environment wherever the remediation is to take place, meaning there can be fluctuations in temperature day by day, as well as from day to night. Due to this exposure, control over the temperature of the culture is minimal. Being exposed to the environment, these bioreactors are also at risk of contamination by airborne microbes. The current belief is that the Biologix bacterial consortium, added as a lyophilized powder to untreated water, can out-compete bacteria present in source water as well as contaminants introduced from the air.

OBJECTIVES

The above information is highly useful and demonstrates that bacteria can be effectively utilized for successful hydrocarbon remediation. However, when attempting to find previous research on field-scale bioremediation where these findings are put into practice, there is a significant reduction in the number of available papers. When trying to find papers evaluating the best way to produce the bacteria for these field-scale treatments, it becomes harder again to find previous research analyzing the requirements for an efficient field-scale bioreactor. So, to address this, the goal of this research was to determine what an ideal field bioreactor would look like. For other large-scale industrial processes, there are numerous bioreactor designs for producing large quantities of bacteria. However, the facilities that these large-scale bioreactors operate in are typically temperature-controlled, have access to sterile water, and are equipped with scientific equipment for testing and analyzing growth. Additionally, they have easily accessible electricity.

When it comes to producing large quantities of bacteria for a remediation project at the site of the spill, there are very few, if any, of those amenities. Building a facility at each spill would be impractical and highly costly, as well as simply not possible at some of the locations spills occur (along train tracks, privately owned land, etc.). Electricity can be found at some locations, and gas or diesel-powered generators can be supplied for other sites for smaller electrical requirements. Due to this constraint on electricity, we cannot operate a large-scale bioreactor with a high electrical demand, which restricts the sterilization methods available for the bioreactor itself, the sterility of the water used in the bioreactor, and the extent to which we can control the bioreactor's temperature.

So, a field bioreactor would ideally not require sterilized water, would not need to be acutely temperature-controlled and should not need to be completely sealed off from the outside environment. Additionally, aeration should be possible, and the air should not require sterilization.

As an objective of this research, we aimed to determine whether the BioLogix 2XP consortium is capable of out-competing contamination introduced via various sources. To evaluate whether the Biologix consortium is truly able to out-compete microbes introduced from the environment, a variety of tests were proposed to determine the minimum amount of sterility and other requirements necessary for an efficient bioreactor to produce large amounts of the desired bacteria. These tests were broken down into four main categories. The first category evaluated what nutrients and conditions provide the quickest growth rate. The second category evaluated what the BioLogix 2XP community looked like in sterile lab settings, and how that composition changed in response to various media and conditions. Category three determined what the largest source of contamination is in our bioreactors, and category four evaluated what the field scale bioreactor community looked like, and how it compared to the lab scale community.

Protocols that may be considered essential for highly academic scientific lab work may not be necessary for these large-scale industrial processes. The goal of these experiments was to answer questions about field and industrial scale applications. As such, there are not the same requirements for highly controlled and manipulated parameters; only the goal of producing large amounts of target bacteria as cost-effectively as possible. This allows bioremediation to be an affordable and effective solution to hydrocarbon contamination. This research aimed to identify the essential components of a well-functioning field bioreactor and those that are unnecessary for industrial bacterial bioremediation.

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CHAPTER 2 - IDENTIFICATION OF THE BIOLOGIX COMMUNITY

INTRODUCTION:

When attempting to use bacteria to address complex issues such as hydrocarbon contamination, it is difficult to find a single bacterium that will be the solution. With these complex pollutants, it is much more effective to utilize a community of bacteria that work synergistically. A community of bacteria will have a much more diverse and extensive range of enzymes and metabolic pathways that will provide a more complete breakdown of the contamination (Sathishkumar et al. 2008). Individual strains or species of bacteria can be selected that excel at degrading certain fractions of a hydrocarbon, so combining multiple species to target as many fractions of hydrocarbons as possible would most likely improve the degradation ability (Ibrar et al. 2022). The Biologix 2XP product utilizes a consortium of bacteria to improve hydrocarbon degradation. This chapter aimed to evaluate the BioLogix consortium to determine the individual bacteria present as well as how the community composition shifts when different media types are used. There are three subsections in this chapter, the first of which is titled “Community Identification – Standard Lab Technique”. This chapter utilized standard lab practices to grow the BioLogix 2XP consortium as the full community. DNA extraction was then performed on these samples followed by 16s amplicon sequencing to determine which bacteria were present and in what proportions. The next section titled “Isolated bacteria identification” used morphologic traits, both macro and micro to differentiate between isolated bacteria. Once duplicate isolated bacteria had been eliminated to the best morphological traits would allow differentiation, the isolated bacteria were grown in nutrient broth, before DNA extraction was performed, followed by 16s amplicon sequencing. The final section of this chapter is titled “Community Identification – Replicated Field Conditions”. This section utilized bioreactors designed to replicate the large-scale field bioreactors and utilized four different media types. The BioLogix 2XP consortium was grown using these replicated field conditions before DNA extraction and 16s amplicon sequencing was performed on samples from these bioreactors. This provided insightful information into how the community changes

depending on the media types as well as how the community in these bioreactors compares to the community grown using standard lab practices.

COMMUNITY IDENTIFICATION – STANDARD LAB TECHNIQUE

Method

Growth Culture

To identify the bacteria present in the Biologix lyophilized product, we utilized DNA extraction and 16s amplicon sequencing. In order to perform DNA extraction, we first cultured the Biologix community. Four 5 ml test tubes were prepared with nutrient broth. Caps were secured over the openings of the test tubes before they were autoclaved at 121 °C for 15 minutes (Lauer et al. 1982). The four tubes were inoculated with the Biologix 2xp consortium. Three of the tubes were labeled and incubated in a rotary incubator for 1 day (A1D), 3 days (A3D) and 5 days (A5D) at 37 °C. The fourth tube was incubated in a stationary incubator for 1 day (NA1D) also at 37 °C. These samples were inoculated at staggered time points so that all four tubes would reach the end of their incubation period at the same time. Additionally, a fifth sample was prepared directly from the lyophilized powder (Lyo) with no culturing. This fifth sample was used to determine if there were any bacteria in the lyophilized powder that were not growing in the nutrient broth used for the other four samples, as well as to determine the proportions of the bacteria present before the community starts to grow.

DNA Extraction:

To extract the DNA from the above mentioned 5 samples, the DNeasy UltraClean Microbial Kit from Qiagen was utilized. To begin this process 1.8 ml of sample was transferred to a 2 ml centrifuge tube from each of the 5 samples. The Qiagen UltraClean Microbial Kit protocol was then followed to obtain the extracted DNA in elution buffer as depicted in Figure 2.1 below (Qiagen 2025).

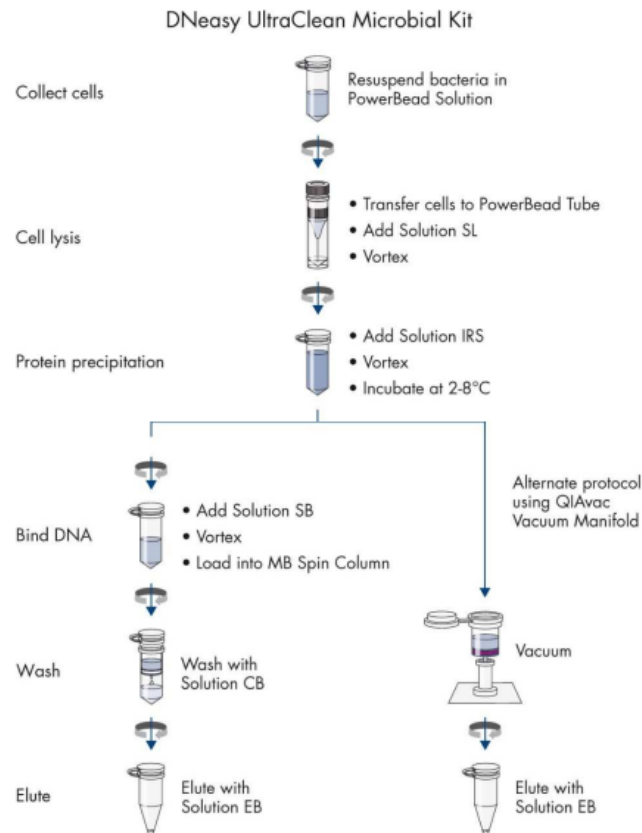


Figure 2.1: Diagram of Qiagen DNA extraction workflow for the DNeasy ultraclean Microbial kit, used for the purpose of extracting bacterial DNA for downstream applications (Qiagen 2025)

16s Amplicon sequencing:

The 16s amplicon sequencing performed by the University of Dalhousie began with PCR-amplification of the amplicon fragments from the DNA. This was done in duplicate with 1:1 and 1:10 template dilutions using the Phusion plus polymerase. Full length 16s fusion primers were then used to prepare the samples for PacBio Vega. The products of the PCR were then visually verified with the help of a Hamilton Nimbus Select robot and Coastal Genomics Analytical Gels. The samples were then pooled and quantified fluorometrically prior to sequencing. The PacBio Vega was then used to sequence the samples. The University of Dalhousie then utilized QIIME 2 and their Bioinformatics pipeline to filter, denoise and remove chimeras (Comeau et al. 2017). Sequencing depth ranged from 18,004 to 28,784

raw reads per sample. After denoising and chimera removal we had 7,733 to 20,807 non chimeric reads corresponding to 43 – 81% retention as summarized in Table 2.1. These percent retention values are lower than the reads from the Illumina-based sequencing used in other chapters however this was full length 16s RNA sequences which provide higher taxonomic resolution.

Table 2.1. Sequencing information for the 16s amplicon sequencing on 5 samples including aerated, non-aerated and directly from lyophilized form.

Sample ID	Raw Reads (input)	Reads After QC (non-chimeric)	% Retained
A1D	28,784	18,122	63.00%
A3D	24,633	12,600	51.20%
A5D	22,517	12,277	54.50%
Lyo	25,641	20,807	81.20%
NA1D	18,004	7,733	42.90%

Results

The results of the 16s amplicon sequencing performed for this experiment found bacteria from the genera of *Bacillus*, *Brevibacillus* and *Pseudomonas* as seen in Figure 2.2. In sample A1D, which was aerated for 1 day, 97.9% of the population was *Bacillus* spp., 1.96% *Bacillus circulans* and 0.11% *Brevibacillus reuszeri*. Sample A3D which was aerated for three days had a population containing 12.16% *Bacillus* spp. and 87.84% *Bacillus clausii*. Sample A5D which was aerated for five days contained 3.66% *Bacillus* spp., 0.09% *Bacillus circulans*, 29.29% *Brevibacillus* spp., 10.05% *Brevibacillus borstelensis* and 56.9% *Brevibacillus reuszeri*. Sample Lyo, which was a sample taken directly from the lyophilized powder consisted of 93.43% *Bacillus* spp., 0.13% *Brevibacillus borstelensis* and 6.4% *Pseudomonas* spp. Lastly, the sample NA1D which was not aerated, and was incubated for 1 day consisted of 73.63% *Bacillus* spp, 18.13% *Bacillus circulans*, 1.39% *Brevibacillus*, 1.09% *Brevibacillus borstelensis*, 4.85% *Brevibacillus reuszeri* and 0.905% uncultured bacterium. Out of these 5 samples, *Pseudomonas* spp. was only found in the lyophilized sample. *Bacillus* spp and *Brevibacillus* spp. were found in all samples

including the lyophilized sample, with the exception of *Brevibacillus* spp. being absent in sample A3D.

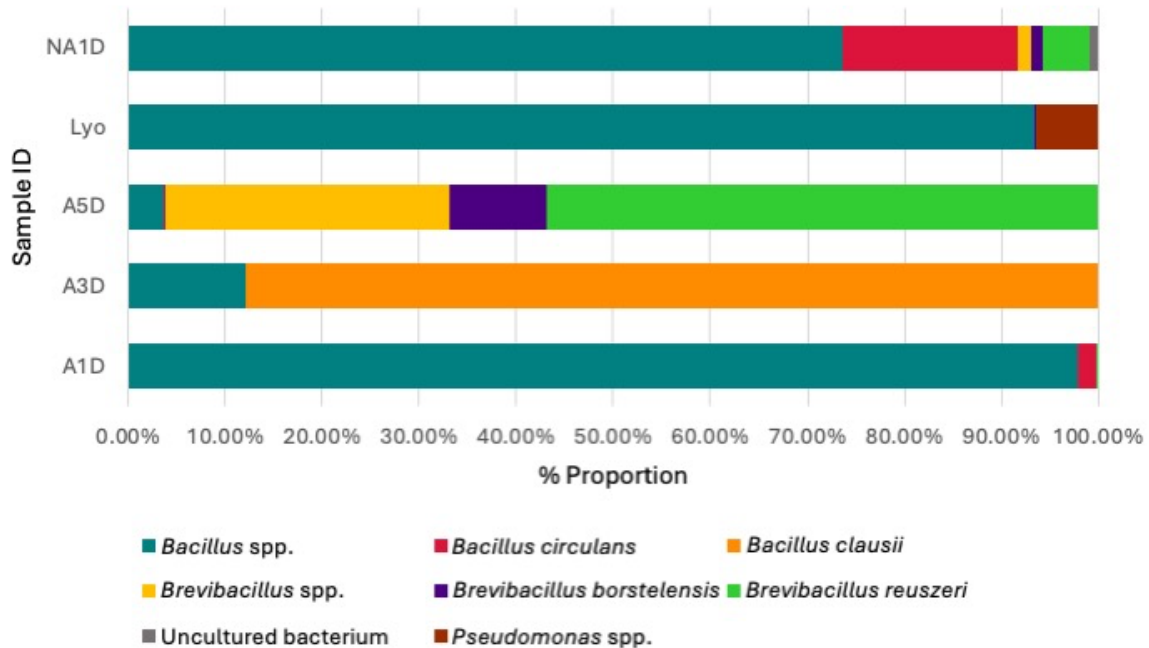


Figure 2.2: 16s amplicon sequencing results evaluating the bacteria present in the BioLogix 2XP product represented as percent of the population in 5 samples of various incubation periods and conditions.

Discussion

The results of this experiment showed that *Bacillus* spp. dominates the population in samples A1D and A3D, however in sample A5D *Brevibacillus* spp. makes up the majority of the population. *Pseudomonas* spp. is only found in the lyophilized sample, this could indicate that *Pseudomonas* spp. is present in the lyophilized product, however it may not be viable and is therefore not showing up in the other samples as the *Bacillus* spp. and *Brevibacillus* spp. population grows exponentially. This could be a result of *Pseudomonas* spp. lacking an endospore, whereas *Bacillus* spp. do form endospores. It is possible the *Bacillus* spp. are better able to survive lyophilization due to this endospore (Palleroni 2005; Logan and De Vos 2009). Alternatively, the *Pseudomonas* spp. may take longer to start growing, allowing *Bacillus* spp. and *Brevibacillus* spp. to get well established and inhibit the growth of

the *Pseudomonas* spp. There has been previous research evaluating the negative impacts of bacillus and pseudomonas on each other, however there is increasing research evaluating mutualism and commensalism between these two genera (Lyng and Kovacs 2023). *Pseudomonas* spp., along with *Bacillus* spp. and *Brevibacillus* spp. are target organisms for this product, so the lack of *Pseudomonas* spp. in the grown cultures is concerning.

ISOLATED BACTERIA IDENTIFICATION

Method

Growth Culture

In an effort to isolate the members of the consortium, any morphologically unique colonies that appeared during any of the other experiments performed were isolated. To isolate the colonies, Nutrient Agar plates were utilized. The unique colonies would be picked and streaked for isolated colonies using a 4-way streak strategy. These plates would be incubated for 24 hours at 37 °C. an isolated colony would be selected from this plate and streaked again for isolated colonies on a new Nutrient agar plate. This plate was then incubated at 37 °C for 24 hours.

Macroscopic and microscopic differentiation

Macroscopic morphologic characteristics will be evaluated and recorded for all isolated colonies. This will include traits such as the margin, elevation, colour and texture of the colonies. Microscopic morphological characteristics will also be identified via Gram staining. Traits such as orientation, shape, Gram stain and presence of endospores will be noted. Isolated bacteria that match for all traits will be considered duplicate samples. These isolated samples will also eventually be analyzed using 16s amplicon sequencing to identify the potential genera and species present (Winand et al. 2019).

Results

To identify the genera and potentially species present, 16s amplicon sequencing was attempted on the isolated bacteria. However, many of the samples failed at the

PCR step of the library preparation for sequencing for unknown reasons. Five of the samples we run successfully, all five of which belong to the genera of *Bacillus*. Despite all 5 of these samples belonging to the genus *Bacillus*, there was both Gram-negative and Gram-positive bacteria present when evaluating the microscopic morphology, suggesting another genus of bacteria is also found in the consortium.

Discussion

The results of this experiment did not provide the information desired from this experiment. However, it was determined that *Bacillus* spp. is present in the consortium, and there is at least one other genus present that is Gram-positive.

COMMUNITY IDENTIFICATION – REPLICATED FIELD CONDITIONS

Methods

Bioreactor set-up

This experiment grew the BioLogix 2XP consortium in bioreactors that resemble field conditions. These bioreactors contained four different media types including: 10:10:10 NPK, Tryptone, Bushnell Haas and yeast extract. These bioreactors were all aerated using an air stone and fish tank aerators to replicate the venturi system used in field bioreactors. The jars used as the bioreactors are large four-liter fermentation jars with a screw on plastic lid as seen in Figure 2.3. These lids had a small hole in the center where a fermentation lock was inserted. These airlocks utilize a curved design and a small amount of water to prevent air from entering from outside the jar, but air inside the jar can be released as the pressure inside increases. An additional hole was drilled into the lids to allow for the airline for air stone to be routed through the lid with a tight seal around the airline to prevent contamination. These air pumps did not have any sort of filtration for the air, so a 0.2-micron syringe filter was added to the airline upstream of the bioreactor lid to filter the air. These air pumps and air stones provided a constant flow of air that was released into the bioreactor in fine bubbles. As the air pressure inside the bioreactor

would increase, the air would be released through the fermentation lock preventing a buildup of pressure inside the bioreactor.

Growth Culture

Each of these jars were filled approximately half full with two liters of the corresponding media. The opening of the jars was covered with tin foil after the media was added to the bioreactors. The Bioreactors with media and tin foil coverings were then run through the autoclave at 15 PSI and 121 °C for 15 minutes to ensure complete sterilization (Lauer et al. 1982). The airlines to be used for air supply were autoclaved as well. The jar lids and airlocks were submerged in 15% v/v bleach solution for 30 minutes as they were not designed to withstand the heat of the autoclave (World Health Organization 2014). The air stones and syringe filters have an assumed sterility while inside their respective packaging. After autoclaving, the airlines were fed through the lids of the bioreactors, an air stone was added to the end inside the jar, and the syringe filter was added inline outside of the bioreactor. The airlock was put in place, the lids were hand tightened onto the jars, and the air pumps were turned on. All four bioreactors were inoculated with 1% w/v Biologix 2XP and were incubated at room temperature.

16s amplicon sequencing/ DNA extraction

DNA extraction was performed following the Qiagen DNeasy ultraclean extraction kit protocol (Qiagen 2025). 16s amplicon sequencing of the V3-V4 region was performed by the University of British Columbia (UBC). UBC generated the amplicons using the primer sequences 16S: 341F CCTACGGGNGGCWGCAG, 805R GACTACHVGGGTATCTAATCC. The library was then sequenced on Illumina NextSeq 2000 P1. QIIME2 version 2024.2.0 along with DADA2 version 1.26.0 via Bioconductor in QIIME was used for analysis. The silva database was then utilized for classification of the sequences. Genus level calls were made at ~97% sequence similarity, and species level calls were made at >99% sequence similarity.

(Sequencing + Bioinformatics Consortium 2024) there was high sequencing depth for all samples with raw reads ranging from 27,776 to 403,036 per sample. After filtering, denoising and chimera removal we were left with 22,756 to 341,514 non-

chimeric reads per sample. This is between 82% and 91% retention as summarized in Table 2.2

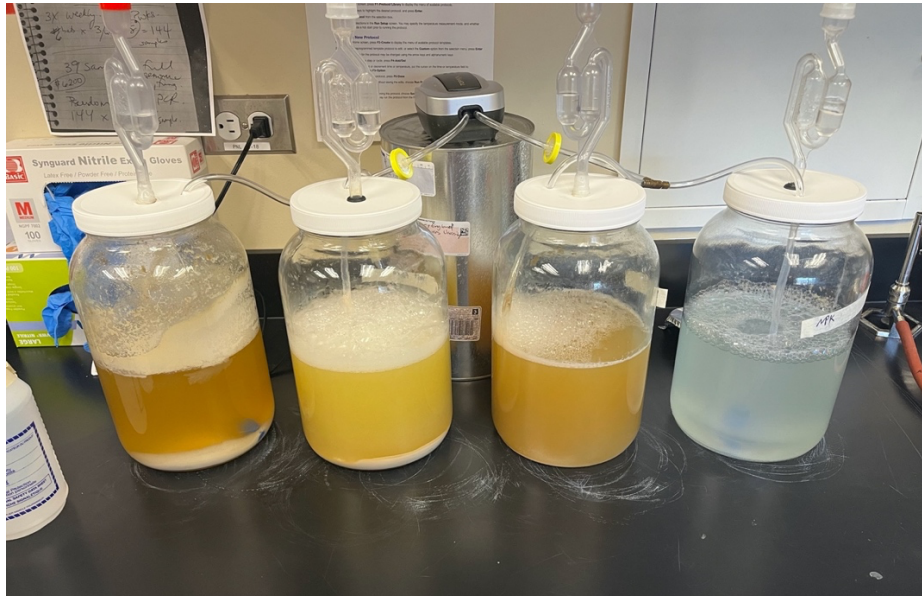


Figure 2.3: Bioreactor setup designed to replicate field conditions, showing air stones (blue), airlines routed through the lids, upstream syringe filter (yellow) behind the central bioreactors, airlocks integrated into the lid centers, and the central air pump.

Table 2.2. Sequencing information for the 16s amplicon sequencing on the air contamination samples

Sample ID	Raw Reads	Reads After QC (non-chimeric)	% Retained
BH	527,075	294,472	55.90%
NPK	440,090	340,088	77.30%
TRY	330,117	260,635	79.00%
YE	338,458	259,603	76.70%

Results

The results of this experiment show that community composition varies between the different media types. The four media types are compared in Figure 2.4. In the Bushnell Haas (BH) bioreactor the majority of the community consists of the genus *Pseudomonas*, at 82.91% of the population. *Bacillus* spp. makes up another 16.05% of the population for the second most prominent genus. This totals 98.96% of the

population being these two genera. *Brevibacillus* spp. makes up 0.53% of the remaining population. The last 0.5% of the population is made up of the genera *Gaiella* spp., *Mycobacterium* spp., *Escherichia-Shigella* spp., *Methylobacterium-methylobacterium* spp. and *Pantoea* spp. in descending order from most prominent to least.

