Engineering a Biofilm for the Biodegradation of Polycyclic Aromatic Hydrocarbons in Estuarine Sediment

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Civil and Environmental Engineering in the Graduate School
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ABSTRACT

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants that accumulate in soils and sediment due to their physicochemical properties. In these environmental matrices, PAHs are predominantly transformed and degraded by native fungal and bacterial communities. However, microbial degradation of PAHs is a slow process that requires engineered approaches in order to improve degradation rates and meet remediation criteria.

Engineered bioremediation approaches consist of altering the microbial community by either increasing cell concentrations of specific microorganisms or by introducing catabolic genes that confer a phenotype that can degrade the target contaminant. These approaches fall under the umbrella term, bioaugmentation. Biostimulation is another method, which includes the addition of nutrients that may be limited to microorganisms and can help increase the overall biomass of the indigenous microbial community, thereby accelerating contaminant degradation. However, biostimulation is not a targeted approach and may stimulate the entire microbial community, not just organisms capable of degrading the target contaminant. This effect could result in a larger prevalence of highly competitive organisms that may suppress preferable organisms performing bioremediation.

Bioaugmentation of sediments is challenging due to constraints surrounding the longevity, stability, and delivery of microorganisms. To address the limitations of this remediation approach, the work within this dissertation outlines an approach for

engineering a consortium of PAH-degrading bacteria coordinated within a sessile community and evaluates the use of an activated-carbon based technology for delivering this consortium to PAH-contaminated sediments.

The first objective was to identify and isolate PAH-degrading bacteria from creosote contaminated sediment. Sediment was collected from sites along the Elizabeth River, VA and a 16S rRNA gene amplicon library was analyzed to generally evaluate the influence of chemical contamination on the bacterial community structure. To detect PAH-degrading organisms within sediment communities, DNA-SIP using uniformly labeled stable isotopes of phenanthrene and fluoranthene were prepared in incubations with Republic Creosoting site sediment. Clones derived from this experiment revealed one prominent degrader of phenanthrene and two prominent fluoranthene degrading bacteria. In an attempt to isolate these and other PAH-degrading organisms for laboratory evaluation, culture-based methods were employed and resulted in the successful isolation of 6 unique bacteria, including one strain which was detected in the DNA-SIP experiments. Overall, it was determined that PAH-degrading bacteria exist in Republic Creosoting site sediments in a low relative abundance compared to other bacteria, which suggests that this site is a good candidate for bioaugmentation.

Most of the research on bioremediation has focused on organisms in isolation and existing in a free-floating, or planktonic, cellular state. The second objective of this dissertation was to coordinate these organisms into a biofilm structure, which provides protection and additional community benefits to participating microorganisms. To this

end, we used a high-throughput, reproducible assay to confirm whether or not isolated bacteria are capable of coordinating within a biofilm. We also used culture-based methods and performed incubations with multiple types of PAHs to determine if the isolated organisms can interact with PAHs of various size and ring number. Finally, we used a metabolic assay for the novel application of assessing the respiration capacity of the isolated PAH-degrading bacteria complexed within the biofilm conformation. We found that all of the organisms isolated were capable of forming a metabolically active biofilm. Many of these organisms demonstrated the ability to degrade phenanthrene and fluoranthene, but only a few showed the potential for degrading pyrene. These results confirmed that the isolated organisms from Republic site sediment can degrade PAHs and form a biofilm structure, which will be beneficial for their application to sediments.

The final aim of this work was to evaluate the use of an activated-carbon amendment technology (SediMite™) for the delivery of a bacterial consortium to PAH-contaminated sediment. While validated for use as a remediation technology and delivery strategy for organisms capable of degrading polychlorinated biphenyls (PCBs), this approach has not yet been tested for use with sediments contaminated with PAHs. We assembled reactors in a factorial design to determine the impact of the SediMite™ amendment and bacterial consortium on aqueous and sediment PAH concentrations of sediment derived from a reference site within the Elizabeth River. Reactors were prepared in quadruplicate and harvested at 1 month and 2 month timepoints. Chemical

degradation in whole sediments was only reliably observed at the high concentrations and followed expected trends with respect to the experimental parameters of SediMite™ and bacterial consortium inclusion. These results suggest that at high chemical concentrations, the joint application of SediMite™ and the bacterial consortium engineered in this dissertation may effectively reduce PAH concentrations in Republic Creosoting sediments.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants that accumulate in soils and sediment due to their physicochemical properties. In these environmental matrices, PAHs are predominantly transformed and degraded by the native fungal and bacterial communities. However, microbial degradation of PAHs is a slow process that requires engineered approaches to improve degradation rates and meet remediation criteria.

Engineered bioremediation approaches consist of altering the microbial community by either increasing cell concentrations of specific, targeted organisms or by introducing catabolic genes that confer a phenotype that can degrade the target contaminant. This approach is called bioaugmentation and is generally applied using the former strategy. Biostimulation is another method, which includes the addition of nutrients that may be limited to microorganisms and can help grow the indigenous microbial community and accelerate contaminant degradation. However, biostimulation is not a targeted approach and may stimulate the entire microbial community, not just organisms capable of degrading the target contaminant, therefore elevating microbial social dynamics that may result in the overall suppression of vital PAH-degraders.

Bioaugmentation of sediments is challenging due to constraints surrounding the longevity, stability, and delivery of microorganisms. To address the limitations of this remediation approach, the work within this dissertation outlines methods for

developing a consortium of PAH-degrading bacteria coordinated within a stable community, as well as a technology for delivering this consortium to creosote contaminated sediments.

This dissertation focuses on developing an engineered bioremediation approach for estuarine sediments contaminated with PAHs using indigenous microorganisms derived from an estuarine site in the Elizabeth River, VA. The overarching purpose of this project is to improve bioaugmentation strategies for sediment polluted with a complex mixture of PAHs.

1.1 Research Objectives

The specific research objectives of this dissertation are to:

1) Identify and isolate PAH-degrading bacteria from PAH-polluted sediments.

2) Engineer a metabolically-active, co-culture biofilm for use as a bioaugmentation strategy.

3) Evaluate the use of activated-carbon amendment for delivery of a PAH-degrading biofilm and PAH remediation.

These aims, described in greater detail below, will answer critical questions about indigenous bacteria that may be used in a bioaugmentation strategy for contaminated sediments at the Republic Creosoting site in the Elizabeth River, VA. This project will also address how bioremediation can be combined with physical treatments

to improve the rate of in situ PAH-degradation and the reintroduction of functionally relevant microorganisms.

1.2 Research Hypotheses and Approaches

The overarching purpose of this dissertation was to develop a bioaugmentation strategy for the remediation of creosote contaminated sediments at the Republic Creosoting (RC) site in the Elizabeth River, VA (ER). It was hypothesized that engineering a bacterial consortium coordinated within a biofilm would be a successful bioaugmentation approach considering the benefits the biofilm structure offers microorganisms. We further explored potential technologies for delivering this optimized biofilm to sediments and hypothesized that an activated-carbon based amendment would be effective as both a delivery mechanism as well as an inherent remediation solution for the heavily contaminated RC sediment.

The first objective was to identify and isolate PAH-degrading bacteria from creosote contaminated sediment at the RC site. It was hypothesized that the RC sediment may have a unique microbial signature due to the high concentrations of PAHs at the site. To determine differences in the sediment bacterial communities at ER sites with varying PAH contamination, sediment was collected from 4 sites and sequenced using the Illumina MiSeq platform targeting the V4 region of the 16S rRNA gene. It was further hypothesized that a successful bioaugmentation strategy for the RC site would utilize indigenous bacteria. To detect indigenous bacteria in RC sediments with the

ability to degrade PAHs prominent within creosote contamination, incubations with labeled isotopes were performed. To utilize PAH-degrading bacteria in a bioaugmentation strategy, culture-based methods were also employed. The combined use of these methods to describe the sediment bacterial community at ER sites and identify and isolate functional PAH-degrading bacteria resulted in the detection of 9 prominent PAH-degrading bacteria and the successful culturing and isolation of 6 of these bacteria. These 9 bacteria were shown to be present at a low abundance in RC site sediment, which suggests this site may be a good candidate for a bioaugmentation approach.

