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In Situ Cultivation of Potential PAH Degrading Bacteria From Coastal Sediment

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IN SITU CULTIVATION OF POTENTIAL PAH DEGRADING BACTERIA FROM COASTAL SEDIMENT

by

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ABSTRACT

Oil spills can introduce potentially carcinogenic pollutants, such as polycyclic aromatic hydrocarbons (PAHs), into coastal environments. Bioremediation uses the natural microorganisms in the environment to remove these pollutants. Traditional studies of these organisms are limited in the types of bacteria isolated due to the limitations of traditional culturing methods. In this study, diffusion chambers were used to culture and isolate potential PAH degrading bacteria from the coastal sediment obtained from the Chandeleur Islands. The diffusion chambers trapped bacteria in agar that contained 1 ppm benzo[a]pyrene. The bacteria were isolated from the diffusion chambers, and the 16S rRNA gene was sequenced to identify the bacteria. Seven unique bacteria isolates were obtained and were found to be genetically similar to bacteria from the *Bacteroidetes* and *Alphaproteobacteria* phyla. It was concluded that the diffusion chamber approach provided an environment that promoted the growth of potential PAH degrading bacteria. Exploration in the use of diffusion chambers should continue in research of PAH biodegradation and the uncultivability of microorganisms.

ACKNOWLEDGMENTS

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INTRODUCTION

In 2010, the Deepwater Horizon Oil Spill released 3.19 million barrels (506 million liters) of oil into the Gulf of Mexico (Barbier, 2015). Although human cleanup efforts and natural processes removed much of the oil, 26% remained in the environment (Atlas & Hazen, 2011). The petroleum that reached coastlines and marshes became incorporated into the estuarine shoreline and sediment. Crude oil contains many pollutants that cause deleterious effects to natural resources. Polycyclic aromatic hydrocarbons (PAHs) are organic molecules characterized by two or more benzene, or aromatic rings, fused together in different shaped clusters (Cerniglia, 1992). PAHs are an interest to researchers due to their potential mutagenic and carcinogenic properties. Benzo[a]pyrene (Fig.1) is one of sixteen PAHs considered a United States Environmental Protection Agency priority pollutant for its high toxicity and carcinogenicity (U.S. Department of Health and Human Services, 2016). PAHs are degraded naturally in the environment by both chemical and biological processes, such as biodegradation by microbes. A

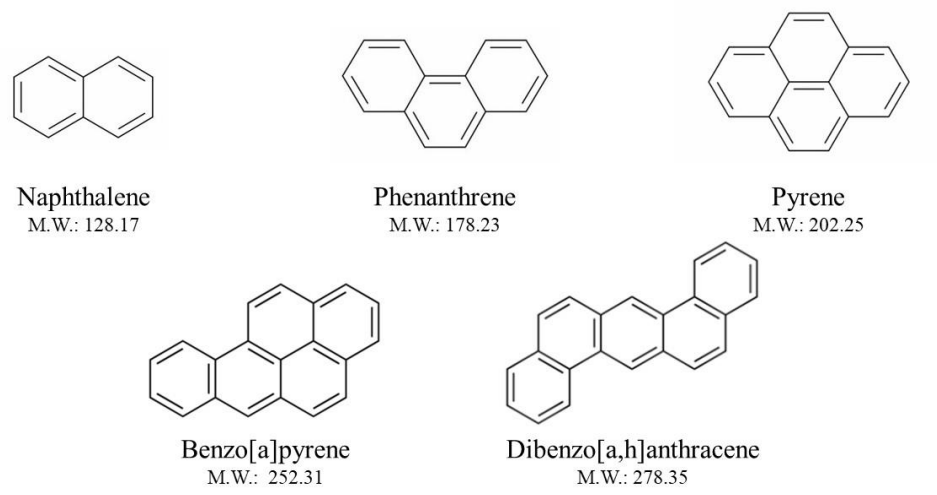

Figure 1: Chemical structures and molecular weights (M.W.) of common PAHs

variety of species of both bacteria and fungi have shown degradation of PAHs. Bioremediation relies on the microbial metabolism or degradation of the pollutants to remove them from the environment. The end goal is to reduce the hazardous compound to a harmless product such as carbon dioxide, water, or ethane (Shahsavari, Schwarz, Aburto-Medina, & Ball, 2019). Bioremediation offers a simple and inexpensive solution to PAH remediation, but the effectiveness is highly dependent on many factors, such as pollutant's concentrations, nutrients, temperature, oxygen, and pH. These components are all unique to an individual location and can impact the microbial community and, therefore, the potential success of bioremediation.

There is a vast distinction between the number of bacteria that are known to exist and those that can be cultured on a petri dish (<1%) (Rappe & Giovannoni, 2003). This distinction is accounted for through the "great plate count anomaly," which describes the difference in the ratio between the cells that form colonies on agar plates and the number of cells counted microscopically (Staley & Konopka, 1985). Today, novel microorganisms are discovered without cultivation by sequencing their DNA, particularly the 16S rRNA gene. Using 16S rRNA gene analysis, new members of microbial communities and their evolutionary relationship can be revealed and analyzed. Many isolated bacteria that have been studied show PAH degrading properties (Marchand, St-Arnaud, Hogland, Bell, & Hijri, 2017; Puškárová et al., 2013; Thomas, Lorgeoux, Faure, Billet, & Cébron, 2016; Yan, Wang, Qu, & Li, 2013), but many potential bacterial species may be missed due to the lack of cultivability. Due to this problem, research has turned toward *in situ* analysis of individual microbes and microbial communities. *In situ* analysis examines the microbes in their natural environment or a simulated environment in the laboratory. The availability of the natural nutrients in the *in situ* environment allows for a greater chance to culture typically unculturable bacteria.