The 10:10:10 NPK (NPK) bioreactor had a population consisting mostly of *Bacillus* spp., with 95.44% of the population being *Bacillus* spp., *Brevibacillus* spp. makes up another 4.49% of the population making it the second most prominent genus. These two genera make up 99.94% of the population. The genera making up the remainder of the population are *Pseudomonas* spp., *Lysobacter* spp., *Klebsella* spp., and *Gaiella* spp. from most to least prominent.

The Tryptone media (TRY) population was majority *Bacillus* spp. with 50.01% of the population consisting of this genus. The genus *Pseudomonas* spp. made up another 48.75% of the population making it the second most prominent genus. These two genera make up 99.91% of the population, with the remainder of the population consisting of the genera *Brevibacillus* spp., unassigned, bacteria, and *Acinetobacter* spp. in descending order of prominence.

The final bioreactor with Yeast extract (YE) media, consisted of majority *Bacillus* spp. with 52.63% of the population consisting of this genus. *Pseudomonas* spp. makes up another 46.9% of the population making it the second most prominent genus. These two genera make up 99.57% of the population, with the remainder of the population consisting of *Pantoea* spp., *Brevibacillus* spp., bacteria, and unassigned. In the four bioreactors, *Bacillus* spp. made up a significant proportion of the population in all media types, with the lowest being 16% in the Bushnell Haas media. *Pseudomonas* spp. also made up a significant portion of the population in all media types except for NPK where *Pseudomonas* spp. only made up 0.05% of the population.

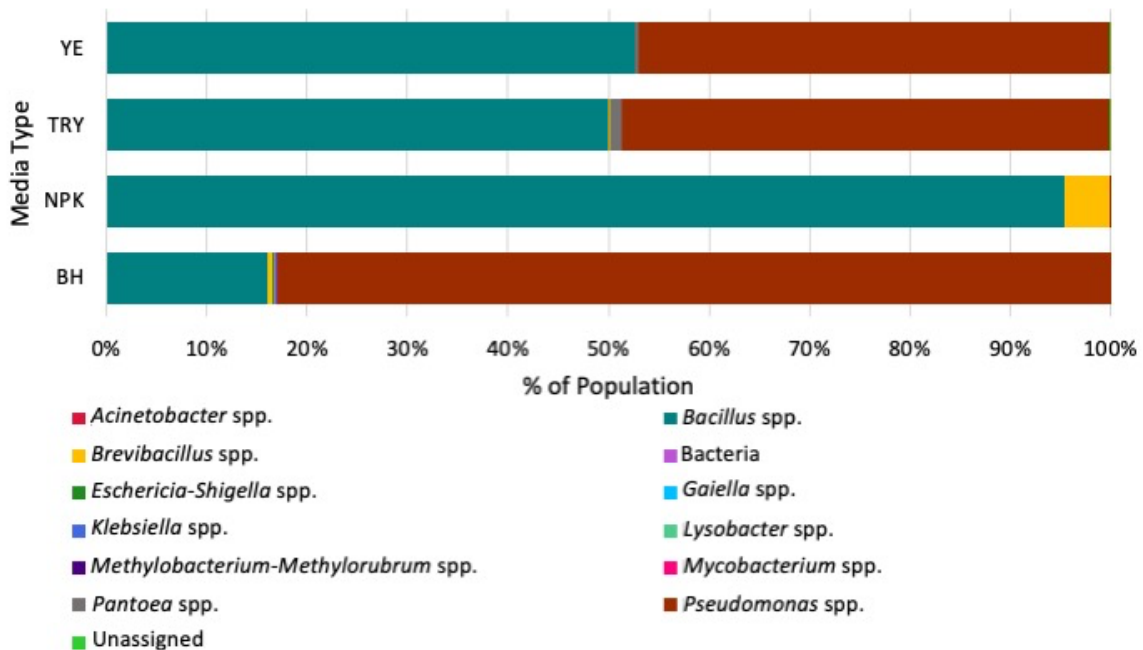


Figure 2.4: Community composition represented as percent along the x-axis for BioLogix 2XP grown in Yeast extract (YE), tryptone (TRY), 10:10:10 NPK (NPK) and Bushnell Haas (BH) on the y-axis.

Discussion

The results of this experiment show that different media types can influence the community of bacteria that grows from the lyophilized Biologix 2xp product. As summarized in Table 2.3, of the 4 media types tested, Tryptone and yeast extract produced a similar community with about 50% *Bacillus* spp. and 50% *Pseudomonas* spp. The Bushnell Haas media shifted the community to be majority *Pseudomonas* spp. with 82.9% of the population. NPK media had the opposite effect and drastically reduced the amount of *Pseudomonas* spp. to 0.05%. As *Pseudomonas* spp. is one of the target genera for this product this result is concerning, however, using a different media type appears to address this concern and increase the amount of *Pseudomonas* spp. present. The NPK nutrient is an agricultural fertilizer and uses urea as the main source of nitrogen. This nitrogen source will most likely favor the *Bacillus* spp. genus as *Bacillus* spp. is more often urease positive, allowing the genus to utilize urea as a nitrogen source (Logan and De Vos 2009). *Pseudomonas*

spp. on the other hand is less commonly urease positive and therefore may not be able to utilize the nitrogen provided in the NPK media (Palleroni 2005). Due to this nutrient lacking a suitable nitrogen source for one of the two target genera, an alternative nutrient source is recommended. Tryptone and yeast extract are both lab grade materials and would be very costly for large scale applications. The Bushnell Haas media however has nutrients to support both *Bacillus* spp. and *Pseudomonas* spp. and is much more affordable to produce on a large scale. As a result, the Bushnell Haas media would serve as a good alternative to NPK as it allows increased growth of *Pseudomonas* spp. and *Bacillus* spp., while being more cost effective than the Yeast extract and tryptone.

Table 2.3: Summary table displaying the bacterium making up the highest percentage of the population to the lowest for each media type.

Percent	BH	NPK	Try	YE
Highest	<i>Pseudomonas</i> spp. 83%	<i>Bacillus</i> spp. 95%	<i>Bacillus</i> spp. 50%	<i>Bacillus</i> spp. 53%
	<i>Bacillus</i> spp. 16%	<i>Brevibacillus</i> spp. 4%	<i>Pseudomonas</i> spp. 49%	<i>Pseudomonas</i> spp. 47%
	<i>Brevibacillus</i> spp. 1%	<i>Pseudomonas</i> spp. 0%	<i>Pantoea</i> spp. 1%	<i>Pantoea</i> spp. 0%
	<i>Gaiella</i> spp. 0%	<i>Lysobacter</i> spp. 0%	<i>Brevibacillus</i> spp. 0%	<i>Brevibacillus</i> spp. 0%
	<i>Mycobacterium</i> spp. 0%	<i>Klebsiella</i> spp. 0%	Unassigned 0%	Bacteria 0%
	<i>Escherichia-Shigella</i> spp. 0%	<i>Gaiella</i> spp. 0%	Bacteria 0%	Unassigned 0%
	<i>Methylobacterium-Methylobacterium</i> spp. 0%		<i>Acinetobacter</i> spp. 0%	
	Bacteria 0%			
Lowest	<i>Pantoea</i> spp. 0%			

CHAPTER CONCLUSION

The results of this chapter reveal that with different nutrients, we see different community compositions. When the consortium is grown in the lab using nutrient broth and standard lab practices, there was no *Pseudomonas* spp. present in any of

the samples other than the sample taken directly from the lyophilized powder. However, when grown in a small bioreactor designed to replicate the field bioreactors *Pseudomonas* spp. was present in three of the four media types. These results provide valuable insight as to the impact of the nutrient. The lab procedures with nutrient broth do not seem to support the growth of the target genera of *Pseudomonas* spp. and *Bacillus* spp. Although *Bacillus* spp. was present in these samples, there was no *Pseudomonas* spp. In the replicated field conditions, we see that *Pseudomonas* spp. was found in three of the four samples. These media types support the growth of the genera *Pseudomonas* spp. and *Bacillus* spp., making all three good options for large-scale application. Of these three, Bushnell Haas provides the most *Pseudomonas* spp., while still supporting the *Bacillus* spp., making it a good option for field applications as *Pseudomonas* spp. is the primary genus desired for bioremediation applications. From these results we also see that 10:10:10 NPK fertilizer is not a suitable option as it does not support the growth of the *Pseudomonas* bacteria as a result of potentially incompatible nitrogen source as well as high levels of EDTA which *Pseudomonas* bacteria are more sensitive to (Palleroni 2005).

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CHAPTER 3 - NUTRIENT AND CONDITION OPTIMIZATION

INTRODUCTION

For the process of bioaugmentation, large numbers of bacterial cells are desired. To achieve these large amounts of cells, we need to determine the ideal growth conditions, so that we can replicate these ideal conditions as closely as possible in the field to achieve the highest rate of growth. Currently, Delta Remediation uses liquid NPK fertilizer designed for agricultural applications. This fertilizer has a 10:10:10 ratio of nitrogen, phosphorus, and potassium, with the main form of nitrogen being urea. This liquid fertilizer is cost effective and is easily added to bioreactors due to it being a liquid. Additionally, dextrose is added to the bioreactors to provide a source of carbon for the bacteria while in the bioreactor.

Like all living things, bacteria require certain nutrients to grow and reproduce (Burrows 1936). Carbon is the backbone of life and is the most abundant element in bacteria. Carbon is required for producing molecules like nucleic acids and proteins, and this carbon can be acquired from many sources, such as the dextrose added to the bioreactors (Bonnet et al. 2020; van Niekerk and Pott 2023). This requirement for carbon is also what makes bacteria so capable of degrading hydrocarbons, since the bacteria will use the carbon found in hydrocarbons as a source of carbon and energy when an alternative source of carbon is not available (Pandolfo et al. 2023).

Bacteria also require a source of nitrogen. Nitrogen allows the bacteria to synthesize proteins which are required for cell functioning. Nitrogen can be found in many different molecules such as urea, ammonia, nitrogen gas, as well as from proteins, or amino acids (Bonnet et al. 2020).

Phosphorus is another important nutrient for bacterial growth and is often a limiting nutrient in the growth of bacteria. Phosphorus is used in DNA and RNA as well as

for the production of ATP which is essential for the functioning of the bacterial cells (Tapia-Torres et al. 2016).

In addition to these main nutrients, there is also many other trace nutrients that bacteria require for growth, such as calcium, iron, chloride etc. These nutrients are required for various cell functions and if these nutrients are absent, it can have a negative effect on the growth and function of the bacteria (Burrows 1936).

This chapter covers four subsections: The first section titled “Optimization of Nutrients for the BioLogix 2XP Consortium” aimed to identify a nutrient source that provided maximum growth while keeping cost in mind. A total of five nutrient blends were tested, including tryptone, yeast extract, yeast flakes, NPK fertilizer and Bushnell Haas media (Bushnell and Haas 1941). These media types have varying nutrient sources and vary in cost. The second subsection titled “Optimization on Optimal Growth and Viable Temperature Range of Biologix 2xp” investigated the temperature range at which we see activity, as well as determined an absolute minimum and maximum temperature where the population no longer increases. Luria-Bertani (LB) (Bertani 1951) media and Bushnell Haas media will be utilized for this experiment, and the BioLogix consortium will be evaluated as a whole in addition to the isolated bacterium being evaluated individually. The third subsection titled “Investigation of Aeration Requirements” will evaluate the impact of aeration on the bacterial culture. As these cultures will be grown in large bioreactors, we want to determine if O₂ needs to be supplemented into the culture to maintain rapid growth. This experiment will be done by growing the bacteria in NPK media with dextrose in two bioreactors, one with aeration, one without. An additional two bioreactors will have yeast flake media, one with aeration one without. The final subsection titled “Evaluation of Anaerobic Abilities of the BioLogix Consortium” will determine the oxygen requirements of the consortium. This will reveal if the consortium is mainly obligate aerobes or if the consortium has the ability to tolerate low oxygen levels. For this experiment, the bacteria will be grown individually as well as the complete

consortium on 2 different media types. LB and Bushnell Haas media will be used, and these plates will be incubated in 3 different conditions: aerobic, anaerobic and CO₂ rich. Each of these subsections will contain their own methods, results and discussion sections, the chapter will then be concluded with a discussion combining the results and findings of all four subsections.

OPTIMIZATION OF NUTRIENTS FOR THE BIOLOGIX 2XP CONSORTIUM

Method

Media Preparation:

For this experiment, two trials were performed, each trial with 4 different media. Trial one used tryptone, yeast extract, yeast flakes, and NPK fertilizer. Trial two substituted Bushnell Haas media for the Yeast flakes, with the other three media remaining the same. The tryptone media was simply tryptone added to water to create the Tryptone media at a rate of 1% w/v. Tryptone is a complex nutrient often used to increase bacterial growth. Yeast extract for this experiment was mixed with water at a rate of 1% w/v to produce the yeast extract media. Yeast flakes are an easily sourced product as it is used as a nutritional supplement for humans and therefore easily found at most grocery stores. Yeast flakes were used as a potential substitute for yeast extract as yeast flakes are much cheaper than yeast extract. Yeast flakes were mixed with water at a rate of 1% w/v to create the yeast flake media. The NPK media utilized agricultural fertilizer with a ratio of 10:10:10 of Nitrogen:Phosphorus:Potassium. This is the nutrient Delta Remediation was using for their field scale bioreactors, as it was easily sourced, and the liquid format of the product made it easy to mix into the bioreactors. The NPK media was created by mixing 1% w/v of NPK fertilizer with water. Lastly the Bushnell Haas media was made in accordance with the recipe created by Bushnell and Haas (1941) (MgSO₄ 0.2 g; CaCl₂ 0.02 g; KH₂PO₄ 1.0 g; K₂HPO₄ 1.0 g; NH₄NO₃ 1.0 g; FeCl₃ 0.05g). This medium was supplemented with 1% w/v dextrose as the original formulation for Bushnell Haas media is designed for testing a bacterium's ability to degrade hydrocarbons, so the nutrient blend is specifically designed to be free of carbon. For

our purpose of growing the bacteria in a bioreactor, we added dextrose as a carbon source to allow the bacteria to grow before being added to the contaminated soil where the hydrocarbon will become the carbon source.

Experiment Set-up:

The experimental procedure for the nutrient testing was as follows. For trial one, four, four-liter fermentation jars were used. These jars came with a screw on lid with a single hole in the center where a simple airlock can be inserted. These airlocks utilize a curved tube with a small amount of water that allows air to be pushed out of the jar but not travel back into the jar. These lids were slightly modified by drilling an additional hole to allow a small airline to be inserted into the jar. These airlines had an air stone on the end inside the jar. The other end of the airline was connected to a small fish tank air pump that provides a steady supply of air into the jar with the air lock acting as an exhaust port. The set up of these bioreactors can be seen in Figure 3.1 below.

These air pumps did not include any type of filtration for the air, so a 0.2-micron syringe filter was slightly modified and inserted into the airline upstream of the lid. Each of these jars was filled approximately half full with 2 liters of media. The opening of the jars with media were covered with tin foil before being run through the autoclave at 121 °C and 15 PSI for 15 minutes to ensure sterility of the media and the jars (Lauer et al. 1982). The airlines to be used for air supply were autoclaved as well. The jar lids and airlocks were submerged in 15% v/v bleach solution for 30 minutes (World Health Organization 2014). The air stones and syringe filters have an assumed sterility while inside their respective packaging. The conditions and media used in each jar is detailed below in Table 3.1. There was one jar of each media type: NPK, Tryptone, yeast flakes, and yeast extract for a total of 4 jars, with Bushnell Haas replacing yeast flakes in trial 2. The airlines were fed through the lids of the bioreactors, an air stone was added to the end inside the jar, and the syringe filter was added inline outside of the bioreactor. The airlock was put in place, and the lids were hand tightened onto the jars. A spectrophotometer was set to 600 nm and was zeroed using DI water prior to every reading (Dalgaard et al. 1994). Trial one of

this experiment was done at 360 nm, which is not the standard wavelength for measuring cell density, however this data was still included as it showed similar patterns to trial two which utilized the wavelength of 600 nm. With these OD readings being collected at two wavelengths, it limited the statistical analysis that could be done between the two trials. To support the Bushnell Haas results, the OD readings that were collected from the controls of 3 later experiments with identical set up to the BH trial were averaged for Figure 3.3 below as well as in Table 3.3, using the standard deviation as error bars. In addition to the average and standard deviation, a Pearsons pairwise correlation matrix was calculated to determine how closely correlated the growth trends from Bushnell Haas media are. The NPK media also is supported with a second set of data collected from a later experiment to provide the average, standard deviation, and correlation.

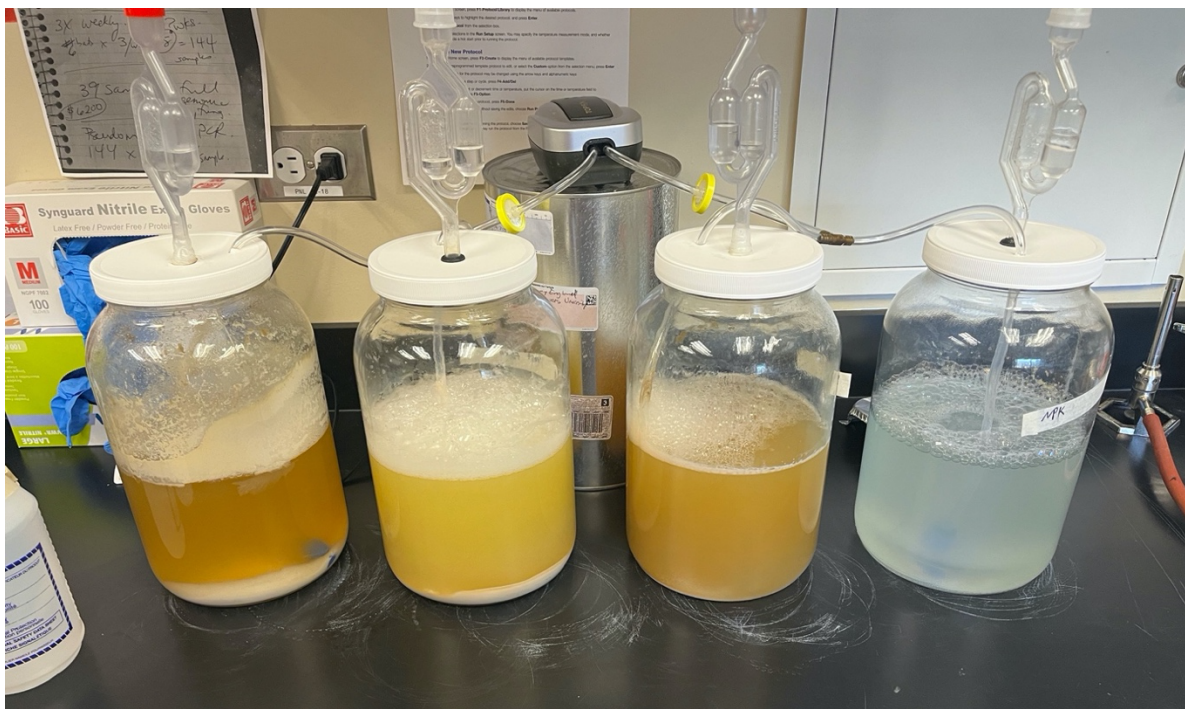


Figure 3.1: Bioreactor setup designed to replicate field conditions, showing air stones (blue), airlines routed through the lids, upstream syringe filter (yellow) behind the central bioreactors, airlocks integrated into the lid centers, and the central air pump.

An absorbance reading was taken of each media type prior to inoculation. This value served as a baseline reading as the various media types had varying absorbance readings. All four bioreactors were inoculated with 1% w/v Biologix 2XP to replicate the inoculum used in field applications. Another absorbance reading was collected immediately after inoculation to get a baseline account for turbidity added by the lyophilized bacteria. Air supply was turned on at this point. Additional absorbance readings were collected every 12 hours for 72 hours. At the end of this 72-hour period, the absorbance values were compared between the 4 media types. To allow for a more representative comparison we first calculated the change in turbidity for each medium by subtracting the original absorbance reading of the uninoculated media from each of the absorbance readings so that we should in theory only be accounting for the bacterial material present in the media, allowing the comparison between media types. With the 72 hours of absorbance readings collected for all four media, we are able to compare the growth curves of the consortium in each medium.

Table 3.1: Nutrient and Condition information for the four bioreactors used in the nutrient optimization experiment.

Bioreactor	Nutrient	Aeration	Inoculation
Jar 1	10:10:10 NPK fertilizer	YES	1% (%w./v.) Biologix
Jar 2	Yeast extract	YES	1% (%w./v.) Biologix
Jar 3	Yeast flakes*	YES	1% (%w./v.) Biologix
Jar 4	Tryptone	YES	1% (%w./v.) Biologix

*Replaced with Bushnell Haas medium in trial 2

Results

In trial one, the experiment was stopped after 48 hours because of the OD values stabilizing or even decreasing in the case of the YF media. Table 3.2 below summarizes the starting OD reading, the peak OD and the final OD reading of the 4 different media. From this table we can see that the greatest OD was achieved using tryptone media, where a peak OD of 1.446 was reached. This was followed by yeast extract, which reached a peak OD of 1.077. Yeast flakes and NPK had similar peak

OD readings of 0.386 and 0.390, respectively, however yeast flakes had a lower starting OD so had a larger increase in OD to its peak than NPK, meaning NPK had the least increase in OD out of all four media types. The tryptone media not only had the highest OD but also reached its peak OD in the shortest amount of time, taking only 12 hours to reach an OD of over 1.000. Figure 3.2 below depicts the trends seen in this trial.

Table 3.2: OD values at time zero, peak OD and the end point of 48 hours for the four media types evaluated in trial one.