The second objective of this dissertation was to confirm the PAH-degrading capabilities of isolated bacteria and to coordinate these organisms into a biofilm structure, which provides protection and additional community benefits to participating microorganisms. The hypothesis driving this objective was that bacteria complexed within a biofilm structure will be easier to deploy to the field and may be more appropriately situated for degrading hydrophobic compounds, due to the surface-bound nature of a biofilm. The methods utilized to confirm biofilm formation potential demonstrated that all 6 isolated bacteria can form a biofilm. We further tested their ability to degrade multiple PAHs and be metabolically active when coordinated within the biofilm structure. To this end, we developed a novel assay for determining the respiratory capacity of each isolate in a biofilm structure.

The final aim of this work was to evaluate the use of an activated-carbon (AC) amendment for the delivery of a bacterial consortium to PAH-contaminated sediment. The hypothesis for this work was that the AC amendment would serve as a structural support for the PAH-degrading biofilm to develop and a stable particle on which to deliver bound organisms to contaminated sediments. Further, it is hypothesized that this technology could serve as a remediation strategy itself, due to the high sorption capacity of AC and its validated use for reducing the bioavailability of hydrophobic organic compounds. To evaluate these endpoints, we established reactors with and without the AC and the bacterial consortium. We measured both sediment and aqueous concentrations of PAHs to determine the efficacy of either treatment application.

2. Background and Literature Review

This chapter includes a presentation of scientific literature as it pertains to the physicochemical properties of polycyclic aromatic hydrocarbons and the remediation of this class of contaminants in sediment. Specific reviews relevant to each research objective will be available in subsequent chapters.

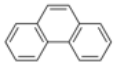
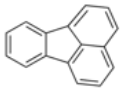
2.1 Polycyclic Aromatic Hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are primarily characterized by the formation and number (3+) of benzene rings[1,2] and are produced via processes that result in the incomplete combustion of organic matter. Volcanic activity and forest fires are examples of natural sources of large concentrations of PAHs. However, the largest emissions of PAHs come from anthropogenic activities such as fossil fuel burning in industrial processes and transportation[3,4]. PAHs are also byproducts of creosote production, which was a commonly used wood preservative until the late-1900's[5]. PAHs constitute approximately 85-90% of creosote by weight, making this matrix an especially potent source of PAH pollution[1,6].

Due to global emissions, PAHs are ubiquitous environmental pollutants that have been detected in every environmental matrix. While deposition of airborne particulate matter[7] and river runoff[8] are common sources of PAHs to soils and sediments, the intentional and unintentional dumping of PAH-containing material is the most prominent source of highly concentrated pollution to these environmental matrices.

The physicochemical properties of PAHs determine their environmental fate. PAHs generally exhibit low water solubility and extreme hydrophobicity, which results in their recalcitrance in sediment and soil[4]. PAH solubility is inversely proportional to the number of aromatic rings within the compound, so high molecular weight PAHs, such as benzo(a)pyrene, are usually strongly adsorbed to organic material (Table 1).

Table 1: Physicochemical properties of common polycyclic aromatic hydrocarbons. Data obtained from United States Environmental Protection Agency's CompTox Dashboard (2019, https://comptox.epa.gov/dashboard).

Chemical Name (CAS No.)	**Chemical structure**	**Molecular formula**	**Molecular weight** (g/mol)	**Log K_{ow}**	**Water Solubility** (mol/L)	**Henry's Law** (atm^*m^3/mol)
Naphthalene (91-20-3)		$C_{10}H_8$	128.2	3.16	5.04×10^{-4}	4.05×10^{-4}
Phenanthrene (85-01-8)		$C_{14}H_{10}$	178.2	4.33	3.18×10^{-6}	5.49×10^{-5}
Fluoranthene (206-44-0)		$C_{16}H_{10}$	202.3	4.88	4.52×10^{-7}	1.17×10^{-5}
Pyrene (129-00-0)		$C_{16}H_{10}$	202.3	4.84	5.48×10^{-7}	1.19×10^{-5}
Benzo(a)pyrene (50-32-8)		$C_{20}H_{12}$	252.3	5.92	3.54×10^{-8}	6.03×10^{-7}

Abiotic factors that affect the fate of PAHs in soil and sediment include adsorption, volatilization, photolysis, and chemical oxidation (Figure 1)[1,9]. However, microbial metabolism is the primary process by which PAHs are transformed or

degraded in the environment[1,9].

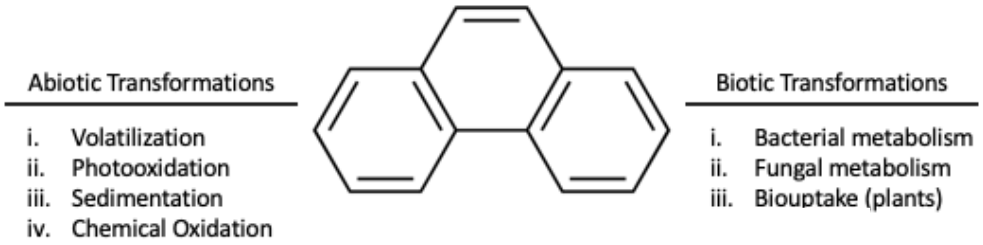


Figure 1: Schematic depicting potential abiotic and biotic transformations of various polycyclic aromatic hydrocarbons.
Currently depicted with phenanthrene. (Adapted from Cerniglia, 1992).

PAHs are concerning environmental pollutants because of research demonstrating carcinogenic, mutagenic, and teratogenic responses in different taxa[1,4,10–12]. Sixteen PAHs are included on EPA's list of priority pollutants due to their known carcinogenicity. Toxicity is positively correlated with increasing molecular weight and the number of benzene rings present within the compound [1,6,10]. PAHs are often distributed within environmental matrices as complex mixtures ranging from low to high molecular weight compounds; combinations of which have been shown to cause synergistic toxicity[13].

Given their persistence in the environment and propensity for causing negative human and ecological health effects, PAHs are an important class of contaminants to remediate from the environment.

2.2 Physical Remediation of PAHs in Soil and Sediment

Soil and sediment are environmental matrices that serve as sinks for large volumes and high concentrations of recalcitrant compounds. The longevity of PAH-contamination is influenced by the composition of the specific chemical mixture, as well as characteristics of the matrices themselves, namely organic matter content and oxygen concentration.

The most common method for eradicating PAHs from sediments is by removing the contaminated media via dredging, which is an extremely costly process[1,4,14]. Dredged material is either incinerated, sent to a landfill, or barricaded behind a contaminant structure, such as a dyke. Dredged material does have the potential to be treated ex situ, but it usually is relocated to an area that poses less risk to human populations. Other issues with dredging are that it can be difficult to reach contaminated material, which may be near obstacles such as private infrastructure. Most importantly, dredging can make otherwise sequestered hydrophobic compounds bioavailable to proximate organisms.

Capping the sediment surface is another common physical remediation strategy for sediment. Capping uses materials, usually sand or clean soil, to form a barrier between contaminated media and the water column. While this approach can be effective at containing material, it does not eliminate the risk of later exposure[14]. Further, sediment caps have been shown to be compromised over time due to the advection of contaminated groundwater and porewater flux[15].

While these two physical remediation strategies are most commonly utilized at sites with extensive chemical pollution, there do exist other treatment technologies that do not require the physical removal or isolation of contaminated material and may be more cost effective. One such alternative treatment strategy is called bioremediation.

2.3 Bioremediation

Bioremediation of chemical pollution is an engineered approach that manipulates the innate chemical degradation capabilities of microorganisms and can be applied in situ or ex situ. Optimizing the use of indigenous bacteria and fungi for PAH degradation is of practical importance because of their important role in the transformation and environmental fate of PAHs. Additionally, bioremediation is a cheaper remediation option than many physical approaches[16], including dredging and capping.

Bioremediation may be achieved through multiple applications. Monitored natural attenuation is the most passive approach, whereby remediation managers monitor environmental conditions and pollution levels without altering the microbial community or environmental conditions. Biostimulation enhances the natural degradation capabilities of microorganisms by adding rate-limiting nutrients (i.e. nitrogen), electron donors (i.e. carbon), or acceptors (i.e. oxygen) to the environment[17].

Bioaugmentation is the practice of modifying the innate microbial community. This may involve increasing the cell concentration of specific microorganisms of interest,

which may not be naturally available within the community and can be introduced from an alternative source. However, the most successful applications of bioaugmentation have focused on autochthonous microorganisms, or those that are already present at the focus site, because they are acclimated to chemical concentrations and other important environmental conditions (pH, nutrient availability, oxygen availability, water saturation, etc.)[18–20]. Autochthonous organisms may be derived from environmental media, modified or researched in laboratory settings, and reintroduced to the site.