The objective of this project was to utilize an *in situ* culture technique, developed by S.S. Epstein (Annette, Kim, & Slava, 2007; S. S. Epstein, 2013; Kaeberlein, Lewis, & Epstein, 2002), that allows for the culture of microbes that would otherwise not grow using typical culture techniques. Diffusion chambers are designed to incubate bacteria by allowing the diffusion of necessary nutrients found in the natural microbial environment through a permeable membrane. By modifying the original method and applying it to contaminated marine sediment, potentially previously uncultivated bacteria may be cultured in the laboratory. These bacteria also have the potential of contributing to the biodegradation of PAHs. Understanding the microbial community and how it behaves in different environments is key to understanding bioremediation and providing solutions to PAH contamination problems when they arise.

LITERATURE REVIEW

Bioremediation and PAH Biodegradation

Bioremediation is the process of using organisms to remove pollutants from the environment. Many different methods, techniques, and organisms have been studied to address various environmental pollutants. Oil pollution, due to human-made disasters such as the Deepwater Horizon Oil Spill, is a significant issue in marine and coastal environments. As oil becomes incorporated into the environment, damaging pollutants such as PAHs, characterized by their fused benzene rings, can disrupt the ecosystem. PAHs are highly carcinogenic and can pose a significant risk to humans in coastal regions.

Microorganism biodegradation is the primary mechanism that removes PAHs from the environment. The degradation process of low molecular weight (LMW) PAHs, such as naphthalene, phenanthrene, and pyrene (Fig. 1), is highly researched. Ring oxidation is done by dioxygenase enzymes that create a cis-dihydrodiol on the PAHs, allowing for further degradation (Shahsavari et al., 2019). The degradation of high molecular weight (HMW) PAHs, such as benzo[a]pyrene and dibenzo[a,h]anthracene (Fig. 1), is not as fully understood when compared to LMW-PAHs. The incorporation of the large molecules into the cell is challenging, limiting the number of species capable of degradation. Research has suggested that fungal degradation of HMW-PAHs into smaller metabolites allows bacteria to complete the degradation process (Shahsavari et al., 2019). Certain PAH degrading fungi can release extracellular enzymes, such as oxidoreductases, laccase, and peroxidases, that contribute to the degradation of PAHs (Balaji, Arulazhagan, & Ebenezer, 2014).

Bioremediation is divided into three different strategies that are used to eliminate pollution in the environment (Megharaj, Ramakrishnan, Venkateswarlu, Sethunathan, & Naidu, 2011). Bioattenuation (natural attenuation) uses the natural microbes in a polluted area to convert pollutants into less toxic substances (Smets & Pritchard, 2003). Biostimulation is a strategy that by providing chemical stimulators and nutrients to the environment, biodegradation is induced using the natural microbial flora (Megharaj et al., 2011). The last strategy used is bioaugmentation. Bioaugmentation introduces new, primed, or pre-adapted organisms into a polluted environment that are best suited for degrading the specific pollutant present (Megharaj et al., 2011). Bioaugmentation has the greatest potential for adaption to any pollution scenario and environment, especially when there is a lack of microorganisms adapted for biodegradation.

Many different factors contribute to the types of bacteria present in an oil-contaminated site and the effectiveness of oil biodegradation. Temperature, pH, oxygen, nutrients, and the bioavailability of PAHs all impact the biodegradation process. Temperature is unique to the contaminated area where higher temperatures increase the solubility of PAHs (Margesin & Schinner, 2001), allowing microbes greater access to the molecules for degradation. The type and concentration of a contaminant in the environment can affect the site pH. For phenanthrene degradation by *Burkholderia cocovenenas*, a pH between 6.5-7 was most favorable (Wong, Lai, Wan, Ma, & Fang, 2002). Very acidic or alkaline environments can decrease the biodegradation activity of microbes. Typically, the biodegradation process of PAHs occurs under aerobic conditions to permit oxidation of the molecules. However, anaerobic degradation does occur and has been seen in marine sediments (Coates, Woodward, Allen, Philp, & Lovley, 1997). The nutrient content of a particular site varies and has a significant impact on the microbial community. Under certain nutrient conditions, one bacterium may flourish, and another will not

be present at all. The bioavailability, or the amount of the chemicals available to the microorganism (Ghosal, Ghosh, Dutta, & Ahn, 2016), is critical to the bioremediation process. PAHs are very hydrophobic molecules with low water solubility and tend to integrate themselves into sediment and soil. The PAHs become less bioavailable to bacteria, which is detrimental to the biodegradation process.

When studying and implementing the bioremediation of PAHs in contaminated environments, it is essential to remember that no two environments are the same. There is a high variance in all these factors that ultimately contribute to the activity of the microbial community and individual bacteria. Isolated cultures in the lab are kept in an environment drastically different from their natural environment, potentially impacting the success of implementing these species into a contaminated site for bioremediation. There needs to be a better understanding of how PAH biodegrading bacteria behave in their natural environment by using *in situ* techniques.

The Unculturability of Microbes

The "great plate count anomaly," first noted by Staley and Konopka (1985), highlights the discrepancy between the number of bacteria that can be observed microscopically and the number of those bacteria that grow on agar media. As microbial biodiversity was further studied using the rRNA gene approach, the discrepancy also appeared to be a diversity gap (S. Epstein, Lewis, Nichols, & Gavrish, 2010). The number of culturable species is significantly smaller than the number of species present in nature. In 1998, there were about 40 phyla divisions identified in the domain *Bacteria*, but 13 of the phyla did not have a cultured specimen (Hugenholtz, Goebel, & Pace, 1998). More recent data suggests that 26 of 52 identifiable phyla do not have a cultured representative (Rappe & Giovannoni, 2003).

Dojka, Harris, and Pace (2000) researched an uncultured bacteria phylogenetic group, WS6. The presence of WS6 organisms was studied in 12 environments, and the result revealed that seven of those environments contained WS6 bacteria, mostly from anaerobic environments (Dojka et al., 2000). The study also expanded the known sequences of WS6 bacteria from three to 60 (Dojka et al., 2000). This study demonstrates the ever-growing knowledge of bacterial phylogenetic diversity, but with a caveat. Because all WS6 representatives are uncultivated, the characteristics of these groups of bacteria are unknown, and more emphasis must be devoted to growing pure cultures.