Media	Time 0	Peak OD	Time 48
NPK	0.259	0.39	0.32
Yeast extract	0.171	1.077	1.077
Yeast Flake	0.107	0.386	0.284
Tryptone	0.196	1.446	1.402

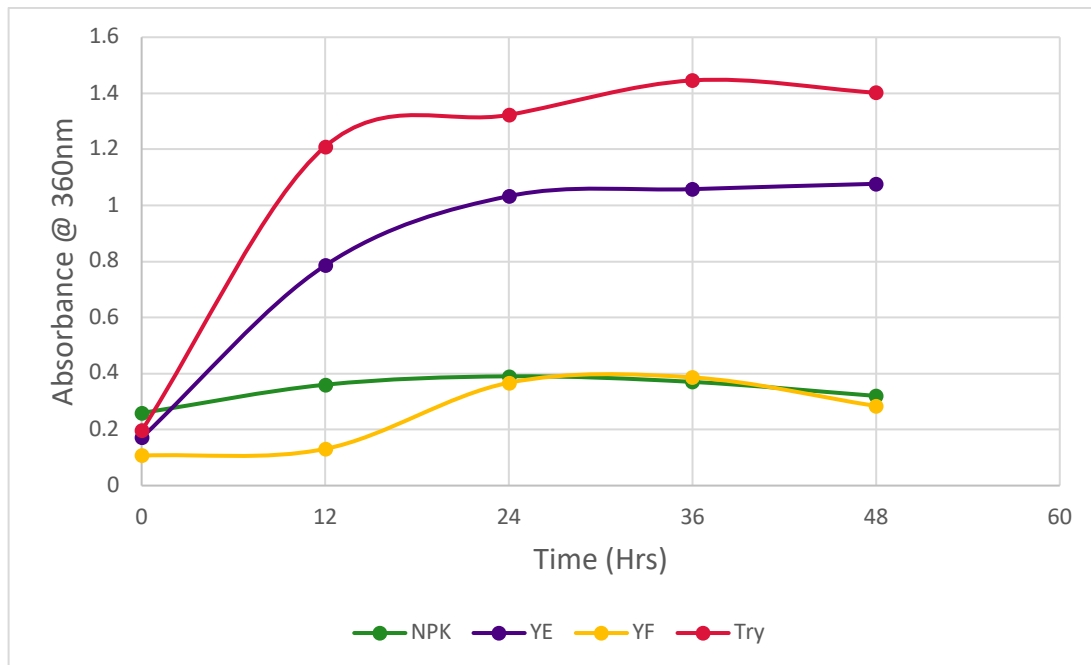


Figure 3.2: Optical density (OD₃₆₀) measurements of bacterial cultures grown in NPK, yeast extract, yeast flakes and tryptone media over a 48-hour incubation period, used to evaluate the medium that provides the greatest increase in OD.

In trial two the experiment ran for 120 hours as slower growth was observed in the Bushnell Haas media. Table 3.3 below summarizes the key points from Figure 3.3. From this table we can see that tryptone had the greatest peak in OD at 1.243. Yeast extract had the next highest OD with a peak of 1.105. Bushnell Haas media reached a peak OD of 0.8995 and lastly NPK reached a peak OD of 0.03. The overall increase in OD for all four media types followed the same pattern as trial one with Tryptone having the greatest increase of 1.243 and NPK having the lowest increase of 0.

In Figure 3.3 the error bars using standard deviation indicate that as time passes the standard deviation increase, suggesting the OD has more variation as time passes. However, despite the error bars showing a larger error, the pairwise correlation matrix showed the lines are highly correlated with correlation coefficients ranging from 0.935 to 0.958 when comparing the BH sample to the other 3 samples from later experiments. The full correlation matrix can be found in the appendix. A pairwise correlation was also calculated for the NPK media using the data from a later trial, revealing that the correlation coefficient between the two trials was 0.996, indicating the data from the two trials was highly correlated.

Table 3.3: Change in optical density (OD₆₀₀) measurements for four different media at 0 hours, peak OD, and 120 hours, including the increase in OD from baseline to peak, with averages used for BH (n=4) and NPK (n=2)

Media	0 hours (start point)	Peak OD	120 hours (End point)	Increase
NPK	0	0.03	0.03	0
Yeast Extract	0	0.976	1.105	0.976
BH	0	0.8995	0.8995	0.894
Tryptone	0	1.243	1.243	1.243

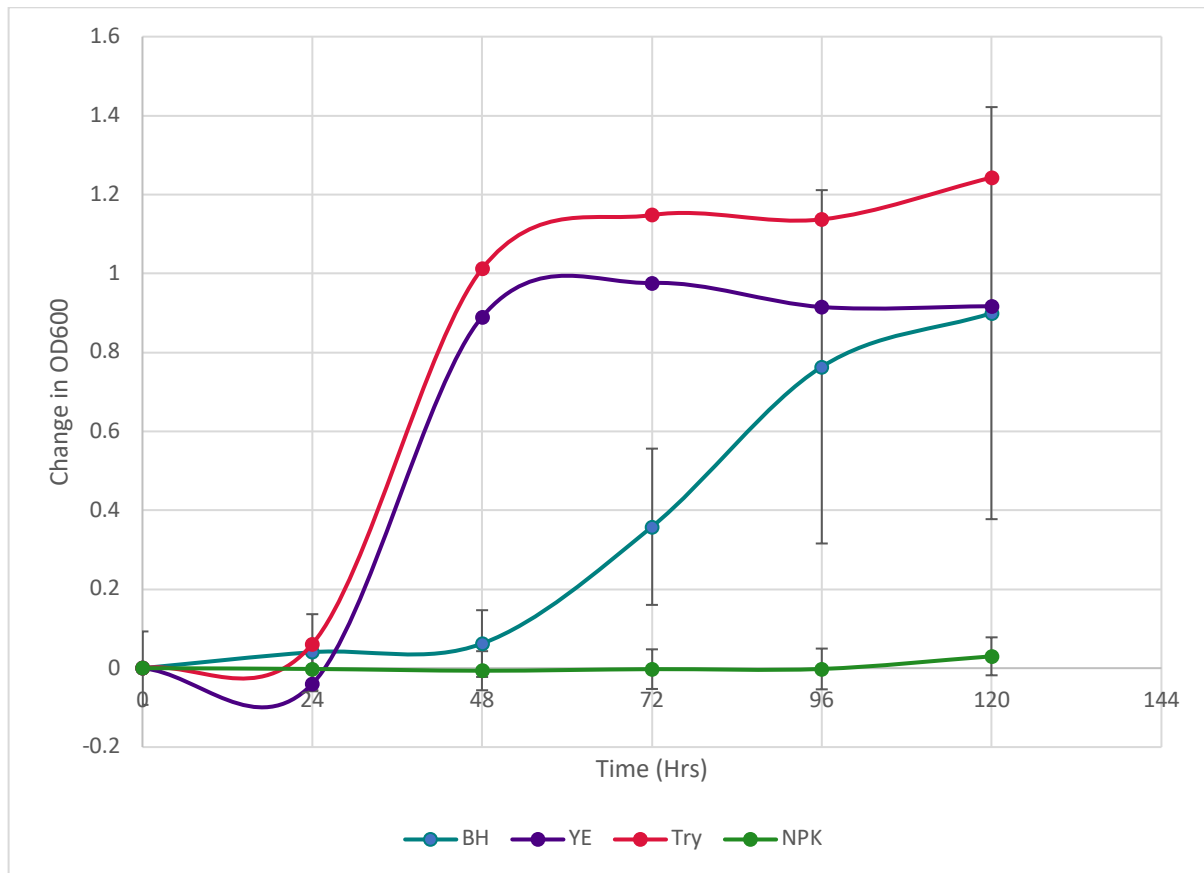


Figure 3.3: Change in optical density (OD600) measurements of bacterial cultures grown in Bushnell Haas, yeast extract, tryptone, and NPK media over a 120-hour incubation period, used to evaluate the medium that provides the greatest increase in OD. Error bars for BH and NPK determined using standard deviation. (BH n=4, NPK n=2)

Discussion

In trial one we compared the four media types of NPK liquid fertilizer, yeast extract, yeast flakes and tryptone. By monitoring the absorbance of these four media types it was revealed that tryptone yielded the largest change in OD which correlates to the highest number of bacterial cells. The next largest change in OD was from the yeast extract media. Both media types are often used in the lab for culturing bacteria, so high OD was expected from these 2 media types. The downside however to these two media is that they are a lab grade material and therefore are expensive. This higher cost makes them unappealing to industrial applications as the amount of

these media that would be required would be extremely costly. To reduce the cost of the media, the other 2 media types of NPK and yeast flakes were evaluated. The NPK media is made using 10:10:10 ratio of Nitrogen:Phosphorus:Potassium. This fertilizer is in liquid form and is used for agricultural applications. The change in OD for this media type over 48 hours showed essentially no change. This limited change in OD is predicted to be a result of two main implications. First, the BioLogix product is intended to be predominantly *Pseudomonas* spp., and not all species of *Pseudomonas* are urease positive. The main source of nitrogen in this fertilizer is urea so as a result does not necessarily have a source of nitrogen available to all the *Pseudomonas* spp. bacteria. Secondly, the NPK fertilizer has high amounts of EDTA, and *Pseudomonas* are sensitive to EDTA as it can provoke cell lysis (Palleroni 2005). The last media type used was yeast flakes, which are a nutritional supplement for human consumption mainly consisting of yeast. This product is available in bulk from many grocery stores and is much cheaper than yeast extract and tryptone. This media was expected to have similar growth to the yeast extract and tryptone media, however it was found that the OD remained relatively low. Although this product was a much more cost friendly option, it did not yield sufficient growth to be a valuable option.

In trial 2 the yeast flake media was removed as it did not appear to be a valuable option, and in its place, we utilized Bushnell Haas media. Bushnell Haas media was specifically designed to have the nutrients required for bacterial growth except for carbon, making this media ideal for testing a bacterium's ability to degrade hydrocarbons. Since the goal of producing these bacteria is to be used to degrade hydrocarbons, this media seemed promising. To supplement the Bushnell Haas media without adding hydrocarbon we utilized dextrose to provide a carbon source for the bacteria while being cultured in the bioreactors. The other three media types were the same as trial one. Like trial one, the tryptone media had the largest change in OD in the shortest period of time. The Bushnell Haas media performed much better than the yeast flakes media and reached a much larger change in OD very

similar to that of the yeast extract. The main difference between the yeast extract and the Bushnell Haas media being that the yeast extract reached a peak OD around 48 hours whereas the Bushnell Haas media didn't reach its peak until around 96 hours. Similar to the first trial, the NPK media showed essentially no increase in OD even after 120 hours of growth. From these findings, it was evident that NPK media was not a viable option as it failed to result in an increased OD, and therefore was not supporting microbial growth. Additionally, these findings show that the Bushnell Haas can support high OD and high microbial growth but has the drawback of taking longer to reach that higher OD. Despite this delayed peak in OD, the cost advantage the Bushnell Haas media has over the yeast extract and tryptone makes it a viable option for industrial applications. By calculating the correlation coefficient of the four separate times a bioreactor used Bushnell Haas media we can support the findings in this chapter that the Bushnell Haas media consistently shows a similar growth pattern. We can also use the two separate times NPK media was used to confirm that the NPK also followed a very similar growth pattern between the two trials supporting the conclusion that NPK media does not support the growth of Biologix 2XP

OPTIMIZATION ON OPTIMAL GROWTH AND VIABLE TEMPERATURE RANGE OF BIOLOGIX 2XP

Methods

To test the temperature range of this consortium Luria-Bertani (LB) agar (Tryptone 10 g; Yeast extract 5 g; NaCl 10 g; Agar 15 g) and broth, as well as Bushnell Haas (BH) agar and broth were utilized (Bertani 1951; Bushnell and Haas 1941). The Biologix 2XP consortium was tested as the combined consortium as well as tested individually to evaluate the previously isolated members of the consortium. These isolated members of the consortium were obtained through out the experiments of Chapter 2. The bacteria were differentiated using macroscopic and microscopic differences and labeled aA, aB, aC, aD, aE, aF, aG, aH and aJ. aI was initially thought to be a unique bacterium but was later determined to be a duplicate and was

therefore removed. The liquid medium was prepared in a 1L flask before being dispensed in 5 ml aliquots into the test tubes. A Kim cap was loosely placed on top of the tubes before they were autoclaved at 15 PSI and 121 °C for 15 minutes (Lauer et al. 1982). Immediately upon removal from the autoclave the Kim caps were firmly secured onto the tubes. The medium was allowed to cool to room temperature before the tubes were inoculated with the corresponding isolated bacterium. The inoculated medium was then placed in an incubator set to the desired temperature and allowed to incubate for 24 hours. The agar plates were also inoculated with the corresponding bacterium and placed in the same incubator with the tubes. After 24 hours, the presence or absence of colonies on the agar was evaluated by visual inspection. For the liquid medium, the absorbance at 600 nm was recorded. The temperature that results in the highest OD after 24 hours was determined to be the ideal growth temperature for the consortium. The temperatures at which no growth was observed after 24 hours was also noted. The cultures were left for an additional 24 hours and were analyzed again using OD₆₀₀ to determine if growth was just slow or if it was unable to grow at these temperatures. With these readings at 2 different time points, we then calculated the change in OD between 24 and 48 hours and used that data to determine if growth was occurring.

Results

Growth in LB broth

Table 3.4 depicts a heatmap of the differences in OD from 24 to 48 hours for each temperature and each bacterium in LB media. Greater difference between 24 and 48 hours indicates higher bacterial growth meaning the values highlighted green exhibited the greatest amount of growth. From this we are able to determine when growth of the bacteria slows down or stops. We were able to determine the overall range of the bacteria is from 12 °C to 54 °C for most of the isolated bacterium. The consortium also showed growth at 56 °C reaching an OD of 0.207, which is over 4x greater change in OD₆₀₀ than any individual bacterium. None of the isolated bacteria or the consortium showed much change in OD at 10 °C indicating this temperature is

too low for bacterial growth. Additionally, multiple of the bacteria appeared to have reduced growth between 14 °C and 18 °C, even when growth was seen at lower and higher temperatures than 14 °C to 18 °C. For detailed description of the growth patterns of each bacterium, refer to Chapter 2 supplemental information in the appendix.

Table 3.4: Heatmap depicting change in OD from 24 to 48 hours for the individual bacteria being grown in LB media at various temperatures

Bacteria	10 °C	12 °C	14 °C	16 °C	18 °C	20 °C	50 °C	52 °C	54 °C	56 °C
aA	0.006	0.067	0.147	-0.114	-0.141	0.549	0.433	0.076	0.013	0.032
aB	0.023	0.245	0.176	0.091	0.261	0.723	0.088	0.11	0.945	0.002
aC	0.015	0.117	0.005	0.241	0.483	0.794	0.534	0.009	0.018	0.025
aD	0.028	0.135	0.177	-0.03	0.18	0.473	0.621	0.31	0.428	0.042
aE	0.018	0.122	-0.002	0.216	0.611	0.672	0.568	0.488	0.021	0.016
aF	0.006	0.086	0.05	0.454	0.478	0.435	0.279	-0.002	-0.002	0.026
aG	0.031	0.152	0.098	0.051	0.295	0.261	0.179	0.144	0.76	0.039
aH	0.037	0.182	0.007	0.036	0.045	0.175	0.427	0.108	0.547	0.067
aJ	0.017	0.196	0.16	-0.005	0.086	0.341	-0.037	0.072	0.754	0.029
consortium	0.016	0.113	0.201	-0.182	-0.109	0.863	0.402	0.254	0.602	0.207

Growth on LB agar

As a secondary measure to establish what temperature growth is no longer present, LB agar plates were used. For the agar plates, it was noted whether or not growth was present or absent.

On LB media, bacterium aA showed growth from 12 °C to 50 °C, with no growth being present at 8 °C, 10 °C, and 52 °C to 56 °C.

Bacterium aB first showed growth at 12 °C up to 52 °C, growth was absent at 8 °C, 10 °C and 56 °C.

Bacterium aC showed no growth from 8 °C to 14 °C, growth was present at 16 °C to 50 °C. and was absent again from 52 °C to 56 °C.

Bacterium aD showed no growth at 8 °C, 10 °C and 56 °C. Growth was present from 12 °C to 52 °C for bacterium aD.

Bacterium aE showed no growth at 8 °C, 10 °C and 14 °C, there was minimal growth present at 12 °C. Growth was present from 16 °C to 50 °C, then absent again at 52 °C to 56 °C.

Bacterium aF showed no growth until the temperature of 16 °C, growth being present from 16 °C to 50 °C, and absent again from 52 °C to 56 °C.

Bacterium aG showed no growth at 8 °C and 10 °C, with growth being present from 12 °C to 52 °C, and no growth at 56 °C.

Bacterium aH showed no growth at 8 °C and 10 °C, then growth being present from 12 °C to 56 °C.

Bacterium aJ showed growth from 12 °C to 52 °C, with no growth at the temperatures of 8 °C, 10 °C and 56 °C.

The consortium has growth present at 56 °C, as well as from 14 °C to 50 °C, and no growth at 8 °C. These are the only temperature data points for the consortium on solid media.

Growth in BH broth

The temperature range of the bacteria was also evaluated in Bushnell Haas medium. Table 3.5 is a heatmap of the changes in OD from 24 to 48 hours for each bacteria at each temperature, similar to what was done above to evaluate the growth when in LB media. From this table we see that the overall temperature range for the bacteria was from 12 °C to 54 °C. Similar to the LB media, growth was minimal at 10 °C indicating the Biologix 2XP consortium struggles to grow at 10 °C. Limited growth was also observed at 56 °C, with the consortium also showing limited growth despite showing growth in the LB media. There was a similar pattern of reduced growth at 14 °C and 16 °C despite growth being present at 12 °C and 18 °C. For a detailed description of the growth patterns of each bacterium refer to Chapter 3 supplemental information in the appendix.

Growth on BH agar

Looking at the agar plates with Bushnell Haas Medium we see that bacterium aA showed no growth at the temperatures of 8 °C, 14 °C, 18 °C and 50 °C to 56 °C. Growth was present at 10 °C, 12 °C, 16 °C and 20 °C.

Bacterium aB showed growth between the temperatures of 12 °C and 50 °C, with no growth being present at 8 °C, 10 °C, 52 °C to 56 °C.

Bacterium aC showed no growth at all temperatures except 16 °C.

Bacterium aD had no growth at 8 °C, 10 °C, 14 °C, 50 °C and 56 °C. Growth was present at 12 °C, 16 °C to 20 °C and 52 °C.

Bacterium aE showed growth at 12 °C, 16 °C and 52 °C, otherwise no growth was present at all other temperatures.

Bacterium aF did not show any growth at any temperature. Bacterium aG showed growth between the temperatures of 12 °C and 52 °C, with no growth being present at 8 °C, 10 °C and 56 °C.

Table 3.5: Heatmap depicting change in OD from 24 to 48 hours for the individual bacteria being grown in BH media at various temperatures

Bacteria	10 °C	12 °C	14 °C	16 °C	18 °C	20 °C	50 °C	52 °C	54 °C	56 °C
aA	0.003	0.117	-0.008	0.052	0.247	0.543	0.02	0.025	0.022	-0.056
aB	-0.011	0.008	-0.075	-0.019	0.323	0.263	0.025	0.004	0.024	-0.057
aC	-0.019	0.008	-0.019	-0.097	0.048	0.749	0.032	0.039	0.043	-0.045
aD	0.003	0.186	-0.032	0.033	0.36	0.601	0.016	0.03	0.015	-0.035
aE	-0.013	0.005	-0.009	-0.058	0.048	0.737	0.025	-0.001	0.032	-0.017
aF	-0.003	0.03	-0.092	-0.05	0.057	0.443	0.004	0.01	0.006	-0.05
aG	0.004	0.116	-0.075	0.092	0.221	0.337	0.003	0.003	0.014	-0.039
aH	-0.001	0.043	-0.03	0.149	0.601	0.752	-0.1	-0.166	0.784	-0.085
aJ	0	0.063	-0.015	0.142	0.24	0.281	-0.012	0.109	-0.027	-0.066
consortium	-0.011	0.089	-0.032	0.123	0.536	0.663	0.461	0.544	0.58	0.021

Bacterium aH showed no growth at the temperatures of 8 °C, 14 °C and 56 °C.

Growth was exhibited at all other temperatures evaluated.

Bacterium aJ showed growth between 12 °C and 20 °C. No growth was present at 8 °C, 10 °C, 50 °C to 56 °C.

The consortium had limited data collected on solid medium, however growth was observed at 14 °C, 20 °C and 50 °C. No growth was observed at 8 °C and 56 °C. No other data points were collected for the consortium on solid medium.

Discussion

Looking at the results from the LB experiments we can see that growth at 10 °C is fairly limited. After 24 hours, the average OD for all 10 samples was 0.0085 indicating that there was limited growth for any of the samples at this temperature. This result is interesting as it shows that none of the individual bacteria have much capacity to grow at the low temperature of 10 °C, however when growing together in the consortium, we see an increase in growth, higher than any one bacterium on its own. The consortium had an OD₆₀₀ of 0.116 after 24 hours and 0.132 after 48 hours. We see a similar pattern at 56 °C, where all individual bacterium other than bacterium aH had negative changes in OD₆₀₀. From these results as well as the data from the solid media, it appears that bacterium aH is the only one capable of growing at 56 °C. An interesting result at 56 °C is that the consortium had an OD₆₀₀ of 0.611 whereas bacteria aH only had an OD₆₀₀ of 0.069. This could indicate that there is one or more additional bacterial strains or species present in the consortium that were not isolated that are capable of growing at 56 °C. Alternatively, there could be an interaction between the other members of the consortium that allows increased resilience to high temperatures when grown as a community. Table 3.6 below shows the lowest and highest temperatures growth was seen for the individual bacteria as well as the consortium in Bushnell Haas media.

Table 3.6: Low and high temperature range of each bacterium as well as the full consortium in Bushnell Haas media.

Bacterium	Low temp (°C)	High temp (°C)
aA	12	50
aB	12	54
aC	16	50
aD	12	54
aE	12	52
aF	16	50
aG	12	54
aH	12	56
aJ	12	54
Consortium	10	56

From these results, it appears that some bacteria are able to grow at lower temperatures, and some are able to grow at higher temperatures. This combination of bacteria that grow better at different temperatures would benefit the consortium when applied in diverse environments to treat hydrocarbons. After 48 hours, the consortium had the highest OD at all temperatures evaluated, demonstrating how the consortium is capable of growth at a wide range of temperatures.

This becomes very important when working in the field to treat contaminated soil as temperatures could climb closer to the top end of these temperatures measured depending on the area you are in and the time of year. On the other hand, having bacteria that can still grow at lower temperatures is a vital trait of the consortium as well because it allows the consortium to remain active later into the fall as temperatures decrease, and become active again earlier in the spring when temperatures are still rising. Combining these low and high temperature range bacteria provides a consortium capable of being productive for a longer period of time each year. This would result in faster remediation as there is reduced periods of inactivity due to less favorable temperatures. This also allows remediation to be done in a variety of locations with differing climates.

When we look at the growth in the Bushnell Haas media, we see some slight differences compared to LB. One difference is that the consortium did not have OD readings significantly higher than the individual bacteria however, it was almost always the highest OD. The OD of the consortium was only surpassed at the temperatures of 20 °C by bacterium aC, and at 54 °C by bacterium aH.

Overall the growth of the bacteria in the Bushnell Haas medium was lower than the growth in the LB medium, this could be attributed to a couple things. If we look back to the results from the media testing, we see that when the bacteria were grown in the Bushnell Haas medium, there was a longer lag phase than the other types of media. This could indicate that the bacteria were not yet at their peak, and the

potential OD could have been more like the LB medium if it was allowed a day or two more to grow.