An alternative form of bioaugmentation is genetic bioaugmentation, which involves genetic manipulation of bacteria, usually by inducing horizontal gene transfer events with the purpose of proliferating mobile genetic elements (i.e. plasmids) that harbor genes conferring for a specialized phenotype[21]. For this strategy to be successful, organisms must remain in the presence of the selection pressure (i.e. contaminant exposure) in order to retain the inserted genetic element. If removed from these conditions, the energetic costs of retaining this genetic addition will outweigh the benefits of expressing that phenotype. Ikuma and Gunsch[22] demonstrated the successful genetic bioaugmentation of *Pseudomonas putida* for toluene degradation from a soil matrix by measuring a negative correlation between conjugated gene copy numbers and toluene concentrations.

In 2001, the United States Environmental Protection Agency reported the use of bioremediation at 104 Superfund sites, with 30% of those sites hosting contamination from wood preservation or petroleum refinery processes, where PAHs were the

predominant class of chemical contaminants[17]. At the time this report was released, 7 sites where ex situ bioremediation strategies (land treatment, slurry-phase bioremediation, and composting) were applied, met remediation targets (usually ~90% reduction).

While microorganisms are particularly effective at degrading recalcitrant compounds, bioremediation is also a limited technology. Degradation rates are dependent on chemical bioavailability, microbial community diversity and distribution, as well as a number of other abiotic factors, which are usually not well understood and need to be assessed on a site-by-site basis. These reasons make bioremediation a riskier technology selection for remediation project managers who need to quickly and efficiently remove large volumes of contaminated material. Strategies to perform precision bioremediation, which involves characterizing the inherent microbial community and site-specific influences on community dynamics, have been previously explored[23] and is a developing field of practice.

The work presented in subsequent chapters of this dissertation focuses on developing a targeted bioremediation strategy for sediment PAH contamination using bacteria assembled in a biofilm structure.

2.3.1 Bacteria

Chemical remediation is largely concerned with detoxifying chemical pollution at sites in order to remove the risks to ecological and human health. Degradation of PAHs

by microorganisms is a natural process that can be manipulated as a remediation strategy. While researchers have identified many genera of fungi and bacteria capable of catabolically transforming PAHs, bacteria are of particular utility because of their ability to completely transform a complex mixture of PAHs to non-toxic byproducts such as carbon dioxide and water.

This process, termed mineralization, occurs with PAHs acting as an electron donor and source of carbon for the eventual production of microbial biomass. PAHs are catabolized intracellularly with the initial cleavage of aromatic rings by dioxygenase enzymes[1,24]. Metabolites proceed through various oxidation steps, forming *cis*-dihydrodiol and catechol as intermediate compounds[3] (Figure 2). While anaerobic degradation of PAHs is possible, aerobic degradation, utilizing oxygen as a terminal electron acceptor, is a much faster and efficient mechanism for PAH biotransformation[25].

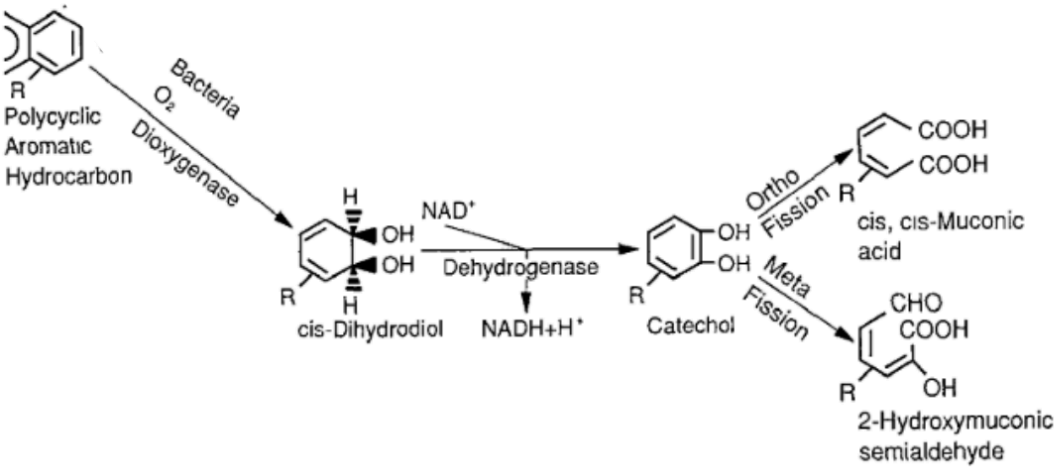


Figure 2: Schematic detailing possible pathways of aerobic bacterial PAH catabolism.
(Adapted from Cerniglia, 1992)

While the rate and extent of transformation of PAHs is largely dependent on the number of rings within the compound, there are a wide variety of bacteria that can catabolize low and high molecular weight PAHs[1]. Such bacteria belong to diverse genera, notably *Pseudomonas*[26,27]*, Sphingomonas*[3,26,28]*, Mycobacterium*[1,20,27,29]*,* and *Alcaligenes*[1,30,31]*.* Research has shown that microbial diversity and abundance is shifted in the presence of chemical pollution and some known PAH-degrading microorganisms may be detected at a higher relative abundance in contaminated sediment than in clean sediment[23].

Generally, bacteria are able to degrade a greater number of low molecular weight PAHs, if they can degrade three- or four-ring compounds[32]. While there are no reported cases of bacteria that can utilize high molecular weight PAHs, such as

benzo[a]pyrene (BaP), as their sole carbon and energy source, the degradation of BaP in the presence of another carbon source, or through cometabolic processes[32], has been demonstrated.

Aside from these inherent catabolic capabilities, bacteria exhibit novel ways of interacting with one another and their environment. Some of these strategies for survival can be beneficial in the context of bioremediation.

2.4 Biofilms

Most of the research on microorganisms has focused on monocultures in a planktonic, or free-floating, cellular state. However, the majority of bacterial life exists in a sessile state[33–37]. This existence strategy is called a biofilm, which is defined as a heterogeneous structure comprised of single or multi-species microorganisms enclosed in a matrix of polymeric material that allows for surface attachment[33,36]. This lifestyle offers protection against dehydration and predation, facilitates enzyme activity and cell signaling, and allows for the sharing of nutrients, resources, and genetic information[35,38–40]. These benefits are largely due to the bacterial secretion of an extracellular matrix (EPS) comprised of water (97%)[33], carbohydrates, proteins, polysaccharides, and other polymers[33,41]. When observing the composition of the biofilm in its entirety, it is comprised of 75-90% EPS matrix and 10-25% cells[36].

The biofilm lifecycle is comprised of three distinct phases: attachment, growth, and detachment/dispersion (Figure 3). Surface attachment is thought to occur through a

build-up of organic material on a surface, and the release of low molecular weight signaling molecules that allow cells to interpret a change in signal diffusion, indicating proximity to a surface[36]. Initial attachment is influenced by the charge and hydrophobicity of the surface, as well as the surface area[33]. After biofilm maturation, cells are released back into the environment via desorption, detachment, or dispersion. Desorption involves the direct transfer of organisms to surrounding environmental media; detachment is caused by external forces like predation, erosion, or scraping; and dispersion is an active process in which regulatory systems enable the release of cells from the biofilm[33].

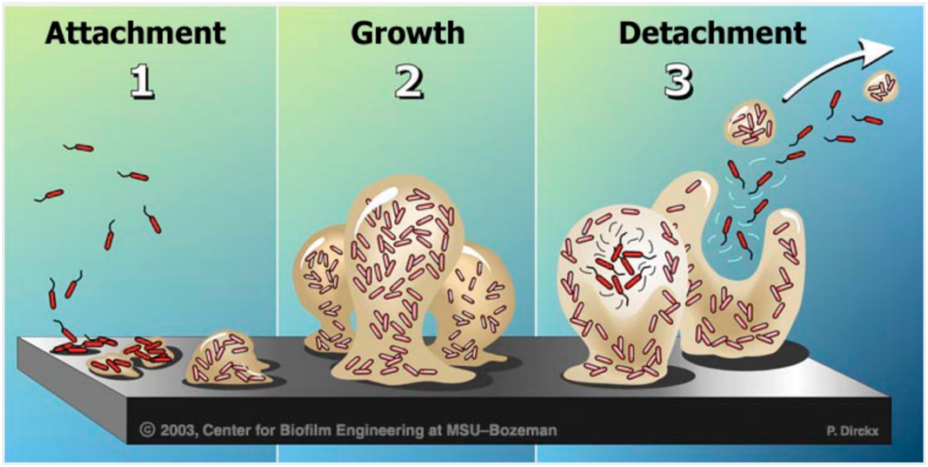


Figure 3: Visual representation of the attachment, growth, and detachment of bacterial biofilm.
Graphic produced by the Center for Biofilm Engineering at MSU-Bozeman, 2003 (http://www.biofilm.montana.edu/biofilm-basics/what_are_biofilms.html).