Progress is being made. The SAR11 clade, a previously uncultured group of bacteria, used to baffle researchers. The bacteria were present in about every marine bacterioplankton community studied using cultivation-independent approaches but were unable to be cultured (Morris et al., 2002). The SAR11 clade was eventually cultured by using marine samples and diluting the samples in a microtiter plate so that each well only contained about 22 cells (Rappé, Connon, Vergin, & Giovannoni, 2002). The supplemented media contained deficient nutrient concentrations, and the dilutions allowed for the isolation of SAR11 clade bacteria (Rappé et al., 2002). The cultivation of SAR11 bacteria represents a significant milestone in the field, but only a tiny minority of all the cells exposed to the same conditions were successfully cultured (Rappé et al., 2002).

Why do bacteria behave like this? Is there a particular mechanism that determines why some bacteria are so difficult to culture, and others will grow on just about anything? S. S. Epstein (2009) proposed a new model that attempted to explain the reasons for uncultivable bacteria. He proposed a “scout strategy” used by bacteria in non-favorable conditions (S. S. Epstein, 2009). The model stated that bacteria can enter a dormancy state in adverse conditions

to increase survival (S. S. Epstein, 2009). Particular "scout" bacteria are stochastically activated and can grow and replicate if the environment is favorable (S. S. Epstein, 2009). Under advantageous conditions, the scouts will begin replicating and can send chemical signals to the dormant cells to "awaken" them (S. S. Epstein, 2009). Epstein (2009) also suggested that in a collaborative microbial environment, a second, different species may act as a scout and release signals to the other species to indicate favorable conditions. The scout model provides a possible explanation of the "great plate count anomaly" and unculturable microbes. If the majority of cells do exist in some dormant state, it would require either a large sample of bacteria to produce enough scouts to be cultivated or enough time for the bacteria to produce scouts that would replicate (S. S. Epstein, 2013). In both cases, the bacteria to appear uncultivable since both scenarios are rare. Epstein's scout model is fairly new and has not been confirmed, but it is supported by evidence in a study where bacteria species *Escherichia coli* and *Mycobacterium smegmatis* "woke up" stochastically (Buerger et al., 2012). Further research is required to understand the mechanisms and reasons for so many unculturable microorganisms, but it is clear that current culture techniques do need reevaluation to account for this large gap.

Diffusion Chambers

Many microbes rely on the nutrients of their natural environment and the microbial community around them to grow, and it is challenging to replicate these conditions artificially. Epstein and his colleagues developed a method that allows the culturing of some of these previously unculturable bacteria using a "diffusion chamber" placed in their natural environment (Kaeberlein et al., 2002). The premise is simple but effective. Agar is placed inside a metal washer and sandwiched between two membranes that allow the diffusion of nutrients into the chamber. In theory, the cells should have all the necessary nutrients to promote growth. In initial

experiments, diffusion chambers, pre-inoculated with bacteria, were placed on top of intertidal marine sediment in a marine aquarium filled with seawater (Kaeberlein et al., 2002). Up to 40% of cells pre-inoculated into the diffusion chamber were recovered, and previously uncultivable bacteria were able to be isolated in pure cultures (Kaeberlein et al., 2002).

The diffusion chamber approach, compared to standard petri dish cultivation, demonstrated not only an increased number of strains cultured, but also an increase in diversity of strains (Annette et al., 2007). Of all the isolates obtained from a freshwater pond sediment sample, 70% were isolated using diffusion chambers, 23% from standard petri dishes, and 7% from utilizing both methods (Annette et al., 2007). Using the diffusion chamber method, isolates were obtained from uncommonly cultured phyla, including *Acidobacteria* and *Verrucomicrobia* (Annette et al., 2007). Interestingly, using multiple rounds of diffusion chamber cultivation promoted the adaption of previously uncultivable microorganisms to in vitro cultivation on petri dishes (Annette et al., 2007).

There are two variations of the diffusion chambers used in experiments. In one, the agar is pre-inoculated with bacteria and placed between two 0.03 µm membranes. In the second, the agar is sterile and placed between a 0.03µm membrane and a 0.2 µm membrane (Fig. 2). The latter allowed for the migration of filamentous bacteria across the 0.2 µm membrane into the agar, trapping them in the diffusion chamber (Gavrish, Bollmann, Epstein, & Lewis, 2008). Filamentous actinobacteria from garden soil demonstrated the ability to penetrate the pores of the membrane with their mycelia and form colonies in the agar (Gavrish et al., 2008).

The diffusion chamber method has also been applied to contaminated soil (Remenár et al., 2015). Nickel contaminated soil was studied using diffusion chambers, leading to the isolation of phylogenetically novel and unculturable species (Remenár et al., 2015). Research

continuing to utilize diffusion chambers to study microorganisms in contaminated environments will answer questions regarding both biodegradation and the uncultivability of microbes. Diffusion chambers may provide a solution to isolating essential microbes for the biodegradation of pollutants, such as PAHs, and allowing them to be further studied. Diffusion chambers will also continue to uncover previously unculturable bacteria, furthering our knowledge of microbial diversity and the great plate count anomaly.

MATERIALS AND METHODS

The basic principles of the methodology were based on the diffusion chamber methods developed by S.S. Epstein and colleagues (Annette et al., 2007; S. S. Epstein, 2013; Kaeberlein et al., 2002). The procedures were modified and adjusted to fit the particular goals and objectives of the current study.