The Results of this section provide valuable information as to the temperatures we can perform bioremediation at. From the results, attempting to operate a bioreactor below 12 °C could have poor results as the bacteria had only slight growth at this temperature. Additionally, above 50 °C there is also a reduction in growth so these high temperatures should also be avoided. This information is valuable as it allows us to know when we should stop operating the bioreactors in the fall, or when we can start them again in the spring. The high range is also valuable so that we can take actions to reduce the temperature of the bioreactors if the water temperature approaches 50 °C. This may not be a concern in Canada; however, Delta Remediation is also starting multiple projects in Kuwait where the air temperature can be 60 °C in the summer. These findings are supported by numerous articles that have evaluated the effectiveness of bioremediation in high and low temperature areas around the world (Das and Chandran 2010; Gomez and Sartaj 2014) (Azubuike et al. 2016; Tekere 2019).

INVESTIGATION OF AERATION REQUIREMENTS FOR THE BIOLOGIX 2XP CONSORTIUM

Methods

Four, four-liter bioreactor jars with lids and fermentation locks were obtained. Two of these jars were filled halfway full with 2 liters of NPK medium. The other two bioreactors were filled halfway full with 2 liters of yeast flake medium as detailed in Table 3.7. The openings of all four jars were covered with tin foil and were placed into the autoclave at 121 °C and 15 PSI for 15 minutes to be sterilized (Lauer et al. 1982). The airlines that were used for aeration were also placed in the autoclave for sterilization. During this time, the lids and fermentation locks were submerged in 15% v/v bleach solution to sterilize these components (World Health Organization 2014). After sterilization was completed, the airlines were inserted into all four bioreactors through a small hole in the lid. An air stone was added to the end

downstream of the lid. The lid was then screwed on fully and the airline adjusted so the air stone was 1 cm off the bottom. The 1 cm height was arbitrarily selected to standardize the air stone height as well as to raise it above potential sediment that could settle out of the media. The fermentation locks were inserted into the center hole in the lid, and the airlock was filled to the fill line with sterilized deionized water. One of the bioreactors with NPK medium and one bioreactor with yeast flake medium had the airlines connected to the fish tank aeration pump. The other two bioreactors had the airline plugged off upstream of the lid. The air stones and airlines were added to these bioreactors to standardize any possible variation in growth resulting from the presence of the air stone. These airlines however were blocked off so no air could enter or exit through these lines. The two bioreactors connected to the aeration pump had a slightly modified 0.2-micron syringe filter placed in the airline upstream of the lid to the bioreactor. At this point the aeration systems were fully set up. The lid was loosened and lifted straight up, and a 1 ml sample was collected from each bioreactor. The absorbance at 600 nm was measured for these samples as a baseline absorbance of the media itself. Next, the bioreactors were inoculated with 0.5% w/v Biologix. Another 1 ml sample was collected immediately after inoculation and the absorbance reading collected to determine how much the BioLogix 2XP lyophilized powder increased turbidity. Absorbance readings were collected every 24 hours for 96 hours. At the end of 96 hours, we were able to determine if aeration resulted in higher turbidity than no aeration for both media types. To standardize the results, the readings from times 0, 24, 48, 72 and 96 were subtracted by the OD reading obtained prior to inoculation. Since we are comparing two media, the spectrophotometer was blanked with Deionized water before an absorbance reading was taken for the uninoculated media. This way we were able to compare the change in OD from the original OD.

Results

In Figure 3.4 we can see that the yeast flake medium with aeration had a change in OD (Δ OD) of 0.776 after 72 hours. The yeast flake medium without aeration started with an Δ OD of 0.280 at time 0, then decreased to -0.344 after 24 hours. After 48

hours the ΔOD of the yeast flakes with no aeration increased slightly to a ΔOD of -0.172 then somewhat plateaued. Due to the change in OD being calculated by subtracting the starting OD of the medias from the OD readings at 24 to 96 hours, some of the change in OD readings resulted in a negative number. Typically, a negative absorbance is the result of an error, however in this case, since this is the change in OD, a negative ΔOD is the result of the OD falling below the starting OD of the media. The NPK media with aeration stayed right around the starting OD of 0.087, with fluctuations of 0.003 or less over the 96-hour period. The NPK medium without aeration started at a ΔOD of 0.109, decreasing to 0.067 after 96 hours. The NPK medium without aeration started at a ΔOD of 0.109, decreasing to 0.067 after 96 hours.

Table 3.7. Nutrient and aeration parameters in four bioreactors.

Bioreactor	Nutrient	Aeration
Jar 1	0.5% NPK w/ Dextrose	Yes
Jar 2	0.5% NPK w/ Dextrose	No
Jar 3	0.5% Yeast Flake	Yes
Jar 4	0.5% Yeast Flake	No

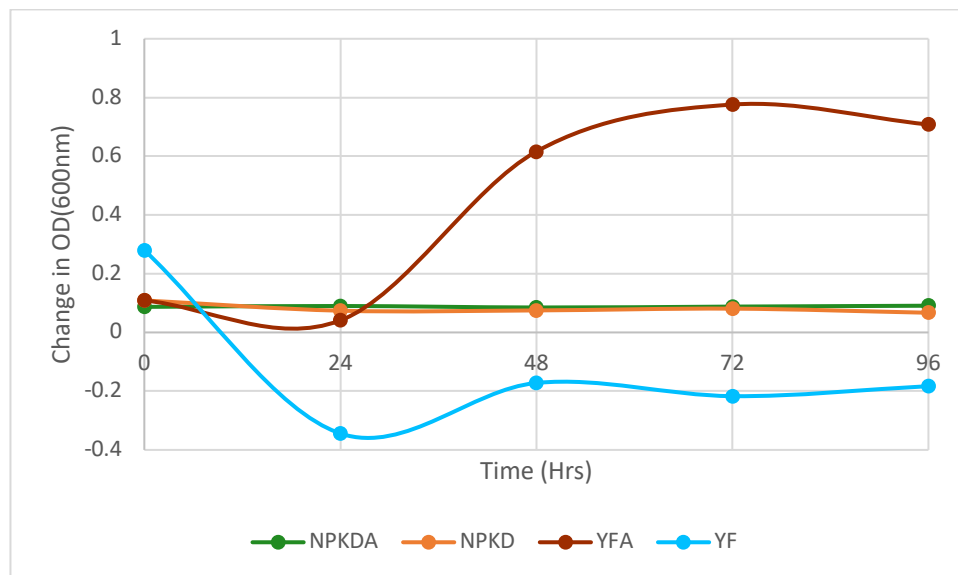


Figure 3.4: Comparison of the change in OD600 determined by subtracting the OD600 of the uninoculated media from the OD600 at all subsequent time points from 24-96 hours for two different media both with an aerated and non-aerated treatment. Note that negative values indicate the OD dropped below the starting value, and does not indicate a negative absorbance.

Discussion

This test indicates that aeration does benefit bacterial growth. Looking at the yeast flake medium with aeration we see that the ΔOD increased from 0.109 at time zero up to a peak of 0.776 after 72 hours. The yeast flake medium without aeration dropped to a ΔOD below 0, and remained below zero for the duration of the experiment, however, there was an increase in the ΔOD from -0.334 to -0.172 from 24 to 48 hours for an increase of 0.162. The yeast flake medium with aeration went from a ΔOD of 0.041 to 0.616 in the same time period, which is an increase of 0.575. This demonstrates that the yeast flake medium with aeration had greater growth than the yeast flake medium without aeration. An interesting result is the large decrease in OD for the yeast flake medium without aeration. A possible explanation for this change is the yeast flakes did not fully dissolve and would slowly settle to the bottom. It is possible that the aeration allowed the yeast flake particles to remain suspended which could be why the OD of the yeast flake medium with aeration only had a slight drop. The yeast flake medium without aeration however would have had no mixing action from the aeration so the yeast flake particles could have settled resulting in the large drop in OD.

The NPK medium did not demonstrate any signs of bacterial growth with or without aeration. If we look back to the results from the media testing, we can see that the NPK did not result in growth during that test either. This test of aeration can be taken into consideration with the media testing supporting the conclusion that NPK medium does not support the growth of the BioLogix bacteria.

ASSESSMENT OF BACTERIAL GROWTH IN AEROBIC, ANAEROBIC AND CANDLE JAR ENVIRONMENTS

Method

To test the anaerobic capabilities of the Biologix 2XP consortium, Bushnell Haas (BH) and Luria-Bertani (LB) agar plates were prepared and divided into 3 equal sections by drawing lines on the bottom of the plate. One of the nine isolated

bacterium previously isolated in chapter 2 was inoculated into a section of a plate allowing three bacteria to be inoculated on each plate. This was done in triplicate for both media types so one set could be placed in an anaerobic (ANO₂) jar, one set could be placed in a candle jar (CO₂) and the last set in an aerobic (O₂) condition. This resulted in a total of nine plates per media type with three for each condition. An additional two plates for each media and each condition were inoculated with the entire consortium using a small spray bottle. 100 ml of sterilized DI water was added to the bottle and 1% w/v BioLogix lyophilized powder was thoroughly mixed into the sterilized water. One spray from the bottle will be applied to three of the plates. The bottle was emptied and rinsed with 70% ethanol before it was refilled with 100 ml of fresh sterilized DI water and inoculated with 0.1% w/v of the Biologix lyophilized powder. One spray from the bottle will be applied to three plates. One of each of these plates was placed in each condition for both media types. These jars were incubated for 24 hours at room temperature. After 24 hours the amount of growth (none, minimal, moderate, significant) was recorded for each isolate as well as for the consortium plates. This provided insight into if the bacteria present in the Biologix consortium are obligate aerobes or if they are still active in anerobic, and low O₂ conditions.

Results

The results of this experiment reveled that all individual bacteria in the consortium were able to grow in the O₂, AnO₂ and CO₂ conditions. The consortium also exhibited growth in all atmospheric conditions. These results were the same on LB media and Bushnell Haas media. Bacterium aE was not seen on the Bushnell Haas media in the O₂ or CO₂ condition, only in the AnO₂ condition, however aE was present in all conditions when grown on LB media. Table 3.8 below depicts the growth of each bacterium, under the O₂, AnO₂ and CO₂ condition for both the LB and BH media.

Table 3.8: Amount of growth observed in three different air conditions

Bacteria	Condition					
	O ₂		AnO ₂		CO ₂	
	LB	BH	LB	BH	LB	BH
aA	++	+	++	+	++	+
aB	++	+	++	+	++	++
aC	++	+	++	+	++	+
aD	++	+	++	++	++	+
aE	+	-	++	+	++	-
aF	++	+	++	+	+	+
aG	++	++	++	++	++	+
aH	+	+	++	+	++	+
aJ	++	++	++	+	++	+
Consortium 0.1%	+++	+	++	+	++	+
Consortium 1%	+++	++	++	++	++	+

*None (-), Minimal (+), Moderate (++), Significant (+++)

Discussion

The results of this experiment were unexpected, as it was not anticipated that all members of the consortium would be facultative. It is possible that all members of the consortium could be facultative, however it is suspected that the method of creating the anaerobic environment was not sufficient and was not an anaerobic condition.

This is also suspected for the CO₂ environment. The lid for the CO₂ environment may not make a sufficient seal and therefore O₂ could leak back inside the jar after the candle has extinguished. In conclusion, the results of this experiment should be retested and confirmed.

CHAPTER CONCLUSION

The goal of this chapter was to test various conditions and nutrients to determine a baseline of how to best produce a fast-growing bacterial culture. These experiments were performed in lab conditions, which may limit the applicability of these results to

field scale operations, however, these results provide a starting point for various aspects of bioreactor set up and requirements. The results of the nutrient test experiment provided vital information on how the nutrient type impacts growth. From this experiment it was evident that the BioLogix 2XP consortium struggled to grow in the NPK medium. This medium was used in field scale applications and would produce bacteria, however, from the results of the nutrient testing experiment, it is evident BioLogix 2xp was unable to grow with NPK medium, meaning contaminating bacteria were likely becoming established and reproducing exponentially. This experiment also revealed that tryptone and yeast extract media yield high amounts of bacteria in the shortest period of time, however these media have higher costs associated with them. Bushnell Haas medium was found to be a balance between cost, and performance, as it yielded high cell density but took longer to achieve this cell density. This chapter also evaluated the temperature range at which the BioLogix consortium was able to grow. This experiment revealed that growth was observed from as low as 12 °C up to 56 °C. This information is useful to establish limits as to when we can have the bioreactor running in the field. These bioreactors do not have the ability to heat or cool the culture, so this information provides us with temperatures at which we may need to stop bioreactor usage. These bioreactors are often operated in locations without a source of electricity so methods of controlling the temperatures of these bioreactors is limited. The next parameter this chapter evaluated was aeration. This experiment aimed to determine if supplying air into the media via a bubbler increased bacterial growth. The results of this experiment revealed that aeration does increase bacterial growth compared to when no aeration is provided. The final experiment of this chapter attempted to evaluate the individual members of the consortium as well as the consortium as a whole to determine their ability to grow in anaerobic, CO₂ rich and aerobic environments. This experiment had unexpected results as all isolated bacteria were able to grow in all three conditions. Although it is possible for all members of the consortium to be facultative, it is unexpected. This could mean there was issues with the anaerobic and CO₂ environment vessels. This information would allow us to determine if providing

oxygen into soil piles or the bioreactor is required to maintain bacterial growth. All in all, this chapter provides key baseline requirements for field bioreactors. The findings of this chapter suggest that NPK medium should not be used in the field bioreactors as it is not compatible with the BioLogix 2XP consortium. Bushnell Haas medium serves as a cost-effective solution that provides high cell density with reduced cost compared to yeast extract or tryptone. The field bioreactor should not be operated at temperatures below 12 °C as bacterial growth is extremely slow or stopped completely. The bioreactor should also not be operated above 56 °C as bacterial growth is again slowed or stopped. Aeration should be supplied into the bioreactor to maintain increased dissolved oxygen levels as it was determined that there was increased bacterial growth when aeration was provided. In short, a field bioreactor should provide aeration, utilize Bushnell Haas media, and be operated in the temperature range of 12 °C to 56 °C to achieve optimal growth while be conscientious of cost.

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CHAPTER 4 - LAB SCALE INVESTIGATION OF CONTAMINATION SOURCES IN THE FIELD

INTRODUCTION

This chapter evaluated three sources of contamination present in the field scale bioreactors in an effort to determine the impact each source of contamination has on the community composition. In the field there are many paths of exposure for contamination to enter the bioreactor and we want to determine if any of these sources are having a detrimental effect on the community. The water used in the bioreactors comes from which ever source is the most accessible. This means that the water could come from a nearby pond or might be delivered by water truck to the location. As a result, the water used in the bioreactor can vary greatly in the amount of contamination that may be present. These bioreactors also use a venturi system to aerate the culture. With this system, air is sucked into the bioreactor through an air tube that is mounted to the top of the bioreactor. This air will be infusing any bacteria or other microorganisms present in the air into the nutrient rich bioreactor. The bioreactors may also be widely open to the environment as seen in Figure 4.1., which allows anything from the air to easily settle into the bioreactor. The results of this chapter provide insight into what sources of contamination have a significant negative impact, allowing these sources of contamination to be addressed. In addition to determining the sources of contamination with a negative impact, the sources of contamination with minimal impact may also be identified. By determining which sources of contamination have minimal impact on the community composition, we can avoid expending resources trying to address these sources and focus our resources on sources of contamination causing significant shifts in the community. This chapter will be divided into four subsections, the first titled "Viability of BioLogix 2XP", which determined the colony forming units per gram (CFU/g) in the BioLogix 2XP lyophilized powder that is used to inoculate the field bioreactors. The second subsection titled "Impact of Contamination from the Aeration System on the BioLogix 2XP Community" evaluated how unfiltered, unsterilized air used to aerate the bioreactors impacts the community composition. This was done by utilizing a set of

bioreactors to compare the community with and without filtered air to determine any differences between the communities. The next subsection titled “Impact of Contamination from Unsterilized Water on the BioLogix 2XP Consortium” will evaluate how water that has not been treated in any way impacts the community composition. This section also used a set of bioreactors to compare the community when sterilized water is used to when non-sterilized water is used to determine the impact of untreated water. The final subsection of this chapter titled “Impact of Contamination from the Open top of the Bioreactors” evaluated the impact on the community from a bioreactor that is not sealed off from the surrounding atmosphere, such as the bioreactor exhibited in Figure 4.1. Again, a series of bioreactors were used to compare the community between a bioreactor open to the air, and another that is sealed off to prevent contamination. From these experiments, information was obtained on what source or sources of contamination need to be addressed, as well as which sources do not have a significant impact and are therefore of lesser concern to address. Each subsection has a discussion on that subsections results. This chapter will then be concluded with a discussion combining the findings from the entirety of the chapter.

VIABILITY OF BIOLOGIX 2XP

Method

Nutrient and Saline Preparation

To determine the number of colony forming units directly from the BioLogix 2XP lyophilized powder, there was no culture media used. To get an accurate count of the live cells in the lyophilized powder, the bacteria could not be allowed to start replicating as that would then invalidate the count as it would no longer be a count of the live cells in the lyophilized product. To prevent growth, 0.9% saline was used as the diluent, and the serial dilution was performed immediately after the lyophilized powder was added to the initial tube of saline. The dilutions were then plated immediately after dilution was completed as to reduce the amount of time passing and the bacteria potentially replicating. 100 ml of 0.9% saline was prepared by

adding 0.9 grams of NaCl to 100 mls of water. The saline was placed on a stir plate and brought to a boil while being stirred, before it was then transferred to the



Figure 4.1: A large metal tank that is being used as a bioreactor, displaying the absence of any kind of barrier to prevent contamination from falling into the bioreactor.

autoclave and sterilized at 121 °C and 15 PSI for 15 minutes (Lauer et al. 1982). The 2 ml centrifuge tubes that were used for the dilution were also autoclaved. Luria-Burtani agar was used in the agar plates for the serial dilution (Bertani 1951). This medium was also autoclaved to sterilize the medium, before it was poured into petri dishes.

Serial Dilution

To perform the serial dilution, 2% w/v BioLogix was added to 1 ml of .9% sterile saline in a 2 ml centrifuge tube. This tube was mixed thoroughly on a vortex before the serial dilution was done immediately after. 100 μ l of this original sample was transferred to a new centrifuge tube containing 900 μ l of 0.9% saline. This tube was mixed thoroughly again using the vortex. 100 μ l was taken from this tube and transferred to another centrifuge tube containing 900 μ l 0.9% saline. This was done a total of 8 times resulting in dilutions from 10^1 to 10^8 . The 10^6 , 10^7 and 10^8 dilutions were plated onto the LB agar plates in triplicate. 100 μ l aliquots were plated from each of the aforementioned dilutions immediately after the serial dilution was completed. The 100 μ l aliquots dispensed onto each plate were thoroughly spread across the surface of the agar using an L-shaped glass spreader sterilized in ethanol and briefly passing through a flame to burn off the ethanol. These plates were incubated at 37 °C for 24 hours. After incubation, a dilution at which between 30 and 300 colonies were present was selected and the colonies counted from each plate. The average number of colonies was calculated from these three plates, which was then used to calculate the CFU/ml, which was used to calculate the CFU/g in the BioLogix 2XP lyophilized powder using equations 1 and 2 respectively (Koch, R. 1883).

Equations:

Equation 1:

$$CFU/ml = \frac{(number\ of\ CFU * dilution\ factor)}{vol.\ plated}$$

Equation 2:

$$CFU/g = \frac{CFU}{ml} \times \frac{volume\ of\ original\ sample}{grams\ of\ Biologix}$$

Results

The original inoculation of the saline solution was 0.028 g, into 1 ml of 0.9% saline. This was then diluted to 10^8 in series. The 10^6 , 10^7 and 10^8 dilutions were plated in

triplicate. The 10^6 dilution plates were counted, and the average CFU was determined to be 31.3 CFU. Using equation 1 above gives us a CFU/ml of 313,000,000 or 3.13×10^8 in the original sample. We then used this CFU/ml value to determine the CFU/g of the BioLogix powder that was added to the original sample. Equation two above was used for this calculation which gave us a CFU/g in the BioLogix powder of 1.12×10^{10} CFU/g in the BioLogix 2xp lyophilized powder as summarized in Table 4.1.

Table 4.1: Average CFU, CFU/ml and CFU/g for the Biologix 2XP lyophilized powder as determined from serial dilution and plate counts.

Average CFU	CFU/ml	CFU/g
31.3	3.13E+08	1.12E+10

IMPACT OF CONTAMINATION FROM AERATION ON THE BIOLOGIX 2XP COMMUNITY

Methods

Experiment Set-up

This experiment evaluated the impact of air contamination on the BioLogix 2XP community. Bushnell Haas medium with dextrose was used as the media for the bioreactors in this experiment (Bushnell and Haas 1941). Two liters of media was prepared in each bioreactor for a total of eight liters. Tap water was used in this experiment to more closely match the water used for field applications, as deionized water is not available in the field. The tops of the bioreactors were covered with tin foil before being autoclaved at 121 °C and 15 PSI for 15 minutes (Lauer et al. 1982). The airlines used for this experiment were autoclaved simultaneously to the bioreactors. The lids and airlocks for the bioreactors were placed in a 15% v/v bleach solution to sterilize them as they cannot withstand the heat of the autoclave (World Health Organization 2014). The bioreactors were allowed to cool to room temperature once they were removed from the autoclave. Once at room temperature, the tin foil was replaced with the sterilized lids. The airlocks were

placed into the lids and sterile tubing was inserted through a small hole in the lid and an air stone connected to the end of the tube inside the jar. The lid was tightened onto the jar and the airline was adjusted so that the air stone was 1 cm off the bottom of the jar as seen in Figure 4.2. To supply air to the bioreactors, a small fish tank aerator was used. This air pump did not have any sort of filtration system that prevents bacteria or other small airborne particles from being introduced to the system. Two of the bioreactors received this unfiltered air and two of the bioreactors received air filtered through a 0.2-micron syringe filter installed in the airline. The first bioreactor received filtered air and was inoculated with 0.5% (%w/v) of BioLogix 2XP lyophilized powder. The second bioreactor received unfiltered air and was inoculated with 0.5% (%w/v) of BioLogix 2XP lyophilized powder. The third bioreactor received filtered air and was not inoculated with BioLogix 2XP, and the fourth bioreactor received unfiltered air, and was also not inoculated. Refer to Table 4.2 for a breakdown of the parameters of each bioreactor.



Figure 4.2: Bioreactor and airline set up to evaluate the effect of aeration on the bacterial community, noting the yellow syringe filter on only 1 of the air supply lines.

Growth Measurements

To monitor the growth of these bioreactors, absorbance at 600 nm was utilized (Dalgaard et al. 1994). An initial OD₆₀₀ reading was collected from all four bioreactors prior to inoculation, as well as immediately after inoculation. Additional OD₆₀₀ readings were collected every 12 hours for 120 hours (5 days). To obtain these OD₆₀₀ readings, a 1 ml micro-pipette sprayed down with 70% ethanol was used (World Health Organization 2014). The lid of the bioreactor would be loosened and lifted just high enough to insert the pipette and draw a sample. The lid was then immediately replaced and tightened.