The biofilm structure is of interest for engineered bioremediation purposes because the surface adherent nature may be advantageous for degrading hydrophobic

compounds that tend to be adsorbed to organic surfaces in sediment environments[10,24]. This strategy is expected to reduce mass transfer limitations common with weakly soluble PAHs to microorganisms. Further, it has been shown that cells are more biologically stable when they exist in an immobilized state[20], which is beneficial for bioaugmentation approaches given that cell density is a large contributor to the success of a technology.

This dissertation will address the potential for PAH-degrading microorganisms to assemble within a biofilm structure and will present a strategic approach to engineering a mixed-microbial biofilm to use as a bioaugmentation strategy.

2.4.1 Mixed-community biofilm

The ultimate goal of bioremediation is to completely mineralize chemical contaminants to inert products, but this can be challenging to achieve when faced with environmental matrices that generally retain complex mixtures of either single or multiple classes of contaminants. This complex chemical signature is unlikely to be removed by singular bacterial genera[20,42].

While many bacteria have adapted mechanisms to utilize PAHs as a source of carbon and energy, their ability to catabolize PAHs of varying ring orientation or number may be limited[1]. However, PAHs generally exist within the environment as a complex mixture of low, medium, and high molecular weight compounds, so it is likely that a consortium of bacteria with diverse catabolic requirements, specifically pertaining to

the degradation of PAHs with different ring numbers and orientation is required in order to have an effective bioremediation strategy that results in chemical mineralization[43].

One study using a consortium of bacteria derived from an enrichment of oil-contaminated sediment, found that isolated bacterial strains were unable to degrade pyrene without the presence of the other bacteria in the consortium, suggesting that either the unculturable bacteria were the primary degraders of pyrene or that the consortium provided catabolic stability[44]. Zafra et al. developed a highly successful mixed-community consortium that achieved 87% of phenanthrene, 48% of pyrene, and 57% of benzo[a]pyrene removal after 14 days of exposure to a 6000 mg/L mixture of the PAHs[45]. That study also demonstrated significantly greater PAH degradation from the consortium than the organisms in isolation.

These studies broadly demonstrate that a consortium of microorganisms may be required in order to address complex chemical contamination in an efficient, effective, and rapid manner.

Further, it has been demonstrated that the biofilm structure, hosting co-communities of bacteria, exhibits distinct characteristics from single-species counterparts[46]. These structures allow for microbial interactions that are not possible while existing singularly or when spatially distant[37]. In the context of PAH-degradation, it has been demonstrated that the occurrence of horizontal gene transfer events increases within a biofilm, compared to planktonic cells[47]. This phenomenon is of interest in bioremediation because many genes required for chemical degradation are located on

mobile genetic elements, such as plasmids, which are transferrable between microorganisms, resulting in the proliferation of a chemical-degrading phenotype.

This dissertation outlines a strategic approach to engineering a mixed-community biofilm optimized for PAH-degradation. Subsequent chapters demonstrate how a biofilm of PAH-degrading bacteria can be developed, applied for PAH bioremediation, and delivered to sediment.

3. Identifying PAH-degrading microorganisms in creosote-contaminated sediment using stable-isotope probes and culture-based approaches.

3.1 Introduction

The Elizabeth River (ER) (Virginia, USA) serves as a model ecosystem for understanding the long-term impacts of polycyclic aromatic hydrocarbon (PAH) contamination[5]. PAHs represent a class of environmental contaminants resulting from the incomplete combustion of organic materials such as wood and fossil fuels. PAH contamination in the ER is largely due to the extensive industrialization of this estuary combined with the improper disposal and storage of coal-tar creosote, which was used extensively during the 1900's as a wood preservative and was produced by multiple manufacturing facilities along the ER. PAHs constitute approximately 85-90% of creosote by weight, making this matrix a potent source of PAH pollution[1,6].

Microbial transformations are the primary processes by which PAHs are degraded or removed from the environment[48,49]. Bacteria of various genera have been reported to degrade multiple PAHs of various ring number and conformation[1], which suggests that enhancing the abundance of these specialized organisms is a reasonable strategy for promoting bioremediation of a complex chemical mixture such as creosote. Bioaugmentation is a specific application of bioremediation in which microbial strains are added to an environment to increase cell concentrations and accelerate contaminant degradation[50]. Promising bacterial candidates for bioaugmentation of

creosote contaminated sites are those that are indigenous to the site, able to degrade multiple PAHs, and can be reliably grown in laboratory conditions.

Identifying relevant PAH-degrading organisms within microbial communities is one of the first steps towards understanding systemic applications of bioaugmentation. In the present work, we used stable isotope probing (SIP-DNA) to identify bacterial degraders of prominent PAHs at creosote contaminated sites, which is a method that allows for the detection of organisms that may not be identified through traditional culture-based approaches. Culturing these or other functional organisms is the next step towards an effective bioaugmentation strategy, which requires increasing cell concentrations and developing an appropriate delivery method.

As part of an engineering scheme to remediate PAH-contaminated sites, this study employed methods to target both uncultivatable and cultivatable bacteria with the ability to degrade PAHs. We utilized stable-isotope probes to identify uncultivated bacteria in environmentally-relevant PAH-contaminated sediments derived from a former creosoting site. In parallel, we employed traditional culture-based approaches to grow and isolate PAH-degrading bacteria. We further elucidated the prevalence of these functionally relevant organisms within the overall bacterial community through microbiome analyses of multiple sites in the Elizabeth River.

3.2 Material and Methods

3.2.1 Chemicals

Phenanthrene (98%), pyrene (98%) and fluoranthene (98%) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Uniformly ^{13}C-labeled versions of phenanthrene and fluoranthene were synthesized previously[51,52] and were a gift of Dr. Michael Aitken (University of North Carolina-Chapel Hill). Pyruvate and acetate were from Sigma-Aldrich (St. Louis, MO, USA). All HPLC solutions were HPLC grade.

3.2.2 Sample Site and Collection

For microbiome analyses, sediments were collected from previously described sites featuring low (King's Creek [KC] & Mains Creek [MC]), moderate (Scuffletown Creek [SC]), and high (Republic Creosoting [RC]) levels of PAH contamination[11,53]. For SIP experiments, a total of 1 kg of sediment was freshly collected from the top 8 cm of five sampling points separated by ~10 m from each other and the shoreline at the RC site. Sediments from each of the five RC locations were then mixed in roughly equal proportion and sieved sequentially through #12 (1.7 mm) and #20 (0.841 mm) metal meshes to remove rocks, shells, leaves, and other debris. RC sediment for bacterial isolations was separately collected at three locations separated by ~3 m, each with visible creosote sheen on the water's surface. Each of these sites was subsampled in triplicate using a sterile soil corer to a depth of ~5 cm, stored in 15 mL Falcon tubes, and transported on ice. When not actively in use, all sediment samples were stored at 4˚C.

3.2.3 Enrichment and Isolation of PAH-Degrading Bacteria From Sediments

Enrichments of PAH-degrading bacteria were performed in medium SRB_{15}, which consisted of the previously described medium sRB2[54] with 1.5% artificial seawater (Instant Ocean, Cincinnati, OH) to mimic Elizabeth River estuarine conditions. RC sediment (~1 g wet weight) was added to triplicate sterile 250mL flasks containing 30 mL of sRB_{15} amended with either crystalline phenanthrene, fluoranthene, or pyrene in quantities exceeding aqueous solubility, and placed on an orbital shaker at ~60 rpm. After 3 weeks, flasks with turbidity or biofilm formation were serially diluted in sterile PBS and plated on sRB_{15} (with no additional carbon source or amended with either 2% acetate or pyruvate) containing 2% agar (Becton, Dickinson and Company, Sparks, MD). After drying 2-3 hours to ensure culture establishment, the plates were sprayed with one of the target PAHs dissolved in acetone[55]. Plates were incubated at 25˚C and monitored daily for zones of clearance indicating PAH transformation. Putative PAH-degrading colonies were struck for isolation and confirmed via respraying with the associated PAH. Strains were maintained on sRB_{15} containing pyruvate and stored at -80˚C in 15% glycerol.