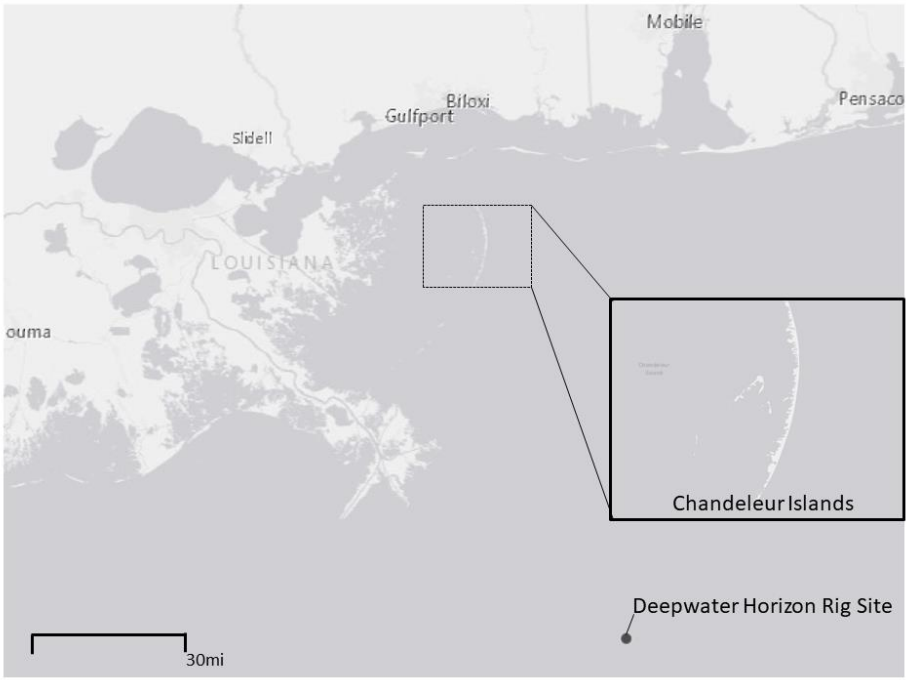

Figure 3: Map of Chandeleur Islands and Gulf of Mexico. (Modified from U.S. Geological Survey)

Subtidal and marsh sediment and water samples from the Chandeleur Islands off the coast of Louisiana (Fig. 2) were previously collected and stored in the lab at 4°C. This sediment was contaminated during the Deepwater Horizon Oil Spill. Every step of the methods was completed using sterile techniques. First, diffusion chambers were constructed using a metal washer, agar, and two filter membranes (Fig. 3). The metal washer had a 2 cm inner diameter. The agar was prepared from a 50:50 mixture of filter-sterilized subtidal and marsh site water, 1.5% Bacto agar, and 1 ppm benzo[a]pyrene. The water and agar were heated to dissolve the

agar and allowed to set between the two membrane filters. A 25 mm, 0.2 µm pore-size polycarbonate (PTCE) membrane filter (Sterlitech, Kent, WA) was attached to the bottom of the washer, and a 25 mm 0.03 µm pore-size polycarbonate (PTCE) membrane filter (Sterlitech, Kent, WA) was attached to the top. The membranes were applied with silicon glue.

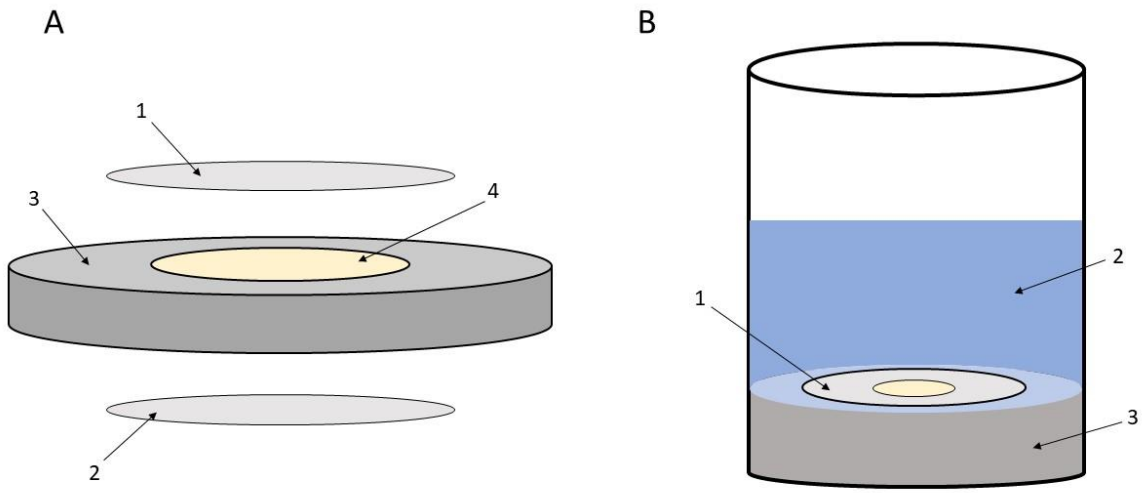

Figure 4: (A) Diffusion chamber design 1.) 0.03µm membrane filter 2.) 0.2µm membrane filter 3.) Metal washer 4.) Agar matrix (B) Experimental container 1.) Diffusion chamber 2.) Site water 3.) Site sediment

Sterile plastic containers were filled with 44-48 g of the collected marsh and subtidal sediment collected from depths between 1-8 cm. The containers were then filled with 70 mL of the filter-sterilized site water. The completed diffusion chambers were then placed in the plastic containers (Fig. 3). The 0.2 µm membrane side was placed on the bottom in contact with the sediment to allow the migration of bacteria into the agar. The diffusion chambers were left in the

in situ environment at room temperature (Fig. 4). During incubation, the chambers were moved every 48-72 hours to prevent any buildup of anoxic conditions.