Table 4.2. Treatment conditions to determine how contaminated water impacts the Biologix consortium

Inoculation/ air filtration	Filtered Air	Unfiltered air
Inoculated	Jar 1*	Jar 2**
Not Inoculated	Jar 3**	Jar 4****

* Will demonstrate what we would expect the Biologix consortium to look like with no contamination.

** Will show the effect contamination from the air has on the Biologix consortium

**** Will show if contamination will occur when the air is filtered without interference from Biologix

**** Will show what is introduced from the air with no interference from the Biologix consortium.

16s Amplicon sequencing

After five days a sample was collected from all four bioreactors and DNA extraction was performed following the Qiagen DNeasy ultraclean extraction kit protocol (Qiagen 2025). 16s amplicon sequencing of the V3-V4 region was performed by the University of British Columbia (UBC). UBC generated the amplicons using the primer sequences 16S: 341F CCTACGGGNGGCWGCAG, 805R GACTACHVGGGTATCTAATCC. The library was then sequenced on Illumina NextSeq 2000 P1. QIIME2 version 2024.2.0 along with DADA2 version 1.26.0 via Bioconductor in QIIME was used for analysis. The silva database was then utilized for classification of the sequences. Genus level calls were made at ~97% sequence similarity, and species level calls were made at >99% sequence similarity.

(Sequencing + Bioinformatics Consortium 2024) there was high sequencing depth for all samples with raw reads ranging from 27,776 to 403,036 per sample. After filtering, denoising and chimera removal we were left with 22,756 to 341,514 non-chimeric reads per sample. This is between 82% and 91% retention as summarized in Table 4.3

Table 4.3. Sequencing information for the 16s amplicon sequencing on the air contamination samples

Sample ID	Raw Reads	Reads After QC (non-chimeric)	% Retained
AC1	27,776	22,756	82.00%
AC2	359,474	320,049	89.00%
AC3	261,933	240,562	91.80%
AC4	403,036	341,514	84.70%

Results

Growth Measurements

Looking at Figure 4.3 we can see that the line representing the bioreactor inoculated with BioLogix 2XP lyophilized powder and aerated with unfiltered air had the largest change in OD₆₀₀. A sudden spike in OD₆₀₀ is observed from 60 to 72 hours, jumping from 0.438 up to 1.274. From there the OD₆₀₀ continued to increase to a peak of 1.619 at which point the experiment was stopped after 120 hours.

The bioreactor that was inoculated and aerated with filtered air demonstrated a steady increase in OD₆₀₀ starting at the 48-hour mark with an increase in OD₆₀₀ of 0.201 from baseline and increasing to 1.038 after 120 hours. This filtered air bioreactor reached a peak OD₆₀₀ that was 0.580 less than that of the bioreactor that was unfiltered. The bioreactor that was not inoculated and was aerated using filtered air had an average OD₆₀₀ of 0.04 from time 0 to 96 hours. At 108 hours the OD₆₀₀ started to trend upwards from 0.104 at 108 hours to 0.414 at 120 hours.

The bioreactor that was not inoculated and was aerated with unfiltered air followed a similar pattern to the filtered uninoculated bioreactor, with a low OD₆₀₀ from time 0 to 84 hours. At time 96 the OD₆₀₀ increased to 0.113, continuing to increase to 0.433 at 108 hours and 0.489 at 120 hours.

16s Amplicon sequencing

Figure 4.4 represents what percentage of the consortium is made up of a specific genus. Looking at AC1 (inoculated, filtered) we can see that the *Pseudomonas* genus makes up 82.97% of the population. *Bacillus* spp. makes up 15.53% of the population. The remaining 1.5% is made up of *Brevibacillus* spp., *Glutamicibacter* spp., *Clostridium_sensu_stricto_13* spp., *Methylobacterium-Methylobacterium* spp. and *Nocardioides* spp. listed in order from highest percentage of the population to lowest.

Sample AC2 (inoculated, unfiltered) also presents a high percentage of *Pseudomonas* spp., with 92.62% of the population being from the genus *Pseudomonas*. *Bacillus* spp. is also prominent in this sample with 6.78% of the population being *Bacillus* spp. The proportion of the *Pseudomonas* spp. population was 0.096 or 9.65% higher in sample AC2 than AC1. The *Bacillus* spp. population was 8.74% lower in Sample AC2 than AC1. The remaining 0.58% of the population in sample AC2 consisted of *Brevibacillus* spp., unassigned, bacteria and *Paenibacillus* spp. in descending order of proportion of the population.

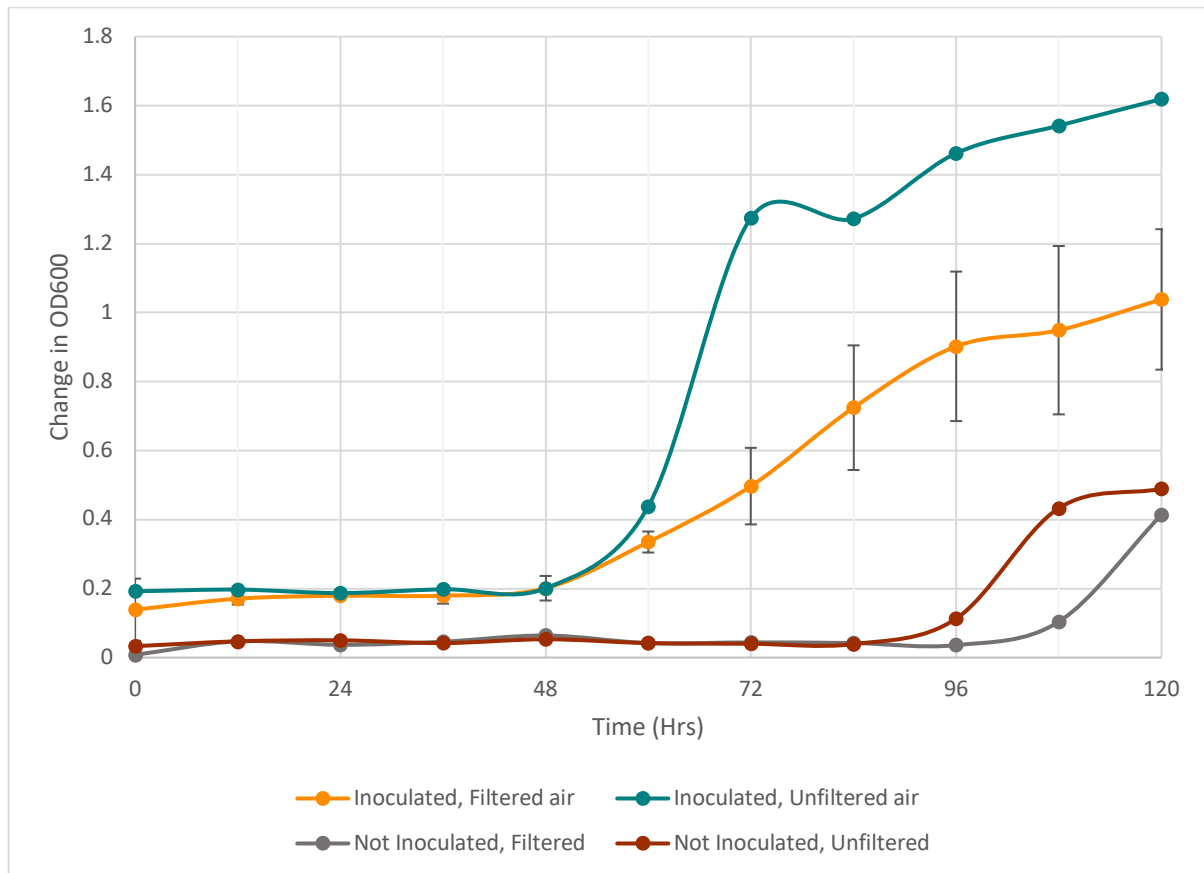


Figure 4.3: Change in OD readings at 600 nm of four bioreactors evaluating the impact of air contamination over a period of 120 hours inoculated filtered bioreactor with error bars calculated using standard deviation with $n=3$ at 12 hour intervals and $n=4$ at 24 hour intervals.

In sample AC3 (not inoculated, filtered) 99.75% of the population is made up of *Pseudomonas* spp. The remaining 0.25% is made up of *Bacillus* spp., unassigned, bacteria, and *Paenibacillus* spp. in descending order of proportion of the population. Sample AC4 (not inoculated, not filtered) had the lowest proportion of *Pseudomonas* spp. with 68.37% of the population being *Pseudomonas* spp. *Bacillus* spp. made up 31.59% of the population, which is the highest percentage of *Bacillus* spp. out of all 4 samples. The remaining 0.041% of the population was made up of unassigned, bacteria and *Cutibacterium* spp. in descending order of proportion. All four samples had *Pseudomonas* spp. making up the majority of the population, followed by *Bacillus* spp. The 2 samples that were inoculated also contained *Brevibacillus* spp.

as the third largest proportion of the population, whereas the 2 samples that were not inoculated did not have *Brevibacillus* spp. present.

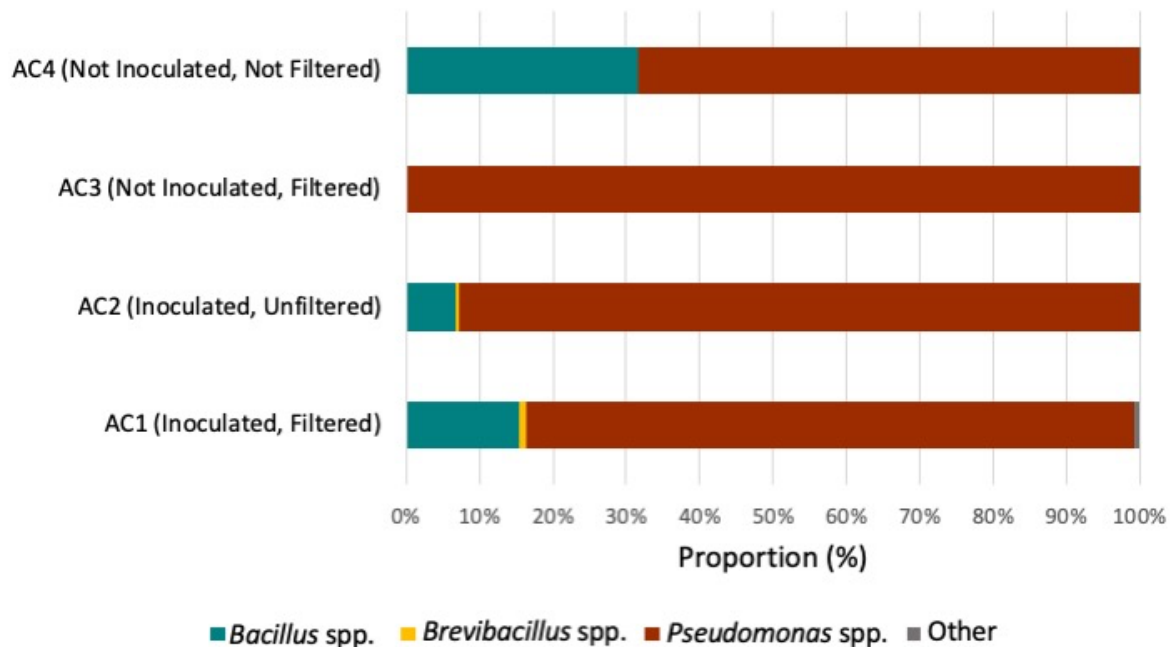


Figure 4.4: Community composition represented as percentages on the x axis from samples collected from 4 bioreactors to evaluate changes in the community as a result of contamination

Discussion

The results of the air contamination experiment revealed that *Pseudomonas* spp. made up the majority of the population in all four bioreactors, with the highest proportion of *Pseudomonas* spp. being in the uninoculated, filtered bioreactor. The Biologix 2XP consortium was designed to have *Pseudomonas* spp. and *Bacillus* spp. present in the mix so *Pseudomonas* spp. being present in the inoculated bioreactors is expected. Additionally, *Pseudomonas* spp. is a ubiquitous environmental bacterium, so it is not surprising that *Pseudomonas* spp. was also present in the unfiltered bioreactors (AL-Saleh and Akbar 2015) (Crone et al. 2020). The presence of *Pseudomonas* spp. in the filtered, uninoculated bioreactor is unexpected as there should not have been bacteria in this bioreactor. This could indicate this bioreactor was potentially contaminated during a sampling period.

If the bioreactor that was filtered and inoculated serves as a baseline for what the consortium looks like when contamination is not present, then we can assume that differences in the proportion of bacteria in the inoculated bioreactors represent how the consortium changes when air contamination is not eliminated. Using this assumption, we can see that there is an approximately 10% larger proportion of *Pseudomonas* spp. in the unfiltered bioreactor. There is also a roughly 9% lower proportion of *Bacillus* spp. in the unfiltered bioreactor. This could indicate that in the presence of contamination from the air, the *Bacillus* spp. population faces increased competition resulting in a lower proportion of the population being *Bacillus* spp. The differences between the two populations are minimal, however the results highlight a decrease in diversity when contamination is present. The 16s amplicon sequencing detected the presence of seven different genera in the filtered bioreactor, however only six genera were detected in the unfiltered bioreactor. Of these six genera only 3 of the genera were also found in the filtered bioreactor. If we look at the unfiltered uninoculated bioreactor, we can see what bacteria become established just from the air and can compare this to the unfiltered inoculated bioreactor as an indication of how the BioLogix consortium altered the growth of the contaminating bacteria. The unfiltered uninoculated bioreactor was not dramatically different from the filtered inoculated population. However, there are some differences in the proportions of the different species. There was a larger amount of *Bacillus* spp. present in the unfiltered uninoculated bioreactor than the filtered inoculated and the unfiltered inoculated, which also reduced the proportion of *Pseudomonas* spp. Between the unfiltered inoculated and unfiltered uninoculated, the only genus of bacteria that was not present in both was *Cutibacterium* spp., which was only found in the unfiltered uninoculated and only made up 0.0009% of the population. Additionally, the genera of *Paenibacillus* spp. and *Brevibacillus* spp. were found in the unfiltered inoculated but not in the unfiltered uninoculated. This minimal difference does not strongly indicate an ability for BioLogix 2XP to outcompete contamination, but it also doesn't support the opposite, that BioLogix is unable to outcompete contamination. Overall, the difference between these populations is fairly minimal, however it is not

insignificant. In the field, a larger volume of air will be pumped into these bioreactors and could increase the differences in the population proportions. Based on these findings, it appears that contamination from the air being used to aerate the culture will have minimal impact on the population and could therefore be a lower priority for finding a solution to completely remove the risk of contamination

IMPACT OF CONTAMINATION FROM UNSTERILIZED WATER ON THE BIOLOGIX 2XP CONSORTIUM

Methods

This experiment evaluated the effect of non-sterilized water on the Biologix 2XP microbial community composition. This experiment started by collecting eight liters of tap water in a sterile vessel and leaving it on the counter with the lid opened for 24 hours. This allowed the chlorine used to sterilize tap water to gas off. After 24 hours, this tap water was used to make Bushnell Haas media with dextrose. A total of four bioreactors were used, with two liters of the tap water going into each bioreactor. When making the media, no heat was used to aid in dissolving the nutrients as to avoid killing off any contaminating bacteria. Two of these bioreactors had tin foil placed over the top, and were placed in the autoclave to be sterilized at 121 °C and 15 PSI for 15 minutes (Lauer et al. 1982). Meanwhile the other 2 bioreactors were left on the counter uncovered. The airlines for this experiment were also placed in the autoclave to be sterilized. The lids and airlocks for the bioreactors were placed in a 15% v/v bleach solution to sterilize them as they cannot withstand the heat of the autoclave (World Health Organization 2014). Once the bioreactors were done in the autoclave, they were left on the counter to cool to room temperature along with the two bioreactors that were not autoclaved so they could stabilize at room temperature if they were not already. Once room temperature had been reached, the tin foil covering the autoclaved jar openings was replaced with the lids. The airlocks were placed into the lids and sterile tubing was inserted through a small hole in the lid and an air stone connected to the end of the tube inside the jar. The lid was tightened onto the jar and the airline was adjusted so that the air stone was 1 cm off the

bottom of the jar. To supply air to the bioreactors, a small fish tank aerator was used. This air pump did not have any sort of filtration system that prevents bacteria or other small airborne particles from being introduced to the system so a 0.2-micron syringe filter was placed in the airline. One of the bioreactors with the unsterilized water along with one of the bioreactors with sterilized water were inoculated with 0.5% w/v Biologix 2XP. The other two bioreactors were not inoculated. Refer to Table 4.4 for a breakdown of the treatments applied to each bioreactor. All four of these bioreactors will receive filtered air continuously for the duration of the experiment.

Table 4.4: Treatment conditions to determine how contaminated water impacts the Biologix consortium

Inoculation/ water	Sterilized water	Non-Sterilized water
Inoculated	Jar 1*	Jar 2**
Not Inoculated	Jar 3***	Jar 4****

* Will demonstrate what we would expect the Biologix consortium to look like with no contamination.

** Will show the effect contamination from the water has on the Biologix consortium

*** Will show if contamination will occur when the water is sterilized without interference from Biologix (negative control)

**** Will show what is introduced in the water with no interference from the Biologix consortium (positive control).

Growth Measurements

To monitor the growth of these bioreactors, absorbance at 600 nm was utilized (Dalgaard et al. 1994). An initial OD₆₀₀ reading was collected from all four bioreactors prior to inoculation, as well as immediately after inoculation. Additional OD₆₀₀ readings were collected every 12 hours for 120 hours (5 days). To obtain these OD₆₀₀ readings, a 1 ml micro-pipette sprayed down with 70% ethanol was used. The lid of the bioreactor would be loosened and lifted just high enough to insert the pipette and draw a sample. The lid was then immediately replaced and tightened. The control treatment of the bioreactor that was sterilized and was not exposed to the source of contamination for all three of the contamination experiments in this chapter as well as the BH treatment from the media testing in the

previous chapter were combined to get an average and calculate standard deviation for the error bars in Figure 4.5. These four bioreactors had the exact same set up so the data was combined to evaluate consistency in the growth patterns. In addition to the average and standard deviation, the correlation coefficient was calculated between all four media types to determine how closely the growth patterns match despite differences in overall optical density.

16s Amplicon sequencing

After five days a sample was collected from all four bioreactors and DNA extraction was performed following the Qiagen DNeasy ultraclean extraction kit protocol (Qiagen 2025). 16s amplicon sequencing of the V3-V4 region was performed by the University of British Columbia (UBC). UBC generated the amplicons using the primer sequences 16S: 341F CCTACGGGNGGCWGCAG, 805R GACTACHVGGGTATCTAATCC. The library was then sequenced on Illumina NextSeq 2000 P1. QIIME2 version 2024.2.0 along with DADA2 version 1.26.0 via Bioconductor in QIIME was used for analysis. The silva database was then utilized for classification of the sequences. Genus level calls were made at ~97% sequence similarity, and species level calls were made at >99% sequence similarity. (Sequencing + Bioinformatics Consortium 2024) there was high sequencing depth for all samples with raw reads ranging from 36,761 to 359,330 per sample. After filtering, denoising and chimera removal we were left with 6,717 to 304,420 non-chimeric reads per sample. This is between 4.2% and 84.7% retention.

Table 4.5. Sequencing information for the 16s amplicon sequencing on the water contamination samples

Sample ID	Raw Reads	Reads After QC (non-chimeric)	% Retained
WC1	350,220	271,139	77.40%
WC2	359,330	304,420	84.70%
WC3	36,761	8,807	24.00%
WC4	159,099	6,717	4.20%

Results

Growth Measurements

Looking at Figure 4.5 we can see that the bioreactor that was sterilized and inoculated starts to increase in OD₆₀₀ before any other bioreactor. At 48 hours the OD₆₀₀ of this bioreactor begins increasing and reaches a peak OD₆₀₀ of 1.038 after 120 hours. The non-sterilized inoculated bioreactor starts to increase in OD₆₀₀ around the time of 84 hours, 36 hours later than the sterilized and inoculated bioreactor. The non-sterilized inoculated bioreactor reached a peak OD₆₀₀ of 1.177 after 120 hours which is 0.138 higher than the sterilized and inoculated bioreactor. The sterilized and not inoculated bioreactor stayed at a low OD₆₀₀ with an average OD₆₀₀ reading of 0.0616 and a standard deviation of 0.0161. The last bioreactor, which was not inoculated and not sterilized also remained relatively flat, with no major increase in OD. This bioreactor had an average OD₆₀₀ of 0.033 with a standard deviation of 0.0164.

16s Amplicon sequencing

Figure 4.6 displays the proportion of the population made up of a given genus of bacteria. The population in WC1 (sterilized, inoculated) is predominately *Pseudomonas* spp., with 59.78% of the population being *Pseudomonas* spp.. *Bacillus* spp. is the next most abundant genera with 38.4% of the population being *Bacillus* spp.. The remaining 1.8% is mostly bacteria from the genera *Brevibacillus* spp. followed by *Massilia* spp., unassigned, *Bacteria* and *Gemmetimonas* spp. The population in WC2 (not sterilized, inoculated) is predominantly made up of *Acidovorax* spp. with 84.6% of the population being *Acidovorax* spp.. *Brevibacillus* spp. is the next most abundant with 7.5% of the population being *Brevibacillus* spp.. *Bacillus* spp. makes up another 7.19% of the population and the remaining 0.5% of the population is *Pseudomonas* spp. The *Pseudomonas* spp. population was significantly different between the two inoculated bioreactors. *Pseudomonas* spp.