3.2.4 PAH Disappearance From Sediments

To determine the rate of PAH disappearance from biostimulated RC sediments for SIP, 100 µg of either phenanthrene or fluoranthene were added in acetone to separate, replicate sterile, glass tubes and the solvent allowed to evaporate. To each

tube was added 0.1 g (dry weight) RC sediment was added in 1 mL of 1.5% Artificial Seawater containing 1 mM Na-K phosphate (pH 7.0) and 1 mM NH_4NO_3. Tubes were shaken at room temperature (~23℃) at 100 rpm for up to 9 days. PAHs were extracted from duplicate sacrificed tubes for each PAH on days 0, 2, 4, 7 and 9 in 5 mL of hexane (VWR; Radnor, PA). After filtering through a 2 µm nylon filter and diluting in 50:50 acetonitrile and water, extracts were quantified using an Agilent/Varian ProStar Modular High-Performance Liquid Chromatography (HPLC) System equipped with three ProStar Solvent Delivery Modules, a 410 autosampler, a 335 UV-detector, a 363 fluorescence detector, and a Supelcosil™ LC-PAH HPLC column (10cm x 4mm, 3µm particle size; Supelco). The program consisted of initial conditions of a mobile phase of 50:50 acetonitrile:water for 5 min followed by a linear gradient to 100% acetonitrile at 25 min with a return to initial conditions after 30 min. PAH concentrations were determined using fluorescence at various analysis windows corresponding to absorption and emission for individual PAHs and five-point standard curves were generated from either a Supelco SS EPA 610 PAH mix (Sigma-Aldrich; St. Louis, MO) or stock solutions of individual PAHs.

3.2.5 Stable-Isotope Probing

Incubations for SIP were set up identically to those for determining compound disappearance as described above, except that duplicate tubes contained either unlabeled phenanthrene or fluoranthene, or [U-^{13}C] phenanthrene or [U-^{13}C]

fluoranthene. Phenanthrene- and fluoranthene-containing tubes were incubated for 4 and 9 days, respectively, before DNA was extracted from the entire sample using either a PowerSoil® DNA Extraction Kit (MoBio, Carlsbad, CA, US; for phenanthrene) or FastDNA Spin Kit for Soils (MP Biomedicals; Solon, OH; for fluoranthene) as per manufacturers' instructions. DNA was also extracted from unlabeled samples sacrificed at regular intervals during the incubation and stored at -20˚C. DNA was separated by density centrifugation using CsCl suspended in TE Buffer (pH 8.0) with no intercalating dyes with a final refractive index (RI) of ~1.403 (density of ~1.74 g/cm^3) as measured by an AR200 Digital Refractometer (Reichert; Buffalo, NY, USA). RI values were acquired using 5 µL of solution on the refractometer, whose effective measurement surface had been artificially reduced in diameter through the application of a custom-cut vinyl sticker. Ultracentrifugation occurred in 6 mL Polyallomer Quick-Seal Centrifuge tubes using a TV-1665 vertical rotor (Sorvall) at 40,000 rpm for 48 hours at 20℃. DNA was recovered after centrifugation from the bottom of tubes by displacement with water and the assistance of a custom 3D-printed tube holder and syringe pump. A total of 24 fractions of ~250 µL each were collected for each tube. After taking a 5 µL sample for measuring RI, the remaining DNA was purified of CsCl by applying the remaining fraction to an EconoSpin™ Spin Column for DNA, centrifuging for 1 min @ 10,000 rpm, washing 2× with 0.5 mL of wash buffer (60% ethanol, 10 mM Tris·Cl [pH 8.0], 1 mM EDTA, 0.1 M NaCl) centrifuging for 1 minute at 10,000 rpm each time while discarding the flow through, spinning an additional time to remove residual ethanol, and eluting the DNA in

2× 50 µL volumes of TE buffer (pH 8.0) through centrifugation. DNA from each fraction was quantified using a NanoDrop 3300 fluorospectrometer (NanoDrop Products; Wilmington, DE, USA) with a Quant-iT™ PicoGreen™ dsDNA assay kit (Thermo Fisher; Waltham, MA, USA).

3.2.6 Identification of 16S rRNA Gene Sequences

PCR of 16S rRNA genes from SIP-derived DNA and isolated organisms was performed using primers 8F[56] and 1492R[57]. Clone libraries of 16S rRNA genes were constructed using the TOPO® TA Cloning® Kit with PCR®2.1 TOPO® (Thermo Fisher; Waltham, MA, USA). Sequencing was performed at the DNA Analysis Facility, Duke University (Durham, NC, USA) using PCR primers or primer sites within the vector and the assembled sequences were identified using Blastn searches of the GenBank nr database[58].

3.2.7 Sediment Microbiome Sequencing & Data Analysis

ER sediments from KC, MC, SC, and RC sites were collected in triplicate subsamples from three locations separated by ~5 m (n=9 total per site). Analysis of sediment bacterial communities were performed using DNA from ~0.12 g (dry weight) of ER site sediment extracted using the PowerSoil DNA isolation kit (Qiagen; Hilden, Germany). To increase DNA yield, samples were heated for 10 m at 65˚C prior to vortexing at step 2 of the manufacturer's protocol and DNA was collected 5 min after addition of the elution buffer to the spin filter. Partial 16S rRNA gene sequences targeting the V4 region of the 16S rRNA gene[59] were obtained using the Illumina MiSeq

platform with 2×250 bp chemistry at the Duke University Sequencing and Genomic Technologies Shared Resource Center (Durham, NC, USA). Mothur[60] was used to demultiplex forward and reverse FASTQ files (Version 1.41.0). DADA2[61] (version 1.6.0) was used to analyze demultiplexed amplicon libraries. The 16S rRNA reference library used during analyses was developed by SILVA[62] (version 132). Packages used for analyses included "phyloseq"[63], "dplyr", and "stringr". Phyloseq objects for each ER site produced from the original pipeline, were filtered to remove amplicon sequence variants (ASVs) with counts above 0.

3.3 Results

3.3.1 16S rRNA Amplicon Sequencing of Elizabeth River Sediment

Amplicon sequencing of partial 16S rRNA bacterial genes was performed to investigate the bacterial community present in sediment at each of the ER sites. Amplicon sequence variants (ASV) categorized by the DADA2 pipeline resulted in the detection of 5,945 ASVs at KC, 6,878 ASVs at MC, 5,355 ASVs at SC, and 6,052 ASVs at RC. We investigated these ASVs within and between sites to determine how many unique ASVs were present in the sediments. KC, the reference site outside of the ER tributary, had 1,919 unique ASVs and the heavily PAH-contaminated RC site, had 1,795 unique ASVs. MC and SC had no unique ASVs, suggesting that all of their ASVs were represented in the other site sediment communities, as well.

To determine if PAH-contamination could be influencing sediment microbial communities, a comparison between the ASVs between sites was performed.

RC and SC, sites with high and moderate PAH-contamination respectively, shared 2,007 ASVs, however all of those ASVs were also detected in MC. Of those 2,007 ASVs, only 975 ASVs were unique to RC and SC when compared with KC sediment. This suggests that sites within the ER tributary may have a unique bacterial signature, regardless of PAH-contamination.

3.3.2 16S rRNA Gene Clone Libraries

DNA extracted from SIP fractions were combined based on measured peaks of "heavy" (comprising ^{13}C isotopically-enriched DNA) or "light" DNA (Figures 4 and 5) and 16S rRNA gene clone libraries were developed for both DNA samples. Approximately 24 clones per labeled compound were sequenced. Duplicate clone libraries of each compound were similar to one another in sequence composition (Table 2).