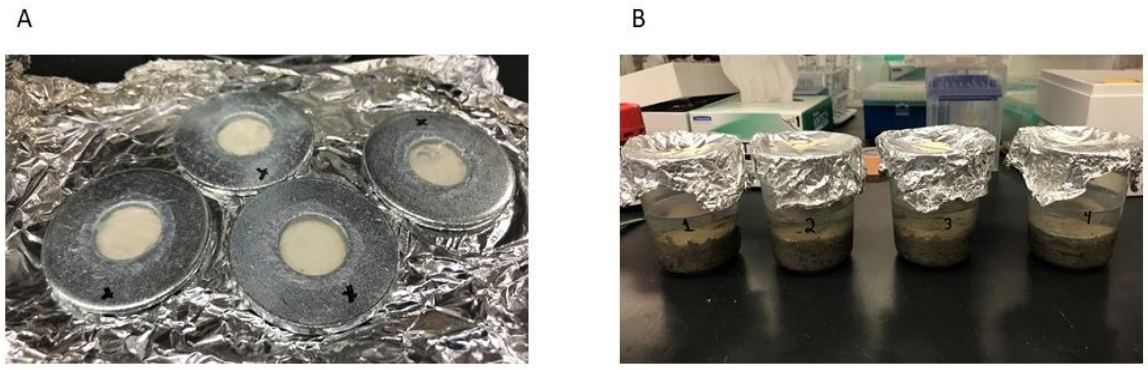

Figure 5: (A) Constructed diffusion chambers (B) In situ environment with diffusion chambers

After four weeks, the agar was removed from one of the chambers. NaCl solution (0.85% saline) was added to the agar in a microcentrifuge tube. The mixture was agitated in order to homogenize the solution. The homogenized agar saline solution was serially diluted to a 10^{-5} dilution factor. A modified M10 media, M10b, was prepared with a decreased salinity to emulate a brackish environment. M10b agar was prepared as followed: 4.0 g/L tryptone, 2.5 g/L yeast extract, 700 mL H_20, 300 ml artificial seawater (35.1 g/L NaCl, 1.5 g/L KCl, 24.7 g/L $MgSO_4 \cdot 7$ H_20, 2.9 g/L $CaCl_2 \cdot 2$ H_20), and 1.5% Bacto agar. The original concentrated solution (50 µL) was plated on an M10b agar plate, and each subsequent dilution (100 µL) was also plated on an M10b agar plate. The plates were incubated for one day at 30ºC. Then, 32 random individual colonies were taken from the serial dilution plates and streaked to single colonies to ensure no contamination. The isolates were placed on a patch plate of M10b agar, and freezer stocks of the isolates were also prepared and stored at -80ºC.

Next, boil preps were prepared for all the isolates. Molecular grade water (100 µL) was added to a 1.7 mL microcentrifuge tube. Using an applicator stick, the isolates were inoculated

into the water. The tubes were heated at 100ºC for 10 minutes. Using the DNA from the boil preps, polymerase chain reaction (PCR) was conducted. The 16S rRNA gene was amplified with an Ex Taq polymerase and Ex Taq buffer (Takara Bio Inc., Japan) using the universal bacterial primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT). Reactions were amplified in a Veriti 96-Well Thermal Cycler (Thermo Scientific, Beverly, MA) using the following program: 94ºC for 3 min, 35 cycles of 94ºC for 1 min, 50-52ºC for 1 min, 72ºC for 1 min, and 72ºC for 10 min last extension. Gel electrophoresis of the PCR product was run on an SB buffer 0.7% agarose gel at 185 V for 30 minutes. A 100 base pair ladder was used, and the dye used to visualize the bands was EZ Vision One. The DNA bands were excised from the gel and collected in microcentrifuge tubes. Following the protocol of the Zymoclean Gel DNA Recovery Kit (Zymo Research Irvine, CA), the DNA was extracted from the gels. The DNA was then quantified using the Qubit Fluorometer Quantitation (Thermo Scientific, Beverly, MA). Boil preps, PCR, and DNA extraction were repeated on failed runs in order to obtain adequate concentrations of DNA.

The samples were analyzed by Sanger DNA sequencing at the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign Facility. The DNA sequences of the 16S rRNA gene fragments were compared using a BLAST search on the National Center for Biotechnology (NCBI) database (Altschul, Gish, Miller, Myers, & Lipman, 1990). Seven unique isolates were identified and prepared for sequencing again for more accurate results. The seven isolates were incubated in M10b liquid media overnight. Following the protocol of the DNeasy Pro Kit (Qiagen, Valencia, CA), DNA of the isolates was extracted. The DNA product was amplified with PCR. The PCR product was run on an SB buffer agarose gel, and the DNA was extracted using the Zymoclean Gel DNA Recovery Kit. The DNA was

quantified using the Qubit system and prepared for Sanger sequencing. Two samples of each isolate were sequenced, one in the forward direction and one in the reverse direction.

The DNA sequences were identified using BLAST, and the most similar sequences were recorded. The sequences and reference sequences were aligned using the multiple sequence comparison by log-expectation (MUSCLE) (Robert, 2004) alignment tool in MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018). A maximum-likelihood, neighbor-joining phylogenetic tree was created using the Kimura 2-parameter model (Kimura, 1980) with 1000 bootstrap re-samplings using the MEGA software.

RESULTS

Recent diffusion chamber studies have demonstrated the potential for diffusion chambers to isolate and culture rare and unculturable bacteria from *in situ* environments (Annette et al., 2007; Gavrish et al., 2008; Kaeberlein et al., 2002; Remenár et al., 2015). In this study, using a modified diffusion chamber method, microorganisms were cultured and isolated in an *in situ* environment from sediment obtained from the Chandeleur Islands in the Gulf of Mexico.