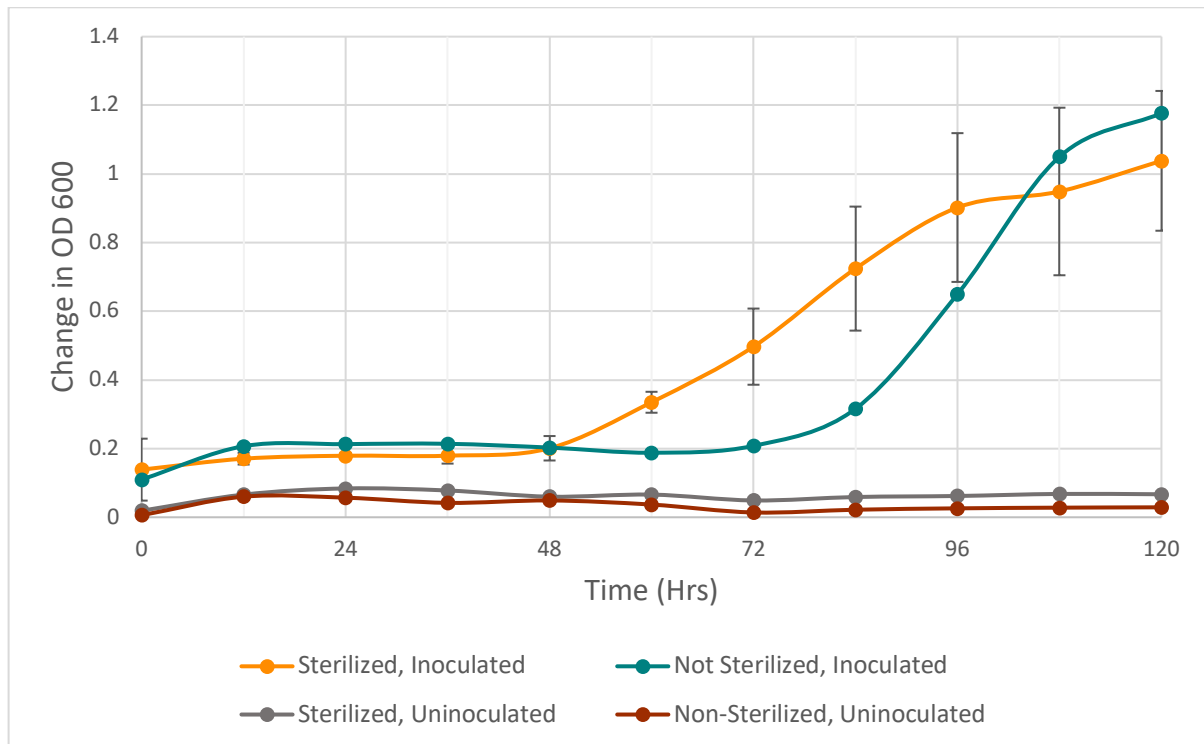


Figure 4.5: Change in OD readings at 600 nm of four bioreactors evaluating the impact of water contamination over a period of 120 hours sterilized inoculated bioreactor with error bars calculated using standard deviation with $n=3$ at 12 hour intervals and $n=4$ at 24 hour intervals.

made up 59.78% of the population in the sterilized bioreactor and dropped to 0.5% in the non-sterilized bioreactor. The sterilized bioreactor had no *Acidovorax* spp. present, and the non-sterilized bioreactor population was 84% *Acidovorax* spp. The WC3 (sterilized, not inoculated) bioreactor had 65% of its population as *Acidovorax* spp.. 17.29% of the population was made of *Glutemicibacter* spp. *Thermomonas* spp. made up another 5.8% of the population, followed by *Cutibacterium* spp. making up 4.4% and *mycobacterium* spp. making up 4.14% of the population. *Eschericia-shigella* spp. made up the remaining 3.25% of the population.

The final bioreactor, WC4 (not sterilized, not inoculated) had *Eschericia-shigella* spp. making up 30.6% of the population. *Acidovorax* spp. was the next most prominent with 26.4% of the population being *Acidovorax* spp. *Nocardioides* spp. made up 23.3% of the population, followed by *Pseudomonas* spp. making up 15.48% of the

population. *Parapedobacter* spp. made up the remaining 4.1% of the population. *Acidovorax* spp. was present in all bioreactors other than WC1 (sterilized, Inoculated). *Bacillus* spp., *Brevibacillus* spp. and *Pseudomonas* spp. were present in both the bioreactors that were inoculated. None of these genera were present in bioreactor WC3 (sterilized not inoculated), and *Bacillus* spp. and *Brevibacillus* spp. were absent in WC4 as well, however *Pseudomonas* spp. was present with 15% of the population being *Pseudomonas* spp. *Escherichia-shigella* spp. was only present in the bioreactors that were not inoculated.

Discussion

From the results of this experiment, we saw a significant difference between the sterilized and unsterilized water. In the bioreactor inoculated with BioLogix 2XP and with sterilized water we can see that 59% of the population was *Pseudomonas* spp., 38% was *Bacillus* spp. and 1% was *Brevibacillus* spp. Since this is the sterilized inoculated bioreactor, we can use this as the control for what the population looks like in sterilized conditions and can use this control to evaluate how the population changes when contamination is introduced. The population in the bioreactor with unsterilized water inoculated with BioLogix had a significantly different population, with the majority of the population consisting of the genus *Acidovorax* spp.. Only 0.5% of this population is *Pseudomonas* spp. whereas in the sterilized bioreactor *Pseudomonas* spp. made up over 59% of the population. Additionally, the genus *Bacillus* spp. made up only 7% of the population in the non-sterilized inoculated bioreactor whereas *Bacillus* spp. made up 38% of the population in the sterilized bioreactor. We can also see that *Brevibacillus* spp. is only present in the bioreactors that were inoculated indicating the *Brevibacillus* spp. is most likely found in the BioLogix product and is not introduced via contamination. There is also no *Acidovorax* spp. found in the sterilized and inoculated bioreactor, however it is found in the three other bioreactors. The number of bacteria found in the sterilized not inoculated bioreactor was very minimal compared to the inoculated bioreactor as the OD₆₀₀ of the sterilized, not inoculated bioreactor had a peak of 0.084. This low OD₆₀₀

in this bioreactor could also explain the poor sequence retention in the 16s amplicon sequencing which had <24% for WC3 and <4% retention for the WC4 bioreactor. Due to these bioreactors having low biomass, a larger percentage of the reads in the sequencing were most likely noise. So, although *Acidovorax* spp. DNA was found in the 16s amplicon sequencing there was a very small amount of the bacteria present. This indicates that *Acidovorax* spp. is most likely not found in the consortium and was introduced via the unsterilized water. This could be a concern as the population of *Acidovorax* spp. drastically outnumbered the *Pseudomonas* spp. and *Bacillus* spp. population.

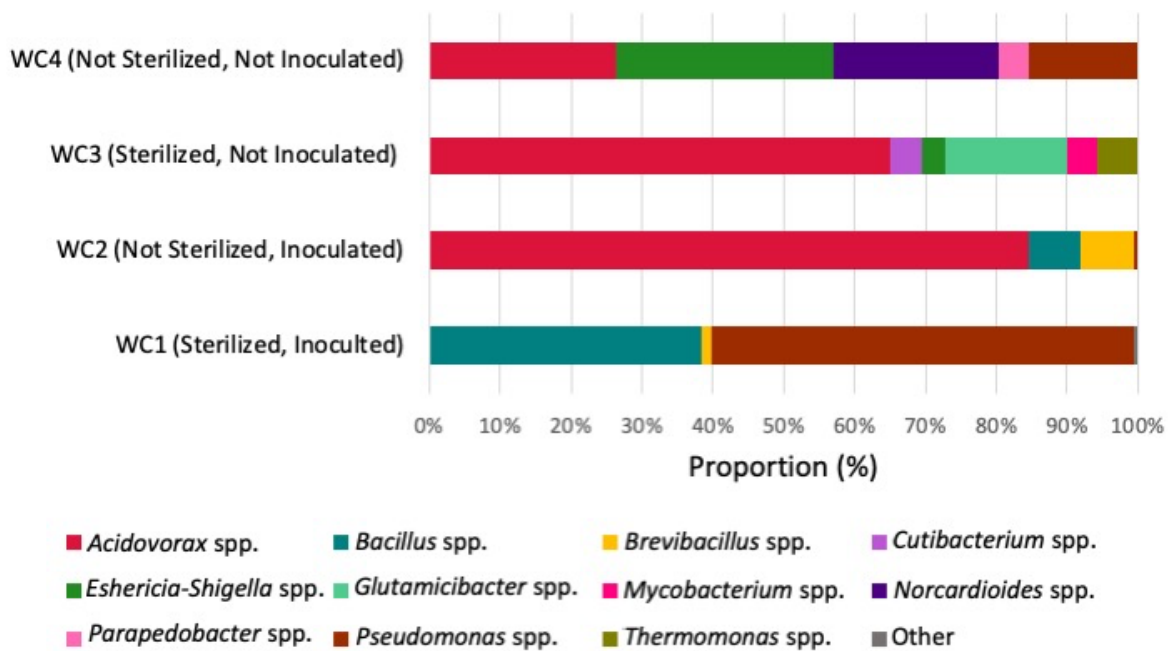


Figure 4.6 Community composition represented as percentages on the x axis from samples collected from 4 bioreactors to evaluate changes in the community as a result of contamination from water

This dramatic shift in population from 0% *Acidovorax* spp. in the sterilized bioreactor to 84.6% in the non-sterilized, inoculated bioreactor could indicate that *Acidovorax* spp. reproduces more rapidly than *Bacillus* spp. and *Pseudomonas* spp. and was therefore able to dominate the community. This is concerning because the target genera for Biologix 2xp is *Pseudomonas* spp. and *Bacillus* spp., and it appears that

those two genera are significantly reduced when the water is not sterilized. This may be something that needs to be addressed for field applications as our bioreactors could be producing a large population of bacteria that are not capable of degrading hydrocarbons and could therefore impede the bioremediation process. Despite the *Acidovorax* genus taking over a large amount of the population in the unsterilized and inoculated bioreactor, if we look at the non-sterilized non-inoculated bioreactor, we can see that there are multiple other genera of bacteria that were introduced via unsterilized water that did not appear in the inoculated un-sterilized bioreactor. In bioreactor WC4 (not sterilized not inoculated), we see that 26% of the population was *Acidovorax* spp., which was 0% of the population in the sterilized inoculated bioreactor. We also see that *Escherichia-shigella* spp. was 30% of the population and *Nocardioides* spp. was 23% of the population in the non-sterilized non-inoculated bioreactor but made up 0% of the population in the sterilized inoculated bioreactor. Interestingly, despite *Escherichia-shigella* spp. making up 30%, *Nocardioides* spp. making up 23% and *Parapedobacter* spp. making up 4% of the non-inoculated non-sterilized population, there was none of these genera in the non-sterilized inoculated bioreactor or in the sterilized inoculated bioreactor. This could indicate that the BioLogix consortium was able to completely out compete these three genera and prevent those genera from growing in the bioreactor. The overall results of this experiment indicate that BioLogix 2xp can potentially outcompete certain contaminants, however there are some contaminants that it cannot out compete. In the inoculated non-sterilized bioreactor, only about 15% of the population is made up of target genera, the remaining 85% is *Acidovorax* spp. This is concerning as only 15% of the biomass we produce would be capable of degrading hydrocarbon. This could have a major impact on the efficacy of the treatment, and as such, further research should be done to evaluate ways of reducing contamination from water, or to increase the competitive success of the BioLogix consortium.

IMPACT OF CONTAMINATION FROM THE OPEN TOP OF A THE BIOREACTOR ON THE BIOLOGIX COMMUNITY

Methods

This experiment will determine how much the bioreactors are impacted by being exposed to the outside environment. For all previous experiments, there was a lid preventing contamination from the surrounding environment. This experiment evaluated the impact of these lids being removed, and the surrounding air and contamination being able to enter the bioreactor through the open top.

Bushnell Haas media with dextrose was used as the media for the bioreactors in this experiment (Bushnell and Haas 1941). Two liters of media was prepared in each bioreactor for a total of eight liters. Tap water was used in this experiment to more closely match the water used for field applications, as deionized water is not available in the field. The tops of the bioreactors were covered with tin foil before being autoclaved at 121 °C and 15 PSI for 15 minutes (Lauer et al. 1982). The airlines used for this experiment were autoclaved simultaneously to the bioreactors. The lids and airlocks for the bioreactors were placed in a 15% v/v bleach solution to sterilize them as they cannot withstand the heat of the autoclave (World Health Organization 2014). The bioreactors were allowed to cool to room temperature once they were removed from the autoclave. Once at room temperature, two of the bioreactors had the tin foil covering the opening replaced with the sterilized lids. The other 2 bioreactors had the tin foil removed, with no lid replacing it, leaving it exposed to the outside air. The airlocks were placed into the two lids and sterile tubing was inserted through a small hole in the lid and an air stone connected to the end of the tube inside the jar as seen in Figure 4.7. The lids were tightened onto the jar and the airlines were adjusted so that the air stones were 1 cm off the bottom of the jar. The bioreactors without lids had the sterile airlines and air stones running over the rim of the jar. To supply air to the bioreactors, a small fish tank aerator was used. This air pump did not have any sort of filtration system that prevents bacteria or other small airborne particles from being introduced to the system so a 0.2-micron

syringe filter was inserted in the airline. One of the bioreactors with a lid was inoculated with 0.5% (%w/v) Biologix 2xp. One of the bioreactors that does not have a lid was also inoculated with 0.5% (%w/v) Biologix 2xp. The other 2 bioreactors, one with a lid and one without, were not inoculated. Refer to Table 4.6 for a breakdown of treatment parameters for each jar.

Table 4.6: Breakdown of treatment conditions to determine how exposure to the environment impacts the Biologix consortium

Inoculation/ Environ.	Closed to Environ.	Open to Environ.
Inoculated	Jar 1*	Jar 2**
Not Inoculated	Jar 3***	Jar 4****

* Will demonstrate what we would expect the Biologix consortium to look like with no contamination.

** will show the effect contamination from the environ. has on the Biologix consortium

*** will show if contamination will occur when no exposure to enviro without interference from Biologix (Negative control)

**** will show what is introduced from the environ. with no interference from the Biologix consortium. (Positive control)

Growth Measurements

To monitor the growth of these bioreactors, absorbance at 600 nm was utilized. An initial OD₆₀₀ reading was collected from all four bioreactors prior to inoculation, as well as immediately after inoculation. Additional OD₆₀₀ readings were collected every 12 hours for 120 hours (5 days). To obtain these OD₆₀₀ readings, a 1 ml micro-pipette sprayed down with 70% ethanol was used. The lid of the bioreactor would be loosened and lifted just high enough to insert the pipette and draw a sample. The lid was then immediately replaced and tightened

16s Amplicon sequencing

After five days a sample was collected from all four bioreactors and DNA extraction was performed following the Qiagen DNeasy ultraclean extraction kit protocol (Qiagen 2025). 16s amplicon sequencing of the V3-V4 region was performed by the University of British Columbia (UBC). UBC generated the amplicons using the primer sequences 16S: 341F CCTACGGGNGGCWGCAG, 805R GACTACHVGGGTATCTAATCC. The library was then sequenced on Illumina NextSeq 2000 P1. QIIME2 version 2024.2.0 along with DADA2 version 1.26.0 via Bioconductor in QIIME was used for analysis. The silva database was then utilized

for classification of the sequences. Genus level calls were made at ~97% sequence similarity, and species level calls were made at >99% sequence similarity.

(Sequencing + Bioinformatics Consortium 2024) there was high sequencing depth for all samples with raw reads ranging from 331,654 to 651,363 per sample. After filtering, denoising and chimera removal we were left with 279,171 to 464,175 non-chimeric reads per sample. This is between 71.30% and 91.10% retention as summarized in Table 4.7.



Figure 4.7: Bioreactor and airline set-up for the evaluation of the effect of bioreactors being open to the surrounding air, note only 2 bioreactors have lids while the other 2 are open to their surrounds

Table 4.7. Sequencing information for the 16s amplicon sequencing on the environmental contamination samples.

Sample ID	Raw Reads	Reads After QC (non-chimeric)	% Retained
EC1	368,396	279,171	75.80%
EC2	389,456	342,708	88.00%
EC3	331,654	302,251	91.10%
EC4	651,363	464,175	71.30%

Results

Growth Measurements

From Figure 4.8 we saw how exposure to the environment effected the growth in these four bioreactors. The bioreactor that was inoculated with BioLogix and was closed off from the environment had an increase in OD₆₀₀ of 0.201 from baseline at the 48-hour mark, after which it started trending upwards. After 120 hours, the OD₆₀₀ had reached 1.038, increasing by 0.837 between hours 48 and 120. The bioreactor that was inoculated with Biologix and was left open to the environment had an OD₆₀₀ of around 0.150 until hour 60 where it increased to 0.278. Between hours 60 and 72 there was a massive jump in OD₆₀₀ from 0.278 at 60 hours to 1.059 at 72 hours. The OD₆₀₀ continued to increase to a max of 1.347 at 120 hours. This bioreactor had a max OD₆₀₀ of 0.308 higher than the max OD₆₀₀ of the closed bioreactor inoculated with BioLogix. The bioreactor that was not inoculated with BioLogix and was open to the environment had an average OD₆₀₀ of 0.021 from time 0 to 84. After 84 hours the OD₆₀₀ started to increase, reaching a peak of 0.349 after 120 hours. The last bioreactor which was closed to the environment and did not get inoculated remained at a low OD₆₀₀ until 120 hours where it had a slight increase to 0.181 from the average of 0.044 over the previous 108 hours.

16s Amplicon Sequencing

From the 16s amplicon sequencing displayed in Figure 4.9, we find that the EC1 (closed, inoculated) population was 56.7% *Pseudomonas* spp. *Bacillus* spp. made up 35.8% percent of the population making it the second most prominent genus. 7% of the population was *Paenibacillus* spp. and the remaining 0.41% of the population was made up of *Brevibacillus* spp., unassigned, and d_bacteria which is represented as other in Figure 4.9. Bioreactor EC2 (open, inoculated) had a population of majority *Pseudomonas* spp., with *Pseudomonas* spp. making up 92.6% of the population. *Bacillus* spp. made up 7.1% of the population, which is 28.7% less of the population compared to EC1. The remaining 0.28% of the population is made up of *Brevibacillus* spp., D_bacteria, unassigned, *Paenibacillus* spp., *Massilia* spp. and *Parapedobacter* spp. in descending order from most prominent to least. Bioreactor

EC3 (closed, not inoculated) had a population that was 99.2% *Pseudomonas* spp. The remaining 0.8% was made up of *Bacillus* spp., unassigned, D_bacteria and *Acinetobacter* spp.

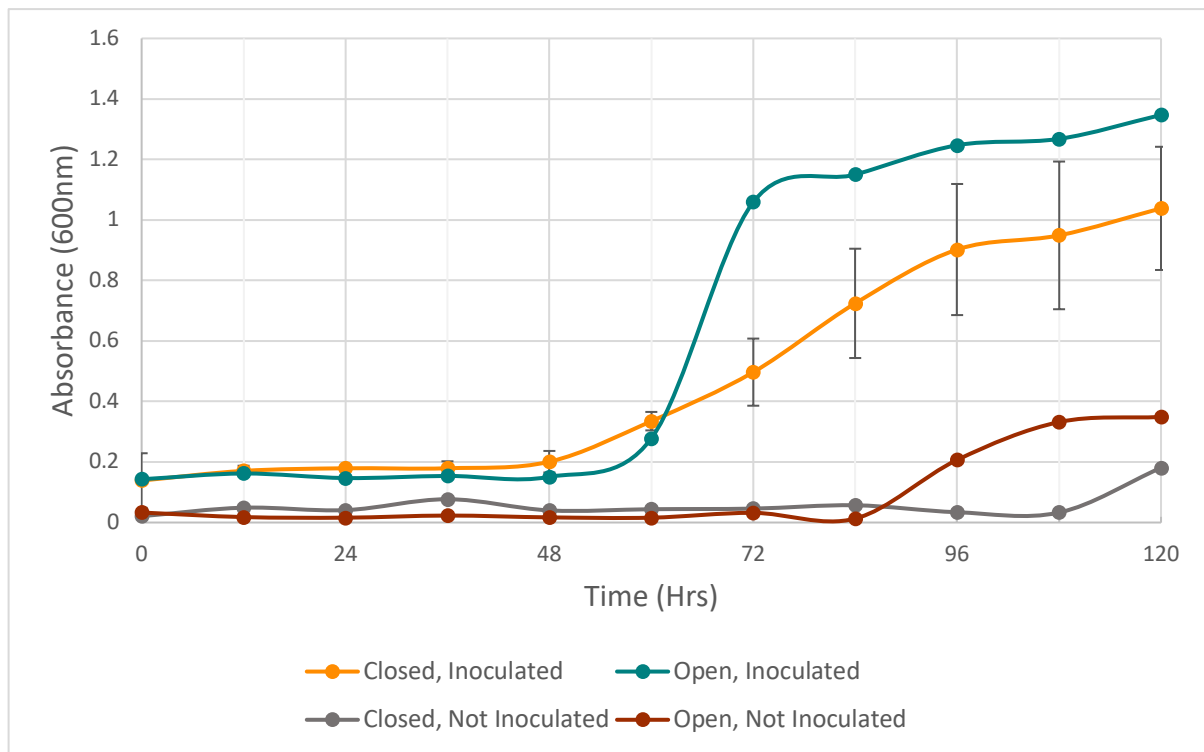


Figure 4.8: Change in OD readings at 600 nm of four bioreactors evaluating the impact of environmental contamination over a period of 120 hours, Closed inoculated bioreactor with error bars calculated using standard deviation with n=3 at 12 hour intervals and n=4 at 24 hour intervals.

The final bioreactor, EC4 (Open, not inoculated) had 44.3% of the population consisting of bacteria from the family Enterobacteriaceae. The next most prominent was *Pseudomonas* spp. with 26.9% of the population. Followed by *Acinetobacter* spp. with 25.5% of the population. The remaining 3.27% was *Enterobacter* spp., *Pantoea* spp., *Bacillus* spp. and D_bacteria. *Pseudomonas* spp. was present in all four bioreactors and was the most abundant genera in all bioreactors other than EC4 where bacteria from the family Enterobacteriaceae were most prominent. *Bacillus* spp. was present in all bioreactors as well, being most prominent in the EC1

bioreactor. *Acinetobacter* spp. was only present in the bioreactors that were not inoculated. The bacteria from the family Enterobacteriaceae as well as the genera *Enterobacter* spp. and *Pantoea* spp. were only present in the EC4 bioreactor that was not inoculated and was open to the environment.

Discussion

Looking at the OD₆₀₀ results we can see that the highest OD₆₀₀ was achieved in the EC2 bioreactor which was inoculated and was open to the environment. This bioreactor reached an OD₆₀₀ of 0.549 higher than that of the closed bioreactor. This could mean there was a different bacterium that is not present in BioLogix that was introduced to the culture from the environment, however looking at the 16s amplicon sequencing data doesn't necessarily support this idea. The only bacterial genera that were present in the open bioreactor that were not present in the closed bioreactor were *Massilia* spp. and *Parapedobacter* spp., and these genera made up less than 0.01% of the population combined. The main shift in community composition is in the genus *Pseudomonas* spp. where we see 56% of the population made up of *Pseudomonas* spp. in the closed inoculated bioreactor compared to the open bioreactor that had 92% of the population made up of *Pseudomonas* spp. There is the potential that a new species of *Pseudomonas* spp. that is not present in the BioLogix consortium was introduced from the environment and was able to thrive in the open bioreactor increasing the proportion of *Pseudomonas* spp. In the open bioreactor we also see a reduction in proportion of *Bacillus* spp. The BioLogix product is intended to have various species of *Bacillus* spp. to improve the remediation efficiency of the consortium. This reduction in *Bacillus* spp. in the open bioreactor could be a concern if the trend is an ongoing reduction of *Bacillus* spp. in the open bioreactor. In the bioreactor with no inoculation, we see the population is made up of bacteria from the genera of *Acinetobacter* spp. and *Pseudomonas* spp. as well as bacteria from the family Enterobacteriaceae.