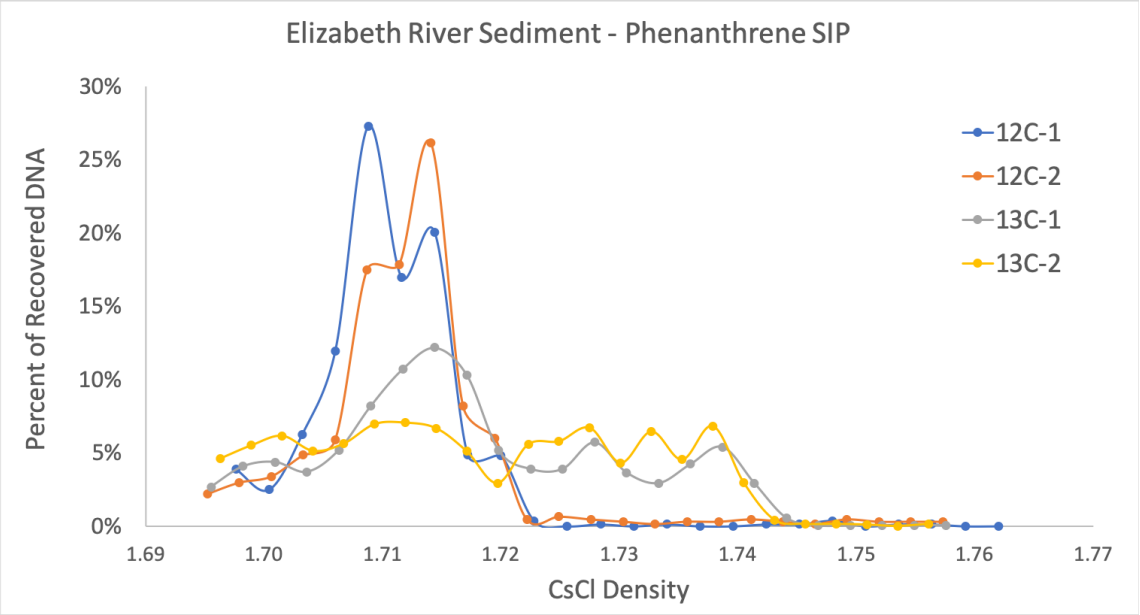


Figure 4: DNA concentrations in 12C- and 13C-labelled phenanthrene fractions of DNA-SIP incubations.
DNA fractions were in the CsCl density range of 1.73-1.745 and light fractions were between 1.7 and 1.72.

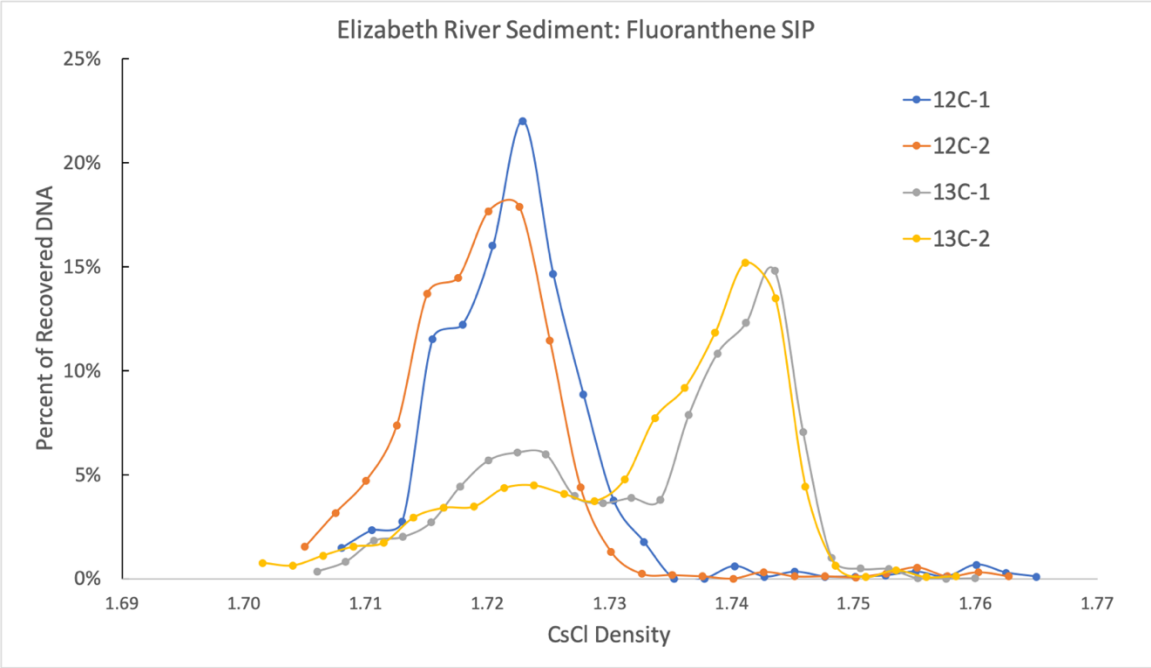


Figure 5: DNA concentrations in 12C- and 13C-labelled fluoranthene fractions of DNA-SIP incubations.
Heavy DNA fractions were in the CsCl density range of 1.73-1.745 and light fractions were between 1.7 and 1.72.

Sequences from the [U-^{13}C] phenanthrene and unlabeled phenanthrene clone libraries shared high similarity with sequences in the *Azoarcus* genus and the *Hydrogenophaga* genus. Sequences from the [U-^{13}C] fluoranthene and unlabeled fluoranthene clone libraries shared high similarity with sequences in the *Croceicoccus* genus.

Table 2: Summary statistics of sequence alignments for clone library replicated from DNA-SIP incubations with [U-^{13}C]phenanthrene and [U-^{13}C]fluoranthene.

[1]Clone library sequence alignments, as determined by the Basic Local Alignment Search Tool (BLAST) (U.S. National Center for Biotechnology Information). Alignments considered to be accurate at genus phylogenetic level.

[2]Total number of clones with alignment to the respective genus.

[3]Percentages were derived using the total number of clones that yielded quality sequences that could be utilized for reference library alignment. Some clones did not yield a significant reference in the database and were excluded from analysis, therefore category totals may not add up to 100% representation of sequenced clone library. Fluoranthene ^{13}C-1 yielded 14 total clones. Fluoranthene ^{13}C-2 yielded 12 total clones. Phenanthrene ^{13}C-1 yielded 18 total clones. Phenanthrene ^{13}C-2 yielded 12 total clones.

PAH	Isotope	Clone Sequence Alignment[1] (Accession No.; Percent Identity)	Total number[2]	Percent of clones[3]
Fluoranthene	^{13}C-1	*Croceicoccus* sp. GM-16 (MH445510.1; 99%)	3	20%
		Croceicoccus sp. D26 (MK100384.1; 99%)	6	40%
		Erythrobacteraceae bacterium LSUCC0212 (KU382408.1; 100%)	1	6%
		Roseovarius sp. (KT185148.1; 100%)	2	12%
		Sphingopyxis sp. (MG099630.1; 99%)	2	12%
		Sphingomonas sp. PQ-4 (MK100384.1; 99%)	1	6%
Fluoranthene	^{13}C-2	*Croceicoccus* sp. (MK100384.1; 99%) Or *Sphingomonas* sp. (KF145129.1; 99%)	7	58%
		Hoeflea sp. (MH929779.1; 95%) (MH929770.1; 95%)	1	8%
		Porphyrobacter sp. (CP016033.1; 98%)	1	8%
		Uncultured bacterium clone (KM851935.1; 97%)	1	8%
Phenanthrene	^{13}C-1	*Azoarcus* sp. (CP022188.1; 100%)	13	72%
		Hydrogenophaga taeniospiralis (AB795550.1; 99%)	2	11%
		Pseudomonas syringae (MK388374.1; 100%)	1	5%
		Uncultured *Comamonas* sp. (JQ860931.1; 93%)	1	5%
Phenanthrene	^{13}C-2	*Azoarcus* sp. (CP022188.1; 100%)	5	42%
		Hydrogenophaga taeniospiralis (AB795550.1; 100%)	4	33%
		Hydrogenophaga cassostreae (CP017476.1; 90%)	1	8%

Amplicon data were also used to determine the relative abundance of the PAH-degrading bacteria detected from the DNA-SIP clones and bacterial isolates in Republic sediment. Generally, PAH-degrading bacteria did not represent a large proportion of bacteria present in ER site sediments (Appendix A, Table 6). The most often detected organism was *Hydrogenophaga* sp., which was found in 5/9 RC samples, 4/9 SC samples, 3/9 KC samples, and 6/9 MC samples. *Croceicoccus* sp. were identified in nearly equal numbers of sediment samples across the sites, with SC and MC samples hosting this organism with the highest frequency in 3/9 samples. *Azoarcus* sp. was represented in 3/9 SC, KC, and MC samples and 2/9 RC samples. Almost all of the species comprised far less than 1% of the overall relative abundance in the samples, if they were present at all. Most of the samples across all four sites that contained at least one organism, contained all three (Appendix A, Table 6). The highest relative abundance in a single sample was *Azoarcus* sp. which comprised 0.02% of one RC sample.