The diffusion chambers were constructed with a 0.2 µm pore size filter on the bottom to allow the bacteria to migrate from the sediment into the agar trap that contained 1 ppm benzo[a]pyrene. After four weeks of incubation, the agar disk was removed from the diffusion chamber and displayed clear signs of microbial growth (Fig. 5). The homogenized agar was

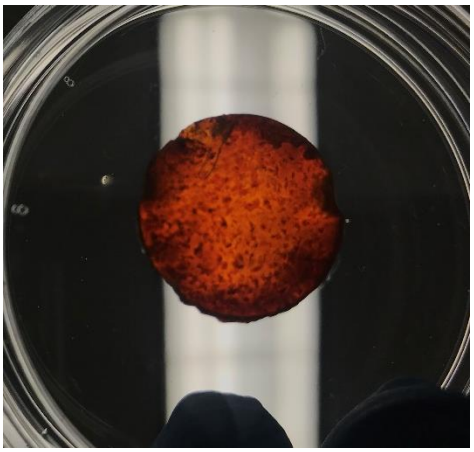

Figure 6: Diffusion chamber agar after 4 weeks of incubation in in situ environment

serial diluted and plated on M10b agar petri dishes. Growth was observed on all serial dilution spread plates (Fig. 6). The M10b agar plates resulted in the growth of red, coral, yellow, white,

and beige colonies. Colonies were randomly selected from all plates, streaked to single colonies, and patched onto a single M10b agar plate.

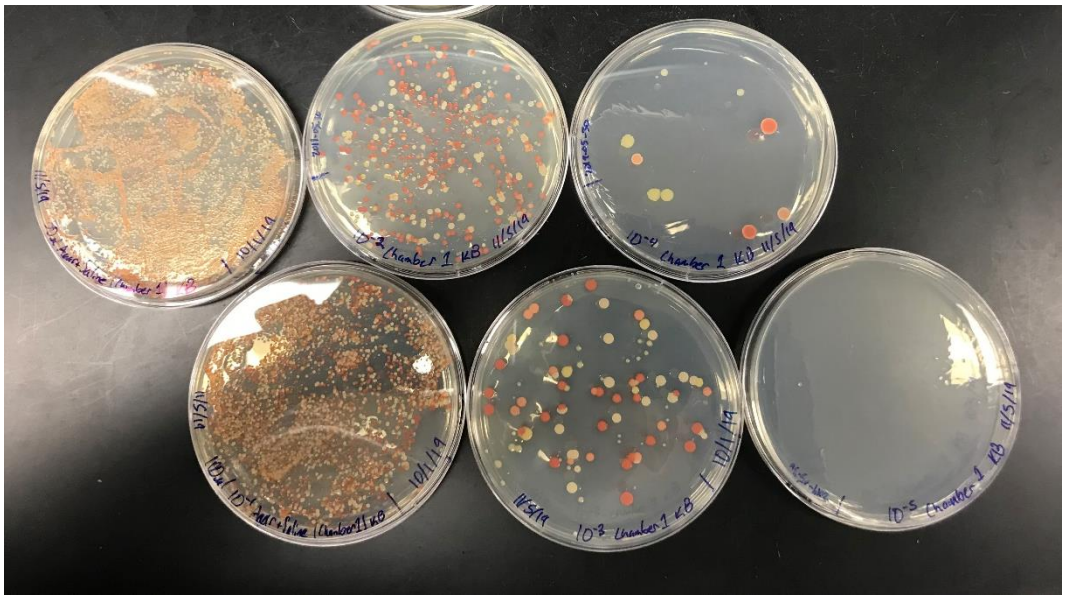

Figure 7: M10b serial dilution spread plates

In total, 32 isolates were obtained as pure cultures that represented seven unique bacterial strains. The diffusion chamber isolates (DCI) 1 through 7 are shown in Table 1 with their closest similarity match to public sequences obtained from the GenBank database using the NCBI BLAST software (Altschul et al., 1990). DCI 2 and DCI 7 are very similar to uncultured bacterium clones. However, from the BLAST search results, DCI 2 has similarity to bacteria from the *Algoriphagus* genus, and DCI 7 has similarity to bacteria from the *Muricauda* genus.

Table 1: Sequencing Analysis of Diffusion Chamber Isolates

Diffusion Chamber Isolate (DCI)	Closest strain in GenBank database (Accession no.)	Similarity (%)
DCI 1	*Algoriphagus winogradskyi* strain ZYF629 (MN491975.1)	98.71%
DCI 2	Uncultured bacterium clone MD06f11_12428 (JQ374746.2)	99.32%
DCI 3	*Paracoccus oceanense* strain YHM127 (MN492473.1)	100%
DCI 4	*Joostella marina* strain AS4G71 (KP706828.1)	100%
DCI 5	*Thalassospira sp*. JDC20 (KT356818.1)	99.70%
DCI 6	*Muricauda ruestringensis* strain YHM200 (MN492627.1)	100%
DCI 7	Uncultured Bacteroidetes bacterium clone SHAB702 (GQ348772.1)	99.58%

The seven unique bacteria were members of two bacterial phyla, *Bacteroidetes* and *Alphaproteobacteria*. The isolates assigned to *Bacteroidetes* were closely associated with the genera *Algoriphagus* (DCI 1, DCI 2), *Joostella* (DCI 4), and *Muricauda* (DCI 6, DCI 7). The isolates assigned to *Alphaproteobacteria* were closely associated with the genera *Paracoccus* (DCI 3) and *Thalassospira* (DCI 5). The phylogenetic relationships between the isolates and representative species are seen in Figure 7.

Research and analysis of the sample of bacteria isolated from the diffusion chambers reveals the potential for PAH biodegradation. *Algoriphagus* is a confirmed oil-degrading bacteria genus (Wang, Zhong, Shan, & Shao, 2014) and has demonstrated proliferation in a bacteria consortia exposed to different PAHs (Ahmad et al., 2019). *Joostella*, a relatively novel genus of the family *Flavobacteriaceae* (Quan et al., 2008), is a promising candidate for biosurfactant production that may be key for bacterial communities to degrade hydrocarbons (Rizzo et al., 2018). Bacteria from the genus *Muricauda* have also demonstrated biosurfactant activity (Cappello et al., 2016). The genus *Thalassospira* has many bacteria that are PAH degraders (Cui, Lai, Dong, & Shao, 2008; Kodama, Stiknowati, Ueki, Ueki, & Watanabe, 2008; Zhao, Wang, Li,

& Mao, 2010; Zhou, Wang, Huang, & Fang, 2016), and a *Paracoccus* bacteria has shown degradation of multiple PAHs, including benzo[a]pyrene (Gan et al., 2018; Teng et al., 2010).