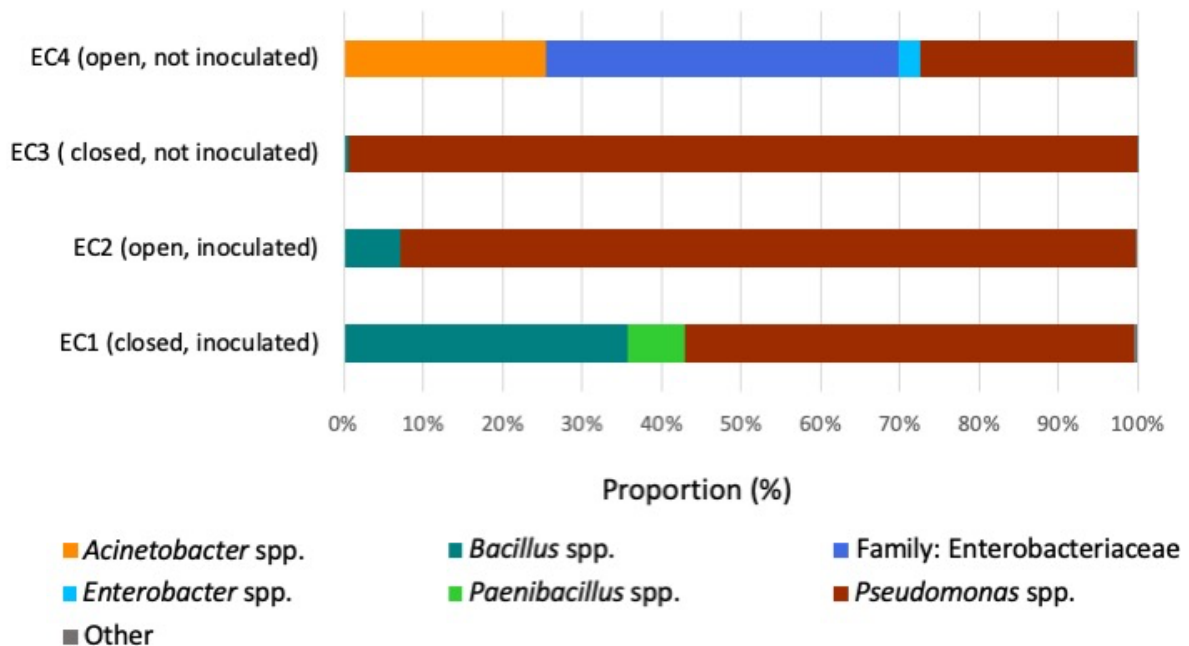


Figure 4.9: Community composition represented as percentages on the x axis from samples collected from 4 bioreactors evaluating changes in the community as a result of contamination from exposure to the environment

This bioreactor represents what the bioreactor would look like when exposed to the environment with no inoculation of specific bacteria. From this bioreactor we can see that the bacteria from the genus *Acinetobacter* make up around 25% of the population in this non inoculated bioreactor, meaning this bacterium came from the environment. When we look at the bioreactor that was inoculated and is open to the environment and therefore exposed to this same bacterium, we see that the *Pseudomonas* spp. out competes the *Acinetobacter* spp., preventing a large proportion of the population from becoming *Acinetobacter* spp., which is an unwanted bacterium for this application. This same trend is apparent for the bacteria from the family Enterobacteriaceae, which makes up the largest proportion of the open non inoculated bioreactor but is present in extremely low numbers in the inoculated bioreactor. This bioreactor also had a large amount of *Pseudomonas* spp., which could support the idea that the inoculated, open bioreactor was contaminated with a species of *Pseudomonas* spp. not found in the consortium, and

that grew more aggressively than the *Pseudomonas* spp. and *Bacillus* spp. introduced via inoculation. This could explain the increased proportion of *Pseudomonas* spp. in the open inoculated bioreactor. The open inoculated bioreactor also has very little *Brevibacillus* spp. compared to the closed bioreactor, which could be an issue if the *Brevibacillus* spp. provide essential products to the bioremediation process. Overall, with environmental contamination we see a shift in the proportion of the genera present in the open inoculated bioreactor. Despite this shift in proportion from the closed bioreactor, there is limited introduction of new genera of bacteria. Small portions of the population are made of genera introduced via contamination however this amount is very small. The main effect from this source of contamination was *Pseudomonas* spp. taking up a large proportion of the population resulting in a decrease in the *Bacillus* spp. and *Brevibacillus* spp.

CHAPTER CONCLUSION

The above three experiments that test the impact of air contamination, water contamination, and environmental contamination provide insights into what source or sources of contamination have a detrimental impact on the BioLogix consortium or have a negligible effect on the consortium. This information will allow us to address the major source of contamination in field scale applications so we can ensure that we are producing an effective consortium for bioremediation applications. From the above experiments, water contamination had the largest impact. The *Acidovorax* spp. population was roughly 85% of the entire consortium, this is a dramatic reduction in the *Pseudomonas* and *Bacillus* bacteria we are trying to produce that have a known ability to degrade hydrocarbons. (Ehis-Eriakha et al. 2020; Ibrar et al. 2022; Chettri et al. 2021). Because of this dramatic change, more research should be done to find cost effective ways of sterilizing large volumes of water that will not affect the growth of the desired consortium. Another potential option to avoid the costly process of sterilizing vast amounts of water could be to produce a seed culture in a smaller volume of sterilized water to allow the desired population to start reproducing rapidly before adding this seed culture to a larger bioreactor with non-

sterilized water. This could possibly give the consortium the head start required to out compete other contaminants. Environmental contamination had the second largest impact on the consortium, however this impact was not an unwanted genus of bacteria taking over, instead it was a shift in the proportions of the desired consortium. This shift in the population resulted in much more *Pseudomonas* spp. than the control bioreactor, and a decrease in *Bacillus* spp. Although this shift may not be beneficial for bioremediation applications, there is not a mass take over by genera that we are not targeting and are unsure of its hydrocarbon degrading properties. Because of this, this source of contamination is less concerning than the water contamination, however, minimizing this source of contamination is much simpler than sterilizing the water. Lastly, the air contamination through the aeration system had the least impact on the population. Similar to the environmental contamination, there was a shift in the proportions of the desired consortium, resulting in more *Pseudomonas* spp. and less *Bacillus* spp. This shift was less dramatic than the shift in the environmental contamination trial. There was roughly a 10% change in the amount of *Pseudomonas* spp. and *Bacillus* spp. This is a small shift, and there is a very minimal introduction of undesired species so this source of contamination could be left unaddressed. Filtering all the air going into these bioreactors would also be fairly costly and difficult due to the volume needed, so due to the minor impact of this source of contamination, it could most likely be ignored, or addressed after the other two sources of contamination have been eliminated or greatly reduced.

Overall, the findings of this chapter highlighted that water contamination can dramatically impact the bacterial community. This is concerning as the goal of these bioreactors is to produce a bacterial culture that will be able to efficiently degrade hydrocarbons. If only 15% of the population is the bacteria proven to be able to degrade hydrocarbons, there is a high risk of the remediation being unsuccessful. The contaminating bacteria have unknown degradation abilities and could therefore be unable to degrade hydrocarbons. This could result in time and resources being

spent to produce a bacterial culture that could have limited effect. Finding a solution to minimize or eliminate the impact of unsterilized water should be a high priority to ensure the quality of the hydrocarbon degrading bacterial community is maintained.

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CHAPTER 5 - *IN SITU* FIELD BIOREACTOR COMMUNITY DETERMINATION

INTRODUCTION

Delta Remediation

For field applications, Delta Remediation uses large vessels to grow the Biologix 2xp consortium to be used for the bioremediation process. These vessels can vary in shape and size which can change their exposure to the outside environment. As mentioned in early chapters, there is also the potential for contamination to be introduced through the water used in the bioreactor as well as from the air used to aerate the bioreactor. With Delta Remediations approach to hydrocarbon bioremediation there is a need to produce a large bacterial culture using the lyophilized BioLogix 2XP product as the seed inoculum. This bacterial community has been proven through years of use at Delta Remediation to be an effective consortium for microbial degradation of hydrocarbons (Delta Remediation Inc. 2025). However, despite the success Delta Remediation has achieved to date, there is a lack of information on how much the mature bioreactor bacterial community resembles the BioLogix 2XP starter inoculum. By evaluating factors that are at play in field settings, there is the potential to create new practices or procedures that maintain the desired community composition in the field scale bioreactors. By determining what factors are having the greatest effect on the community, efforts can be taken to remedy these negative influences, and generate a purer culture. By creating a system that produces a bacterial community as close to the Biologix 2XP consortium, without requiring highly sterile conditions, we ensure the amendment produced is of high quality and will degrade the hydrocarbons as intended while still being cost effective. If the required level of sterility is high, this would in turn increase costs of the process. Creating a system that produces the target consortium also reduces the risk of the culture being taken over by unwanted bacteria that can foul the bioreactor, requiring the bioreactor to be shut down by fully draining it and starting it again with fresh nutrients, water and BioLogix 2xp inoculum. This restarting of the bioreactor increases costs as the bioreactor takes multiple days to reach high cell density after a full restart, which can be costly when it delays the

completion of the project. Additionally, it is risky not knowing if the bacteria that have been produced are actually capable of degrading hydrocarbons.

Churchill Manitoba – Contaminated Site

This chapter evaluated the bacterial community in a large-scale field bioreactor to determine what the community looked like when there is no form of sterilization involved. This project took place in Churchill, Manitoba and required just under 93000 liters of bacterial culture to be produced. Churchill is located in northern Manitoba on the shore of the Hudson's Bay and is not accessible by road. To access Churchill, one must either travel by train, or plane. The location of this contaminated soil was on the northern end of Churchill, between the Hudson's Bay and Churchill river and is the site of the Churchill marine tank farm. This location has been a storage facility for fuels like jet fuel and diesel for over 50 years and provides the fuel for the airport, trains, ships and the local population. At one point, Churchill was a main port for Canadian grain heading to international markets, so there was a demand for large amounts of fuel at this location for refueling ships as well as the trains hauling the grain to the port. To accommodate this demand there were numerous high volume tanks used to store fuel. The grain export through Churchill eventually stopped and the demand for fuel decreased and so over the years, these massive tanks have been removed and the land is being slowly remediated. This facility now only has rail cars and a few smaller tanks to store the various fuel types. However, over the 50 years this site has been in operation, accidental releases, leaks and spills of these fuels have occurred, with little done in the way of remediation. As a result, the soil and ground water in this area is highly contaminated and this contamination is migrating through the ground water. Delta Remediation has performed 2 treatments to date on the site. One involving excavation of soil and treatment in soil piles, and the second involving 106 injection wells to treat the ground water. This second treatment is the project focused on in this chapter

Due to the constraint on accessibility to this location, many of the typical methods of remediation would have been extremely costly. The commonly used dig and dump method for example would have required excavating the contaminated soil, loading it onto a train, and transporting it to the city of Thompson over 400 km away, then disposing of it in a landfill. The volume of soil that would have needed to be transported would have been extremely costly. Delta Remediation was able to offer a much more affordable option as they only needed to transport the nutrients and lyophilized bacteria. These materials are considerably lighter and lower in volume than the soil that would have needed to be transported. In addition to reduced cost of transportation, another cost savings realized by utilizing Delta Remediations process was that Deltas process allowed the soil under the train tracks to be treated without disruption of tracks. The dig and dump method would have required the tracks be removed and reinstalled after the project was completed. With deltas process, trains were able to come and go with no issue for the entire duration of the project. As a result, the Delta process was much more affordable than other options.

Field Bioreactors

The 93000 liters of bacterial culture required for this project was produced using two bioreactors as seen in Figure 5.1 below. These bioreactors were 7000 liters and 9000 liters in volume. The bioreactors were started by first filling them with water from a nearby pond. Nutrients in the form of dextrose and 10:10:10 NPK fertilizer were added to the bioreactors. Following addition of the nutrients, the bioreactors were inoculated by adding the BioLogix 2XP lyophilized powder. Venturi systems were connected to 1 horsepower submersible pumps, with an airline going from the venturi out of the top of the bioreactor and hanging over the side. After start-up of these bioreactors, the bacterial culture was allowed to grow for 2-3 days to reach high cell numbers. After these 2-3 days, 50% of the bioreactor volumes could be drawn from each bioreactor and transferred to a trailer with six 1000-liter tanks as seen in Figure 5.2. These tanks would be filled half full of bioreactor fluid and then topped up with water to the full 1000 liter volume. The bacterial amendment was

then injected into the ground through a series of injection wells that had been installed prior. The amendment would be injected using a small 1-inch gas powered pump with the goal of injecting 200 Liters into each well. Some wells easily accepted 200 L or more, while others would surface vent almost immediately. Injection was stopped as soon as the amendment started flowing out at the surface of the soil (surface venting). Each day 6000 liters of amendment from the bioreactors was injected into the ground until the total of 93000 liters was reached. Each day after the injections had been completed, the 50% volume drawn from the bioreactors would be replaced with fresh water from a pond, and additional NPK fertilizer, dextrose and Biologix 2XP would be added. The next day 50% of the volume would be used and again would be refilled at the end of the day. This was repeated every day for the duration of the project.



Figure 5.1: Churchill 9000 liter bioreactor, exhibiting high foam production as a result of aeration and bacterial growth, noting the green airline providing air to the venturi system.



Figure 5.2: The 1000 liter tanks used for transporting the bacterial amendment to each injection well in Churchill, Manitoba

Objectives

To relate the findings of the previous chapters to the field, this chapter evaluates the community composition of these field scale bioreactors. The bioreactor that was sampled for this experiment was the large 7000 L bioreactor. This bioreactor had an approximately 45 cm diameter opening in the top. Used the unsterilized pond water and used a venturi for aeration that had no form of filtration or sterilization for the air.

The results of this experiment provided insight into if the community composition remains the same for extended periods of time, while constantly being diluted with contaminated water. As well as if the community composition of the field bioreactor compares to the lab bioreactor. The lab bioreactor was grown in sterile conditions so it provided a comparison for what the community could look like. This allowed for analysis of the community composition of the field bioreactor and determined if there were species of bacteria growing that we did not intentionally inoculate.

After looking at the result of this chapter, the findings from chapter 4 provided suggestions as to what sources of contamination should be addressed.

METHOD

Bioreactor start up:

To start the bioreactor, water started being pumped into the 7000-liter tank. While the tank was filling, dextrose and 10:10:10 NPK fertilizer was mixed with water in a 1000-liter tank so it could be dissolved before being pumped into the large bioreactor. The nutrients and water were pumped into the bioreactor, then the 1000-liter tank was filled with additional water and the Biologix 2XP lyophilized powder was added. After ensuring everything was dissolved and no lumps remained, this tank was again pumped into the bioreactor. The bioreactor was filled to 7000 liters and the venturi system was turned on. The first sample was collected immediately after the bioreactor was filled to 7000 liters. 1.5 ml samples were collected in 2 ml centrifuge tubes from the field bioreactor, and these samples were collected every five days for the duration of the project, resulting in a total of 4 samples. These samples were sealed and labeled, and DNA extraction was performed as soon as possible, within a couple hours of collection.

DNA extraction

DNA extraction was performed at the Churchill northern studies center. The Qiagen DNeasy DNA extraction kit used, and extraction was done as per the Qiagen protocol (Qiagen 2025). The extracted DNA was stored in a freezer at -20 °C until the end of the project when the samples were transported back to Thompson Rivers university, and then further shipped to the University of British Columbia.

16s Amplicon sequencing

DNA extraction was performed following the Qiagen DNeasy ultraclean extraction kit protocol (Qiagen 2025). 16s amplicon sequencing of the V3-V4 region was performed by the University of British Columbia (UBC). UBC generated the amplicons using the primer sequences 16S: 341F CCTACGGGNGGCWGCAG, 805R GACTACHVGGGTATCTAATCC. The library was then sequenced on Illumina

NextSeq 2000 P1. QIIME2 version 2024.2.0 along with DADA2 version 1.26.0 via Bioconductor in QIIME was used for analysis. The silva database was then utilized for classification of the sequences. Genus level calls were made at ~97% sequence similarity, and species level calls were made at >99% sequence similarity.

(Sequencing + Bioinformatics Consortium 2024) there was high sequencing depth for all samples with raw reads ranging from 272,742 to 335,778 per sample. After filtering, denoising and chimera removal we were left with 240,104 to 257,374 non-chimeric reads per sample. This is between 74.70% and 88.00% retention as summarized in Table 5.1.

Table 5.1. Sequencing information for the 16s amplicon sequencing on the large scale bioreactor samples.

Sample ID	Raw Reads	Reads After QC (non-chimeric)	% Retained
SR1	335,778	250,696	74.70%
SR3	310,755	257,374	82.80%
SR4	272,742	240,104	88.00%

RESULTS

The results of the 16s amplicon sequencing are displayed in Figure 5.3 providing a visual for the genera of bacteria detected from each sample. The y axis displays the sample names SR1, SR3, and SR4. This figure shows only the genera that made up more than 1% of the population. These samples are from the 7000 L bioreactor used in Churchill Manitoba and were taken every five days. SR1 was taken the day the bioreactor was started and SR4 was one month later. SR2 sample was lost due to the sample leaking out or evaporating and was found to be empty when attempting to send to UBC for the 16s amplicon sequencing. Looking at SR1 we can see that the largest proportion of the population is from the family Enterobacteriaceae, with 23.14% of the DNA being associated to the family plus 14.72% being associated with the genus *Enterobacter* spp., 4.67% from the genus *Citrobacter* spp. and 4.95% being the genus *Raoultella* spp., all of which are from the family Enterobacteriaceae. This is a total of 47.47% of the population being from the family Enterobacteriaceae. The other genera making up larger proportions of the population are *Aeromonas*

spp. with 10.74% of the population, *Pseudomonas* spp. with 9.41%, *Dysgonomonas* spp. with 7.68% and *Acinetobacter* spp. with 7.43%

The SR3 sample was collected 10 days after SR1, this sample had a reduction in the amount of Enterobacteriaceae present compared to sample SR1. 11.63% of the population was determined to be from the family Enterobacteriaceae, around 12% less than SR1. The genera *Raoultella* spp., *Enterobacter* spp. and *Citrobacter* spp. made up 7.19%, 6.28% and 2.29% of the population respectively. This adds up to 27.40% of the population compared to 47.47% in sample SR1. The most prominent genus shifted to *Lactococcus* spp. with 29.63% of the population being from this genus. *Leuconostoc* spp. is the second most prominent genus making up 28.10% of the population. Other genera of note for this sample are *Corynebacterium* spp. which increased from 0.23% in sample SR1 to 4.30% in SR3, *Pseudomonas* spp. decreased from 9.41% in SR1 to 1.18% in SR3, *Aeromonas* spp. decreased from 10.74% to 0.05% and *Clostridium_sensu_stricto_1* spp. increased from 0.15% in SR1 to 3.98% in SR3.

SR4 sample is taken 15 days after SR1 and 5 days after SR3. This sample saw the Enterobacteriaceae family increase in proportion again. 16.00% of the population was determined to be in the family Enterobacteriaceae, with the addition of *Raoultella* spp. making up 35.40%, *Citrobacter* spp. making up 1.72% and *Enterobacter* spp. making up 1.44%. This is a cumulative proportion of 54.56% of the population being from the family Enterobacteriaceae. *Raoultella* spp. was the most prominent genera, with the family Enterobacteriaceae being the second most prominent. *Prevotella_9* spp. was the next most abundant with 11.93% of the population consisting of this genus, followed by *Lactococcus* spp. with 11.55%. Another genus of note includes *clostridium_sensu_stricto_1* spp. which increased to 7.39% in SR4, from 3.98% in SR3 and 0.15% in SR1. *Gluconobacter* spp. increased from 0.30% in SR3 to 3.78% in SR4. *Leuconostoc* spp. decreased from 28.10% in SR3 to 0.93% in SR4 and *Raoultella* spp. increased from 7.19% in SR3 to 35.4% in SR4.

Of these three samples, SR1 had the smallest number of different genera with 66 different genera being detected. SR3 had the highest number of different genera with 98 different genera being detected and SR4 was in between with 80 different genera detected.

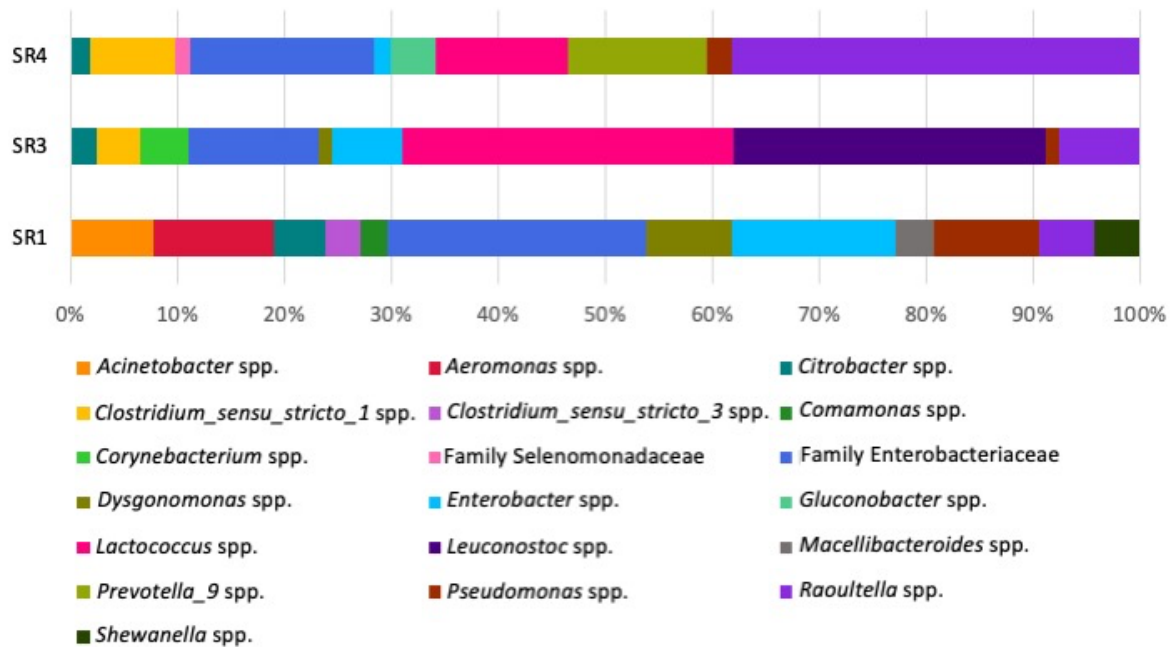


Figure 5.3: Population proportions determined via 16s amplicon sequencing of a community over a four-week period. Percent of the population along the x axis.