3.3.3 Culture Based Microbial Isolations

Of the 67 bacterial isolates obtained on media with pyrene, fluoranthene, or phenanthrene, seven isolates were unique (Table 3). The majority of isolates obtained from the fluoranthene plates were phylogenetically identified as *Novosphingobium pentaromativorans.* There was a similar lack of diversity in the isolates obtained from the phenanthrene-sprayed plates.

Table 3: Summary statistics of bacteria isolated from PAH-enriched creosote contaminated sediment from the former Republic Creosoting Co. site (Elizabeth River, VA).

Note that bacteria were not isolated from pyrene enriched sediment, despite incubations.

[1]Organism sequence alignments, as determined by the Basic Local Alignment Search Tool (BLAST) (U.S. National Center for Biotechnology Information). Alignment labelled "other" represents unspecific alignment to multiple genera.

[2]Total number of organisms with alignment to the respective genus.

[3]Percentages were derived using the total number of isolated organisms that yielded quality sequences that could be utilized for reference library alignment. Some isolated organisms did not yield a significant reference in the database and were excluded from analysis, therefore category totals may not add up to 100% representation of sequenced clone library. Seventeen apparently unique isolates from phenanthrene treatments were sequenced. Forty-three apparently unique isolates from fluoranthene treatments were sequenced. Pyrene treatments did not yield individual isolated organisms viable for sequencing.

PAH	Isolate Sequence Alignment[1] (Accession No.; Percent Identity)	Total number[2]	Percent of isolates[3]
Phenanthrene	*Hydrogenophaga taeniospiralis* (MH259966.1; 99%)	6	35%
	Sphingobium RA C03 (CP016456.1; 99%)	4	24%
	Novosphingobium indicum (NR_044277.1; 100%)	2	12%
	Sphingobium sp. NBRC (AB681371.1; 100%)	2	12%
Fluoranthene	*Novosphingobium pentaromativorans* (KY511052.1, CP009291.1; 98%)	23	54%
	Stenotrophomonas sp. (KY996876.1; 99%)	1	2%
	Alcaligenes faecalis (MK583955.1; 96%)	1	2%

3.4 Discussion

3.4.1 Amplicon Sequencing Library

While utilizing microbial community sequencing technologies has been suggested as a primary method for identifying target bacteria for bioaugmentation

treatment strategies[23], this approach largely neglects important aspects required for the biodegradation of PAHs, namely organism viability and functionality. Further, while next-generation sequencing analyses may be best used as a tool for generally exploring trends within or between the microbial communities, they may not detect critically functional bacteria performing PAH-degradation at contaminated sites. This was demonstrated in this work, wherein bacteria identified in the DNA-SIP enrichments from RC sediment were not detected in the majority of sediment samples sequenced. However, it is worth noting that often minor members of microbial communities play key roles in the degradation of xenobiotic contaminants.

This work further explored how PAH-contamination may impact the sediment microbial community at sites within the ER tributary and one reference site. Based on the sequence alignment and amplicon library search with clones produced from the DNA-SIP incubations, there did not appear to be an enrichment of the PAH-degrading bacteria identified in this work at sites with PAH-contamination. While 2,007 ASVs overlapped in the heavily contaminated RC and SC site sediments, these ASVs were also detected in the uncontaminated MC sediments, indicating that these organisms were not influenced primarily by PAH concentrations. Further, over 900 of these ASVs were also in KC sites, signifying that many of those ASVs are not specific to the ER tributary, either. These data suggest that for the sediments collected for this work from the RC site, known PAH-degrading organisms are not enriched at the site, suggesting that either abiotic or nutrient conditions are not ideal for them to thrive.

In a further analysis of the 20 most abundant bacteria within the sediment amplicon libraries (Table 6), there were not obvious shifts in the abundance of the bacterial microbiome towards PAH-degrading organisms at the RC site. Within the most abundant bacteria at RC were present *Pseudomonas* sp., *Sphingomicrobium* sp., and *Polycyclovorans*, but these organisms were not represented in large percentages within every sediment sample collected within the RC site and they were also detected in other ER site sediments. This finding suggest that PAH-degraders may be widespread through the Elizabeth River system and dispute previous work identifying diverse PAH-degrading bacteria[29] existing in significantly greater abundance at the RC site than at other ER sites[23]. However, as noted previously, it is likely that these organisms could thrive under improved environmental conditions that stimulate microbial activity.

3.4.2 Functional PAH-Degrading Bacteria

The results obtained in this study demonstrate that both culture-based and culture-independent methods must be employed in order to obtain a more accurate representation of bacterial candidates for use in a bioaugmentation approach. While DNA-SIP incubations revealed three prominent bacteria capable of degrading phenanthrene and fluoranthene, we were further able to isolate and identify six additional PAH-degrading bacteria from RC sediment. In this work with culture-based isolation methods, we successfully isolated one of the target organisms, *Hydrogenophaga taeniospiralis*, identified in the DNA-SIP incubations. A percent identity

matrix based on sequence alignment (Appendix A, Table 7) indicates the genetic similarities between the *Hydrogenophaga taeniospiralis* clones and cultures which confirms the close relatedness of the isolated organism and that enriched in the DNA-SIP incubations. This further validates the functionality and prevalence of this organism at the RC site.

Since autochthonous bacteria are preferred organisms to use for bioaugmentation, culturing relevant organisms for the target contaminant is of vital importance. There was minimal diversity in the bacteria isolated from RC sediment using the methods employed, with the majority of isolates from fluoranthene-sprayed agar plates aligning to the *Novosphingobium pentaromativorans* taxonomy. This organism was previously shown to degrade HMW PAHs of environmental concern, including benzo[a]pyrene[64,65]. Also isolated was *Novosphingobium indicum*, which has been reported to utilize both fluoranthene and phenanthrene as its sole sources of carbon and energy[66], as seen in this work. Furthermore, this organism has been isolated from Elizabeth River sediments in previous work exploring bioremediation of PAH-contaminated media[29]. Members of the *Sphingomonads* have been previously identified to degrade PAHs of various conformations[37,67,68], so it is unsurprising that these organisms were also isolated from our samples.

Alcaligenes faecalis has been previously isolated in phenanthrene-enriched conditions[1,31,69], but fewer studies report it as an organism capable of degrading fluoranthene[70]. Under our laboratory conditions, *A.faecalis* showed potential as a

degrader of pyrene and other HMW PAHs, although further experimentation will be required to determine this capability.

Hydrogenophaga taeniospiralis has been previously shown to degrade benzene[71], but has been notably identified in river sediment with PCB contamination[72] and is considered to be a psychrotroph (surviving in cold temperatures) capable of utilizing biphenyls as a sole carbon and energy source[73]. Our work confirms that the strains isolated from ER with similarity to *Hydrogenophaga taeniospiralis* actively degrade phenanthrene, as determined by the biomass incorporation of carbon-labeled phenanthrene.

Stenotrophomonas sp. have been previously isolated from soil and has been shown to utilize pyrene as a sole carbon and energy source[74]. *Stenotrophomonas* sp. have also been found to significantly degrade phenanthrene, pyrene, and HMW PAHs such as benzo[a]pyrene[74–76].

One limitation of this study was the method employed for isolating PAH-degrading bacteria. Metagenomic analyses of Republic sediment did not indicate a significant abundance of *Sphingomonas* sp., however our method of enriching sediment with select PAHs resulted in the isolation of viable organisms belonging to this taxonomic group. This is interesting because previous reports indicate that PAH enrichment commonly selects for bacteria belonging to the *Alcaligenes, Pseudomonas,* and *Sphinogmonas* genera[37]. It is possible that in producing stressful conditions, enrichment with the chosen PAHs significantly reduced microbial diversity. Further, we

utilized one type of medium at a single salinity using only three PAHs and two additional carbon sources, which may have further selected for organisms capable of competing under such nutrient conditions.

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4. Engineering an Optimized Consortium of PAH-Degrading Bacteria in a Biofilm Structure.