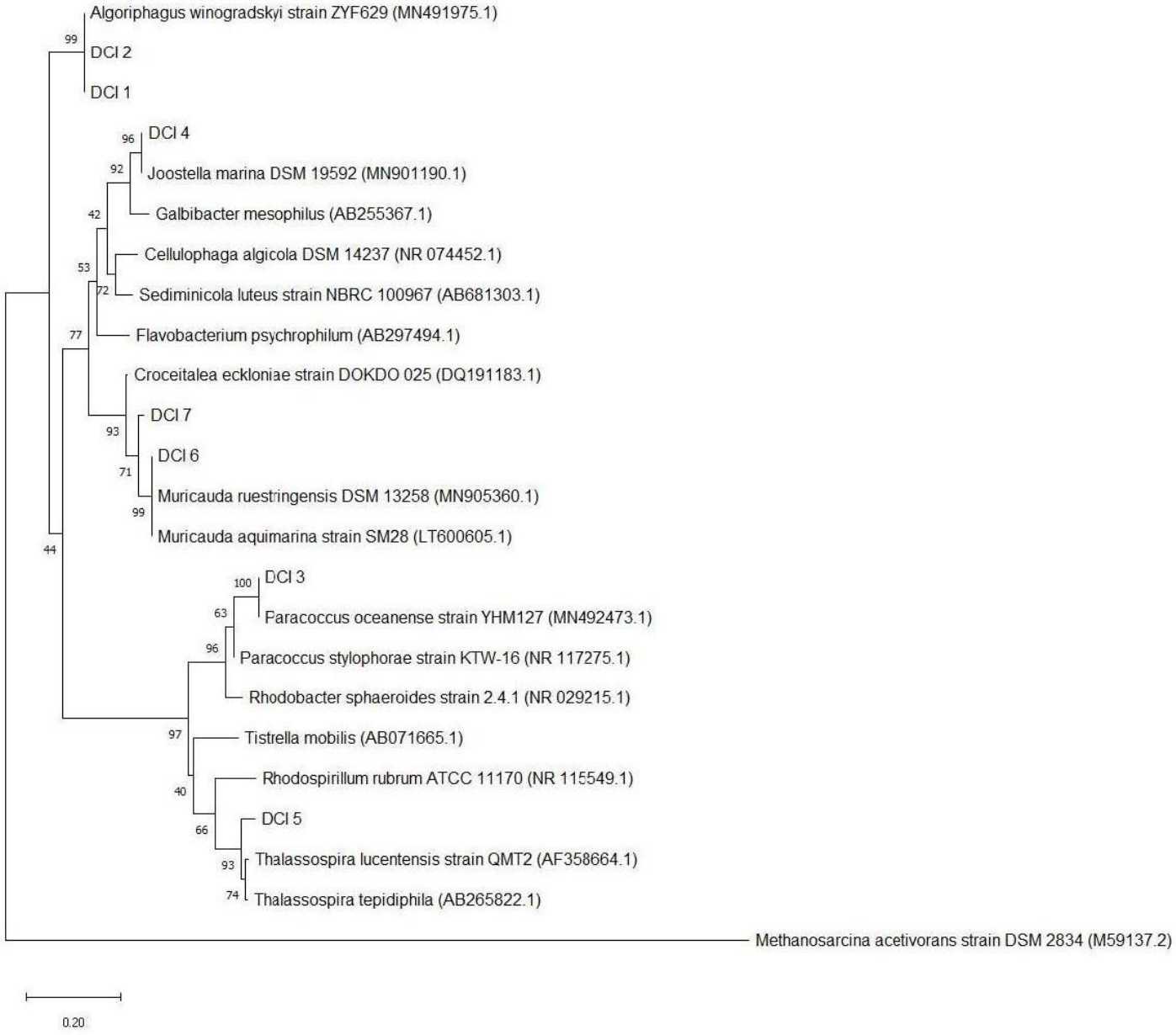

Figure 8: Phylogenetic tree derived from 16S rRNA gene sequences of diffusion chamber isolates and reference strains, as determined by the Kimura 2-parameter model. The GenBank sequence association numbers are shown in parentheses. The sequence of Methanosarcina acetivorans strain DSM 2834 was used as an outgroup. The numbers at the branch nodes are bootstrap values based on 1000 re-samplings for maximum likelihood. Bar, 0.20 nucleotide substitution per site.

DISCUSSION

The bacterial community is highly dependent on the conditions of its environment. Diffusion chambers provide a method of culturing that attempts to mitigate the bacterial environment as a factor in the growth of the bacterial community. The permeable membranes give microorganisms access to all the nutrients of the *in situ* environment, promoting the growth of those bacteria dependent on those nutrients that would not grow otherwise (Kaeberlein et al., 2002). The simplicity and effectiveness of the diffusion chambers have been demonstrated in multiple studies (Annette et al., 2007; Gavrish et al., 2008; Kaeberlein et al., 2002; Remenár et al., 2015).

This study attempted to utilize the diffusion chambers as a method to culture potential PAH degrading bacteria. Sediment contaminated from the Deepwater Horizon Oil Spill was obtained and using a diffusion chamber, bacteria were cultured and isolated to pure culture from the sediment. Seven unique bacteria isolates, from the phyla *Bacteroidetes* and *Alphaproteobacteria*, were sequenced and matched to the closest known bacterial strain in the GenBank database (Table 1). The extracted DNA of the recovered isolates closely matched the genera *Algoriphagus* (DCI 1, DCI 2), *Joostella* (DCI 4), *Muricauda* (DCI 6, DCI 7), *Paracoccus* (DCI 3) and *Thalassospira* (DCI 5). The results reveal that the bacterial isolates are known to be potential participants in the biodegradation of PAHs.