DISCUSSION

16s Amplicon sequencing

The results of this experiment show the bioreactor population was dominated by contaminating bacteria, with very small proportions of desired bacteria being present. *Pseudomonas* spp. made up 9.41% of the population when the bioreactor was first started, however it dropped to 1.18% at 10 days and then increased to 2.18% after 15 days. *Bacillus* spp. made up less than 1% of the population in all three samples with the highest amount of *Bacillus* spp. being 0.26% of the population in SR3. This means less than 10% of the population was made up of desired genera of bacteria. This could pose a major issue when trying to do

bioremediation in the field. In addition to the low proportions of desired bacteria in these samples, it is also concerning that the number of different genera increased as time went on. This indicates the BioLogix 2XP consortium is not able to outcompete this contamination, and new genera are continually becoming established in the bioreactor. A solution for this detrimental contamination should be implemented to increase the proportion of desired bacteria in the consortium.

Corrective Actions as Suggested by Chapter 4

To determine what the main source of this contamination is we utilized the findings from chapter 4 which tested different sources of contamination individually to determine which source resulted in the greatest impact to the consortium. The chapter 4 data revealed that water contamination had the largest impact on the community. The water used in chapter 4 was tap water left out for 24 hours, so it could gas off chlorine as well as have bacteria from the air build up in the water. The water used in the SR bioreactor was obtained from a nearby pond. This pond was not in any way cleaned or filtered or sterilized, so the number of bacteria already present in the pond would have been much higher than the bacteria found in the tap water which had been previously filtered and treated. Even with the tap water which would have been low in bacteria compared to the pond water, the BioLogix 2XP consortium still failed to outcompete the contamination. This suggests that contamination from water needs to be addressed to ensure a large community of the desired bacteria is produced. However, the issue arises that the cost of sterilizing the volumes of water required for large scale industrial bioremediation could quickly become cost prohibitive. Alternatively, further testing on creating a stronger seed inoculum may allow the BioLogix 2xp consortium to get a head start on the contamination. Further research would need to be done to evaluate the community composition when using a seed inoculum to determine if this provides an adequate advantage for the BioLogix consortium to dominate the community. The findings from chapter 4 indicated that air contamination and environmental contamination

had a much smaller impact on the consortium, meaning water contamination should be the focus for ongoing improvements to the bioreactor process.

Microbial Bioremediation:

The goal of this chapter and the chapters before is to improve Delta Remediations process to in turn improve the quality of the degradation. The target consortium for this application contains *Pseudomonas* spp. and *Bacillus* spp. as these genera of bacteria have been successfully used for bioremediation in many studies. For example, Subathra et al (2016) found that out of 113 isolates, *pseudomonas* spp and *bacillus* spp were the most prominent genera accounting for 38.94 and 35.39% of isolates respectively. In a field trial by Ehis-Eriakah et al (2020) *pseudomonas* was again found to be the dominant genera. These genera are thought to be so effective at hydrocarbon remediation for multiple reason. For one, these two genera have extremely diverse metabolic capabilities and are highly adaptable to their environment (Chettri et al. 2021). A second potential reason for these genera's wide usage in hydrocarbon remediation is their ability to produce biosurfactants. These biosurfactants improve the degradation rate, are less toxic than man-made surfactants and are biodegradable (Ehis-Eriakha et al. 2020; Ibrar et al. 2022; Yakimov et al. 2007).

In addition to these genera of bacteria, there are many articles supporting other genera of bacteria that have also been shown to be effective for hydrocarbon remediation. Table 5.2 below provides a summary of bacteria along with the key features of these bacteria.

Many different genera of bacteria can be utilized for degrading hydrocarbons, however there are also many bacteria unable to degrade hydrocarbons. Ensuring the Delta bioreactors are producing a consortium of bacteria that will be able to degrade hydrocarbons effectively is the highest priority. With the results of this chapter, it is evident that the bioreactor is producing a consortium of bacteria that may not be able to effectively degrade hydrocarbons. However, even though the bioreactor didn't maintain the Biologix 2xp consortium, there were three other genera

found in the bioreactor that have been used successfully to degrade hydrocarbons. This could be a result of using the pond water directly next to the contaminated site, there is a possibility the contaminated ground water is reaching the pond and encouraging hydrocarbon degrading bacteria to grow in this water. This may not always be case with the water being used for the bioreactors, so actions to maintain the Biologix 2xp consortium the bioreactor is inoculated with should still be taken.

Table 5.2: Compilation of various genera of bacteria that have shown the ability to degrade hydrocarbons

Genus/Species	Typical Hydrocarbons Degraded	Features/Notes	Key Citations
<i>Pseudomonas aeruginosa</i>	Alkanes, PAHs, diesel, crude oil	High efficiency, biosurfactant production, consortia member, metabolic versatility	(Pandolfo et al. 2023) (Subathra et al. 2013)
<i>Pseudomonas putida</i>	Alkanes, crude oil, diesel	Effective in soil and water, biosurfactant and chemotaxis, widely studied	(Pandolfo et al. 2023) (Subathra et al. 2013) (Bekele et al. 2022)
<i>Bacillus subtilis</i>	Diesel, crude oil, PAHs	Biosurfactant & endospore formation, versatile, consortia member	(Pandolfo et al. 2023)
<i>Bacillus cereus</i>	Diesel	Robust diesel degrader, found in diverse environments	(Pandolfo et al. 2023)
<i>Stenotrophomonas maltophilia</i>	Diesel, aromatic hydrocarbons	Single and consortia cultures, broad substrate range	(Bekele et al. 2022) (Pandolfo et al. 2023)
<i>Achromobacter xylosoxidans</i>	Diesel, PAHs	Significant growth in hydrocarbon-rich environments	(Bekele et al. 2022) (Pandolfo et al. 2023)
<i>Providencia rettgeri</i>	Diesel	Identified in possible consortia, moderate degrader	(Pandolfo et al. 2023)
<i>Enterobacter</i> sp.	Diesel, crude oil	Found in consortia, moderate efficiency	(Ahmed and Fakhruddin n.d.)
<i>Acinetobacter</i> sp.	Diesel, crude oil, PAHs	Consortia member, moderate degrader	(Ahmed and Fakhruddin n.d.)
<i>Rhodococcus</i> sp.	Alkanes, high MW PAHs	Specializes in short-chain/complex hydrocarbons, forms biofilms	(Pandolfo et al. 2023)
<i>Alcanivorax</i> , <i>Marinobacter</i> , etc.	Marine oils, alkanes, PAHs	Specialized marine bacterium, dominates after marine oil spills	(Yakimov et al. 2007) (Ibrar et al. 2022)
<i>Micrococcus</i> , <i>Corynebacterium</i> , <i>Flavobacterium</i> , <i>Moraxella</i> , <i>Vibrio</i> , etc.	Crude oil, diesel	Isolated from contaminated soil, moderate capacity	(Sathishkumar et al. 2008) (Subathra et al. 2013)

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CHAPTER 6 - CONCLUSION

The aim of this research was to determine how to maximize the efficiency of producing a bacterial culture in a remote field setting, in a way that is cost effective while still achieving the desired hydrocarbon degradation. By determining the key factors needed to produce the desired microbial community, it will provide Delta Remediation with valuable insight into actions that can be taken to improve their process. The Delta process is already successful; however, this research was done in an effort to provide insight into the factors at play within these field bioreactors. This will allow for increased reproducibility of results and less chance of error as a result of a lack of understanding of the mechanisms at play in the field bioreactors. To address this question, a series of experiments were done as detailed in the previous 4 chapters. The first of these chapters, Chapter 2, evaluated various nutrient types and how these different nutrients effected the bacterial community. Chapter 3 provided additional information on the nutrients. This chapter evaluated the growth pattern for each nutrient type to determine which nutrient type provided the optimal growth. Chapter 3 also evaluated the temperature range, as well as optimal temperature for each isolated bacteria as well as the consortium. Additionally, the effect of aeration was evaluated along with the aerobic and anaerobic abilities of both the consortium and the isolated bacteria. Chapter 2 evaluated the community and allows us to compare those findings to Chapter 3 which allows us to select a nutrient source that is cost effective, yields high numbers of bacteria and produces the desired community composition. With these two chapters evaluating the bacterial community and various conditions in the lab, the next chapter, Chapter 4, aimed to evaluate multiple sources of contamination that are present in field applications in an effort to determine which sources of contamination may be detrimental to the community and which sources may not be as impactful. This chapter utilized the nutrient that was determined to be the best option in Chapters 2 and 3. From the Chapter 4 results we were able to see that some sources of contamination had drastic impacts on the community such as the water, whereas the aeration and being open to the environment had less of an

impact. Lastly, Chapter 5 evaluated the community composition from a field bioreactor that was in operation for a little over a month. From the results of this chapter, we are able to make suggestions based on the previous three chapters on how to improve the efficiency of the field bioreactor. As well as how to better target the desired community and which sources of contamination should be addressed to further aid in producing the desired community.

Key findings

Evaluating all four of the chapters together provides many insights into adjustments that need to be made to the field bioreactor. From the findings of these chapters, it is evident that contaminated water has a detrimental effect on the community, and addressing this issue should be high priority. Sterilizing the water would be the most effective option however in these field settings sterilizing the water is highly costly due to the large volumes of water needed. Alternatively, further research should be done to evaluate if there are other options for producing a target community without expensive sterilization methods. One option that could potentially allow for the production of a target community could be utilizing a seed culture that is produced using sterile conditions and goes through a scale up process to the full bioreactor. This may give the target consortium a competitive advantage allowing the consortium to maintain high proportions of the community. However, this method would be more time consuming and may not work with the daily harvesting of the bioreactors. A second alternative could be utilizing a selective media that inhibits nontarget bacteria from growing. One option for this could be utilizing a hydrocarbon as the carbon source. Although this may not stop all contamination, it should promote only bacteria capable of utilizing hydrocarbons as a carbon source becoming established. So, although contamination may still be present, it may not be as big of an issue as the contamination should also be capable of degrading hydrocarbons. Additionally, this research found that the aerating the bioreactors results in increased cell counts, and therefore the bioreactors should continue to be aerated, and the dissolved oxygen level should be monitored to ensure adequate

aeration is being provided. These bioreactors should also utilize Bushnell Haas media as this media showed promising results for being low cost and still yielding high cell counts and supporting the target genera of *Pseudomonas* spp. and *Bacillus*. This is a finding that Delta Remediation has already taken action on, replacing their previously used NPK medium with a nutrient source very similar to Bushnell Haas with minor alterations for increased applicability at field scale. To maintain optimal growth, these bioreactors should be operated between 14 °C and 54 °C as this was determined to be the range the Biologix consortium was capable of growing in.

The findings of these chapters are valuable to the field of bioremediation as they provide insight into factors that need to be considered for field applications, as well as some that do not need as much consideration. By evaluating the requirements of a field bioreactor and determining that some standards followed in lab settings do not need to be followed in the field there is potential for cost reduction of field bioreactors. As well as simplification of the process in field settings. Producing thousands of liters of biological amendment using lab standards would increase the cost of this amendment significantly, potentially making bioremediation cost prohibitive. By determining what factors may not be as influential to the final product we can reduce the cost of bioremediation making it a more viable option. As suggested in Chapter 4, contamination from water had the largest impact on the microbial community. However, the contamination from aeration and from being open to the environment had much less of an impact on the community. Shifts in the population were still present with these two sources of contamination, however, they did not experience non target bacteria making up majority of the population. Because of this, Chapter 4 recommended that water contamination be addressed. The first option is to sterilize the water by heating it or filtering it, however this would be costly for large volumes of water. Alternatively, Chapter 4 suggests utilizing a seed culture started in sterile water, that is used to inoculate the large bioreactor. This option needs additional research and experimentation to evaluate the effect of

this option. This option could potentially give the BioLogix consortium a competitive advantage, instead of adding it directly to the bioreactor where it may take longer to start growing exponentially due to the fact it is in a lyophilized form. The contaminating bacteria may be able to start reproducing exponentially sooner since they are not lyophilized, potentially giving the contamination an advantage over the target consortium.

Strengths and weaknesses

A particular strength of this research was the opportunity to do this research alongside Delta Remediation, a bioremediation company that has been doing microbial bioremediation for the past 10 years. By working with an industry partner with 10 years of experience in hydrocarbon bioremediation, I had the opportunity to experience first-hand how field bioremediation was done. By working on bioremediation projects with this company, I had the chance to experience the difficulties faced in the field and the unique chance to attempt to address these issues in the field, as well as the ability to go back to the lab and perform additional small-scale tests to help find the best solutions. A drawback to this research, however, is the variability between sites. This variability could change the results of these experiments depending on the location of these projects. It would be beneficial to continuously collect data from field projects wherever they may be to build up a database of information that could potentially reveal consistent findings across all sites.

Current Knowledge

Current knowledge on the subject provides plenty of information on different bacteria that are capable of degrading various hydrocarbons or other contaminants. The genus *Pseudomonas* has repeatedly been highlighted as having a high potential for bioremediation due to its high metabolic adaptability (Pandolfo et al. 2023; Subathra et al. 2013). *Bacillus* genus has also been highlighted for its potential in bioremediation due to multiple factors such as metabolic adaptability, formation of

endospores potentially making them more persistent in the environment, as well as some species ability to produce biosurfactants that can increase the bioavailability of the hydrocarbons to all species present (Logan and De Vos 2009; Hossain et al. 2022; Brinda et al. 2024). Many other species of bacteria have also been found to have high potential in hydrocarbon remediation, with some showing high potential for specific hydrocarbons that can be more difficult to degrade. For example, the bacterium *Pseudomonas benzopyrenica* has demonstrated the ability to degrade Benzo (a) pyrene which is a 5 ring polyaromatic hydrocarbon (Dong et al. 2023). In addition to many species of bacteria being identified and studied in regards to their bioremediation applications, there has also been research linking certain genes to the ability to degrade certain hydrocarbons (Ehis-Eriakha et al. 2020). This information can provide insight into a bacterium's potential for hydrocarbon remediation. Evaluation of the range of temperatures remediation can occur at has been studied in field pilot study's as well as in the lab. Pilot scale field trials have been performed all around the world, from short and cold seasons such as the Canadian arctic (Sanscartier et al. 2009; Gomez and Sartaj 2014) to long and hot seasons such as Kuwait (Gallego et al. 2022). Studies on different types of bioremediation have been conducted such as biopiles, bioaugmentation, biostimulation etc. demonstrating the wide range of options for bioremediation, and highlighting how the overall process of bioremediation is also highly adaptable (Lemming et al. 2010; Azubuike et al. 2016; Lukić et al. 2024). Research on the amount of various nutrients needed to degrade a given amount of hydrocarbon have been conducted (Crone et al. 2020). There is a vast amount of research determining that bioremediation is possible and that it can work very well. Despite all this research, from my review of the literature, I found it very difficult to find research on how to produce the required bacterial culture. Many research articles make brief mention of utilizing a commercial product, or the amendment being transported to site, or major bioreactor set ups being utilized. Although using commercially produced amendment manufactured in highly controlled systems is an option, it is limited in its application due to the cost of transporting these products to the

locations of the contaminant. Therefore, my research aimed to work towards a field bioreactor that would allow for the production of these cultures at the location of the contamination. This not only reduces the cost of transportation, it would also allow for the harvest of the culture during its exponential phase and immediately be applied to the contamination without any need to preserve the culture for transportation.

The significance of this research is improving the knowledge base around using a field bioreactor for bioremediation. This research provides insights into some of the issues that need to be addressed for field bioreactors that have a primary focus on producing biomass. This research differed from most research as it does not strictly follow aseptic techniques, as these techniques are meant for lab research and may not be required for industrial processes where producing scientific results is not the goal. Although reproducibility is still important, scientific lab results are not required for these applications. Additional research is needed to determine the most practical methods of dealing with the issues found in these trials, but this research has provided insights into which sources of contamination are of higher concerns and provides direction as to the next questions that could be investigated further.

Bioremediation utilizing bacteria has major potential in the future as a method of remediating contaminated sites. Microbial bioremediation is growing in popularity due to many reasons, including it being a cheaper option, more environmentally friendly, its ability for complete degradation of many different contaminants and the adaptability of the process (Nwankwegu et al. 2022; Mandal et al. 2014).

Bioremediation can be used for soil, water and even air pollution. It can be adapted to many different contaminants such as hydrocarbons, solvents, textile dyes, glycol, wastewater etc. (Yakimov et al. 2007; Nwankwegu et al. 2022). The adaptability of bacteria as well as the massive diversity in metabolic pathways of bacteria makes bioremediation highly adaptable. Many countries around the world utilize bioremediation to treat contaminated soil, recognizing that treating the soil is a much

more sustainable option for dealing with contamination. In Canada, the majority of contaminated soil is excavated and hauled to landfills. For example, in Saskatchewan, the only government approved method of reclamation is excavation of the contaminated soil and hauling it to landfill (Government of Saskatchewan 2012). At these landfills, the soil is mixed in with all kinds of other contaminants with no plan on treating the soil. This process not only leaves the soil untreated, it makes it much more difficult to treat in the future as it is now mixed with many different toxic substances. Additionally, the barriers used in Canadian landfills to prevent leaching of contaminants are designed to last a few hundred years, however the true lifespan of liners is much shorter. In the article by Sun et al. 2019, they suggest that these barriers can start to fail in as little as 10 years, with the leak rate increasing over time. Landfills are not a permanent solution for soils disposed of at the landfill. In the future these barriers could fail, releasing a blend of toxic components into the environment. Treating the soils initially is a much safer option, and treatment of these soils initially is much more manageable compared to the soil in landfills that has become contaminated with a slew of different contaminants.

Because of the potential of bioremediation and the growing popularity of the process, continued research on the process should include studies on consortia of bacteria in order to evaluate blends of bacteria that produce the best degradation. This research should also include testing on many different contaminants to evaluate the range of the bacterial consortia. Additional research to design systems that allow manipulation of various parameters on a large scale should be considered. For example, testing ways of providing oxygen throughout soil piles to maximize aerobic metabolism of the bacteria could be investigated. Continuing this research on designing an efficient and effective field bioreactor would be beneficial for bioremediation. As mentioned earlier, it would be beneficial to collect as much data as possible from any site that these field bioreactors are used to work towards revealing patterns between these sites as well as highlighting which factors are highly dynamic between sites. By designing a highly effective field scale bioreactor

that allows for the production of target species at the site of contamination, massive amounts of bacteria can be applied to the contamination creating a very effective process for removing these toxic compounds from the environment. By treating these sites we make the environment healthier which in turn makes us healthier.

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APPENDIX

Chapter 3 Supplementary Information

LB broth growth

The OD for aA increased at all temperatures from 10 °C to 56 °C with the exception of 16 °C and 18 °C where the OD decreased. Additionally, the increase in OD for the temperature of 10 °C, 12 °C, 54 °C and 56 °C were very minimal.

Bacterium aB had an increase in OD at all temperatures from 10 °C to 56 °C.

Minimal increase in OD was observed at the temperature of 56 °C with an increase of only 0.002 and 10 °C where the OD increased by 0.023.

Bacterium aC increased in OD at all temperatures between 10 °C and 56 °C.

Minimal increase was observed at temperatures of 52 °C and above where the OD increased between 0.009 and 0.025. The lowest increase in OD was observed at 14 °C with an increase of only 0.005 OD.

Bacterium aD exhibited an increase at all temperatures between 10 °C and 56 °C other than 16 °C where the OD decreased by 0.03. Minimal increase was observed at 10 °C (0.028) and 56 °C (0.042)

Bacterium aE exhibited growth at all temperatures between 10 °C and 56 °C with the exception of 14 °C where the OD decreased by 0.002. Minimal growth was observed at 10 °C, 12 °C, 54 °C and 56 °C.

Bacterium aF grow at all temperatures between 10 °C and 50 °C, and minimal growth at 56 °C. A decrease in OD was observed at the temperatures of 52 °C and 54 °C of 0.002. Minimal growth was also observed between 10 °C and 14 °C.

Bacterium aG increased in OD at all temperatures from 10 °C up to 56 °C. Minimal increase in OD was observed at temperatures of 10 °C and 56 °C, increase in OD at 12 °C to 16 °C was also relatively low.

Bacterium aH increased in OD at all temperatures between 10 °C and 56 °C.

Minimal increase in OD was observed from the temperatures of 10 °C to 18 °C with the exception of 12 °C where the OD increased by 0.182.

Bacterium aJ grew at all temperatures between 10 °C and 56 °C other than 16 °C and 50 °C.

The Consortium exhibited an increase in OD at all temperatures other than 16 °C and 18 °C. The lowest increase in OD was observed at 10 °C where the OD increased by 0.016.

BH broth growth

Bacterium aA had an increase in OD at the temperatures of 10 °C to 54 °C with the exception of 14 °C where there was a decrease in the OD. There was minimal growth at 10 °C where there was only a minor increase in OD of 0.003 from 24 to 48 hours.

Bacterium aB had an increase in OD at the temperature of 12 °C as well as between 18 °C and 54 °C. The lowest increase in OD was at 12 °C.

Bacterium aC also increased in OD at 12 °C and between 18 °C and 54 °C, with decreases in OD at 10 °C, 14 °C, 16 °C and 56 °C. The lowest increase was at 12 °C.

Bacterium aD exhibited increasing OD from the temperatures of 10 °C to 54 °C with the exception of 14 °C. The lowest increase in OD was at 10 °C where OD only increased by 0.003.

Bacterium aE increased in OD at the temperatures of 12 °C, 18 °C – 50 °C, and 54 °C, decreasing in OD at 10 °C, 14 °C, 16 °C, 52 °C and 54 °C. The lowest increase in OD was at 12 °C with an increase of 0.005.

Bacterium aF grew at 12 °C as well as from 18 °C to 54 °C. The lowest increase in OD was at 50 °C with an increase of just 0.004.

Bacterium aG had an increase in OD at all temperatures between 10 °C and 54 °C with the exception of 14 °C where there was a decrease in OD. The lowest increase in OD was 50 °C and 52 °C, both temperatures with an increase in OD of 0.003.

Bacterium aH had an increase in OD at the temperatures of 12 °C, 16 °C – 20 °C, and 54 °C. The lowest increase in OD was observed at 12 °C with an increase of just 0.043.

Bacterium aJ had an increase in OD at the temperatures of 12 °C, 16 °C – 20 °C, and 52 °C. The lowest increase in OD was at the temperature of 12 °C.

Lastly, the consortium had an increase in OD at the temperatures of 12 °C and 16 °C to 56 °C. The lowest increase in OD was at 12 °C with an increase of only 0.089

Correlation Matrix Supporting Figure 3.3

	Inoculated, Filtered air	Sterilized, Inoculated	Closed, Inoculated	BH
Inoculated, Filtered air	1			
Sterilized, Inoculated	0.998033619	1		
Closed, Inoculated	0.996866022	0.994895463	1	
BH	0.958521864	0.953063023	0.935648881	1