As previously stated, diffusion chamber experiments have been conducted in two ways. The first uses two 0.03 µm pore-size membrane filters with pre-inoculated agar inside the diffusion chamber. The second uses one 0.03 µm and one 0.2 µm pore-size membrane filters with sterile agar inside the chamber. The latter allows the bacteria to migrate into the agar and

proliferate. In this study, the latter method was used, where the agar contained 1 ppm benzo[a]pyrene. The benzo[a]pyrene in the agar was a way to replicate a PAH contaminated environment in the diffusion chamber. The idea being that only those bacteria adapted for PAH degradation would be captured in the agar of the diffusion chamber. It has been well researched that increased oil prevalence in the environment results in an enrichment of the oil-degrading bacteria in the microbial community (Beazley et al., 2012; Hazen et al., 2010; Kostka et al., 2011). The results support that the agar was successful in promoting the growth of potential PAH degraders. The phyla *Alphaproteobacteria* and *Bacteroidetes*, which are known to contain PAH degraders, were exclusively cultured in the experiment. The genera found that were most similar to the isolates were all found to be connected to PAH degradation in the literature. Most notably, *Algoriphagus* (Wang et al., 2014) and *Thalassospira* (Cui et al., 2008; Kodama et al., 2008; Zhao et al., 2010; Zhou et al., 2016) are known PAH degraders. Also, *Paracoccus* has shown the ability to degrade benzo[a]pyrene (Gan et al., 2018), and is effective in bioaugmentation experiments to remove PAHs from contaminated soils (Teng et al., 2010). Had the diffusion chamber been pre-inoculated with bacteria or lacked benzo[a]pyrene in the agar, different bacteria, not associated with PAH degradation, may have flourished. The application of diffusion chambers to the study of PAH biodegradation is promising and should continue to be explored.

One of the potential solutions the diffusion chamber method provides is to the uncultivability of microbes. Two isolates cultured were closely associated with uncultured bacterium clones (DCI 2, DCI 7). However, most of the bacteria isolates recovered were previously cultured bacteria, discovered using standard culture techniques (Bruns, Rohde, & Berthe-Corti, 2001; Fu et al., 2011; Kodama et al., 2008; Nedashkovskaya et al., 2004; Quan et al., 2008). In the experiment, the agar collected from the diffusion chamber was subsequently

cultured on standard petri dishes. Perhaps, potential isolates were lost in the switch from the diffusion chamber to the petri dish. Research on diffusion chambers supports that multiple rounds of culturing in diffusion chambers are needed to culture certain bacteria (Annette et al., 2007). Had the bacteria been directly isolated from the diffusion chambers using subsequent rounds of diffusion chamber incubations, the diversity and recovery of uncultivated microbes may have been higher.

Overall, the results of this study demonstrate the ability of the diffusion chamber to culture and isolate potential PAH degrading bacteria. As an explorative study, many questions remain unanswered and can be explored in future works. The bacteria cultured in this study grew in the presence of a single PAH, benzo[a]pyrene. Exploration of different PAHs and at different concentration in the agar may be useful in isolating other bacteria capable of PAH biodegradation. The use of multiple rounds of cultivation in the diffusion chamber may also provide more isolates that may be unique or previously unculturable. Further studies of the individual bacteria recovered from this experiment may prove useful in understanding the greater role of the bacterial in the microbial community. Future applications of the diffusion chamber and the isolates obtained from this study will prove useful in contributing to furthering the knowledge of PAH biodegradation and the uncultivability of microbes.

CONCLUSION

PAHs are considered one of the top priority pollutants in the United States due to the carcinogenicity to humans. To ensure a positive future for our ecosystems, solutions to PAH pollution are needed. Bioremediation provides a simple solution that has proven effective against various pollutants. Continued research on the different microbes that participate in bioremediation will foster improved efficiency in the cleaning up of contaminated environments and will provide solutions to new environmental problems as they arise. One obstacle that impacts almost all microbial studies is the uncultivability of microbes. The majority of bacteria are unculturable, and therefore their properties are still a mystery. There is a possibility that many of these unculturable bacteria may be crucial to biodegradation processes, but they simply work behind the scenes. It is of utmost importance that new, especially unculturable bacteria, continue to be discovered to advance bioremediation technologies.

Diffusion chambers have demonstrated success in culturing new bacteria and recovering greater microbial diversity. Using diffusion chambers, the goals of this study were to reveal if new potential PAH degrading bacteria could be cultured and isolated from contaminated sediment. To accomplish this objective, diffusion chambers were constructed with 1 ppm benzo[a]pyrene in the agar. The chambers were placed in an *in situ* environment and cultured over four weeks. The bacterial isolates recovered were isolated to pure culture, and the 16S rRNA gene was sequenced and analyzed.

Seven bacterial isolates were identified that matched to bacteria that have shown direct and indirect involvement in the biodegradation of PAHs. Phylogenetic analysis revealed that the isolates were from the phyla *Bacteroidetes* and *Alphaproteobacteria*, both known to contain

PAH degrading bacteria. Although no new or unculturable bacteria were uncovered in the experiments, the results provide positive evidence for the usefulness of diffusion chambers in future applications in the study of bacterial biodegradation. The diffusion chamber design and the benzo[a]pyrene agar exclusively cultured bacteria associated with PAH biodegradation. The bacteria uncovered in this study are likely essential participants in the biodegradation of PAHs and should be researched and tested further. Future work in the field of biodegradation can use this study as an example of the effectiveness of diffusion chambers in culturing bacteria.

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