4.1 Introduction

PAHs are ubiquitous environmental contaminants primarily produced through the incomplete combustion of organic matter, such as fossil fuel burning in industry or transportation[3,4]. Many of these compounds have been shown to be carcinogenic, mutagenic, and teratogenic in different taxa[1,4,10–12]. PAHs are primarily characterized by the formation and number (2+) of benzene rings[1,2]. Toxicity is positively correlated with increasing molecular weight and the number of benzene rings present within the compound [1,6,10]. High molecular weight (HMW) PAHs, exhibit low water solubility and extreme hydrophobicity. This physicochemical property impacts the environmental fate of these compounds, with many PAHs strongly adsorbed to soils and sediments, resulting in chemical recalcitrance within these environmental matrices[4].

In situ bioremediation is a cost effective and minimally invasive approach for treating chemically contaminated sediment that involves augmenting or stimulating the native microbial community to degrade target contaminants. In contrast to common physical remediation strategies such as dredging or capping sediment, bioremediation offers a long-term solution to removing recalcitrant environmental pollutants[14,16]. While abiotic chemical transformations commonly affect the environmental fate of PAHs, biological metabolism is the primary mechanism by which PAHs are transformed and

removed from sediment[1], suggesting that targeted bioremediation approaches can be utilized to reduce PAH concentrations.

Most of the research on microorganisms has focused on monocultures in a planktonic, or free-floating cellular state, however, the majority of bacterial life exists in a sessile state[33–37]. This existence strategy, called a biofilm, is defined as a surface-attached heterogeneous structure comprised of single or multi-species microorganisms enclosed in a matrix of extracellular polymeric material[33,38]. This lifestyle offers protection against dehydration and predation, facilitates enzyme activity and cell signaling, and allows for the sharing of nutrients and genetic information[35,38–40].

A bacterial biofilm exhibits distinct characteristics from its free-floating counterparts[46]. Biofilms allow for microbial interactions that are not possible while existing singularly or when spatially distant[37]. The biofilm structure is of particular interest for engineered bioremediation purposes because surface adhesion may be beneficial for degrading hydrophobic compounds that tend to be adsorbed to organic surfaces in sediment environments[10,24]. This existence state can reduce mass transfer limitations common with weakly soluble or effectively insoluble PAHs to microorganisms. Further, biofilms may also be resilient to dynamic estuarine conditions including tidal disturbances and water saturation variance.

An engineered mixed-community biofilm that may be used in a successful bioaugmentation technology for a creosote contaminated site would include bacteria

capable of catabolizing PAHs of various molecular weight and conformations, without antagonistic effects between the microbes[77]. Furthermore, this strategy should demonstrate the ability to produce sufficient biomass for the maintenance and spread of the biofilm and maintain a metabolically-active and cooperative community of PAH-degrading organisms[78].

Measuring the energy utilization or respiratory capacity of bacteria capable of degrading PAHs can provide insight into the activity of these organisms in the presence of contamination. Metabolic rates can be determined by measuring bacterial biomass production[79] or the change in concentration of by-products of respiration over time (i.e. electron donors or acceptors)[80,81]. Metabolic assays provide insight into microbial activity, substrate kinetics, and if there are nutrient limitations within a given system[81]. In this work, we utilize a high-throughput technology with the novel application of assessing bacterial biofilm respiration rates. The metabolic scope for the bacteria used in this study has yet to be determined, providing insight into how some of these prominent PAH-degrading organisms may be utilizing substrates and contributing to substrate and population dynamics in nature.

This work was performed using a biofilm composed of previously characterized bacteria with the known ability to degrade multiple PAHs prevalent in estuarine sediments from a coal-tar creosote contaminated site in Elizabeth River, Virginia. We validated the potential for these organisms to complex within a biofilm structure in both

monoculture and co-culture conditions and evaluated the community's structural dynamics of these units using genomic technologies.

4.2 Methods

4.2.1 Bacteria Evaluated

Six bacteria were isolated from sediment obtained from a former coal-tar creosote manufacturing site in the Elizabeth River, VA. Methods of isolation and the bacteria used in the following assays are described in Aim 1 and were taxonomically identified as *Novosphingobium pentaromativorans*, *Alcaligenes faecalis*, *Novosphingobium indicum*, *Sphingobium* RA C03, *Hydrogenophaga taeniospiralis*, and *Stenotrophomonas* sp.

4.2.2 Assessing Multiple-PAH Degradation with Spray Plating

Isolates were tested for their ability to degrade multiple PAHs utilizing the spray plate technique[82] over sRB_{15} and 2% agar (Becton, Dickinson and Company; Sparks, MD) supplemented with sodium pyruvate as an additional carbon source. After drying 2-3 hours, the plates were sprayed with each of the target PAHs dissolved in acetone[55]. Plates were incubated at 25˚C and monitored daily for zones of clearance indicating PAH transformation.

4.2.3 Quantifying PAH-Degradation using High Performance Liquid Chromatography

Incubations of organisms with PAHs were performed in 10mL sterile glass Hungate tubes capped with crimped Teflon stoppers. Phenanthrene (98%), pyrene (98%) and fluoranthene (98%) were acquired from Sigma-Aldrich (St. Louis, MO, USA). PAHs were dissolved in acetone and added to Hungate tubes at a final concentration of 1000 ng/µL (ppm). The solvent was allowed to volatilize in a fume hood, such that crystalized PAHs remained in the tubes. Bacteria were added to 1 mL sRB_{15} medium (Aim 1) with supplementation of sodium pyruvate, at a concentration of 10^5 cells/mL, as determined by counts of colony forming units (CFUs) on sRB_{15} agar plates. Abiotic controls included sRB_{15} with the PAH mixture and water with the PAH mixture. Reactors were assembled in 4 replicates per treatment and incubation was controlled at 25˚C on an orbital shaker table (~60 rpm) for 7 days.

To harvest PAHs for quantification, hexane (VWR Chemicals BDH) was added in a 5:1 ratio to media and reactors were vigorously vortexed for 20s to allow for complete dissolution of PAHs into the solvent. After filtering through a 0.2 µm nylon membrane filter (Merck Millipore, Ltd.; Burlington, MA) and diluting in 50:50 acetonitrile and water, extracts were quantified using an Agilent/Varian ProStar Modular High-Performance Liquid Chromatography (HPLC) System equipped with three ProStar Solvent Delivery Modules, a 410 autosampler, a 335 UV-detector, a 363 fluorescence detector, and a Supelcosil™ LC-PAH HPLC column (100mm X 4.6mm, 2.6 µm particle size; Supelco). The

program consisted of initial conditions of a mobile phase of 50:50 acetonitrile:water for 5 minutes followed by a linear gradient to 100% acetonitrile at 25 minutes with a return to initial conditions after 30 minutes. PAH concentrations were determined using fluorescence at various analysis windows corresponding to absorption and emission for individual PAHs and four-point standard curves generated from a Supelco SS EPA 610 PAH mix (Sigma-Aldrich; St. Louis, MO).

4.2.4 Assessing Biofilm Development

Bacteria were grown in sRB_{15} media supplemented with sodium pyruvate, diluted in fresh media to a cell concentration of 10^6, as determined by CFU counts, and added to replicate wells at a volume of 400µL. Abiotic controls included water and sRB_{15}. Microtiter plates (Corning; were incubated at 25˚C on an orbital shake (~60RPM) for 7 days. Planktonic bacteria or free-floating cells were removed by washing the wells with deionized water and removing the supernatant. Adhered cells were stained using 0.1% w/v crystal violet for 10 minutes at room temperature[83]. Biofilm development was quantified by resuspending dyed cells in a 30% acetic acid solution and transferring the volume to a new microtiter plate for optical density measurements as determined by absorbance at a wavelength of 600nm using a fluorescence plate reader (FLUOstar Optima, BMG Labtech; Cary, NC).

4.2.5 Biofilm Bioenergetic Assessment

Using a plate count technique, approximately 10^6 cells of each tested bacterium previously isolated from PAH-contaminated RC site sediment (Aim 1) were added to Seahorse XF24 Cell Culture Microplates (Agilent, Santa Clara, CA) in 400 µL aliquots of sRB_{15} medium supplemented with sodium pyruvate. Plates were incubated in a temperature-controlled environment at 25˚C for 7 days to allow for biofilm maturation. A blank treatment group comprised exclusively of growth media, sRB_{15}, was similarly prepared in each plate. Prior to plate reading, planktonic cells were removed from the microplate by performing a triplicate rinsing with deionized water. Fresh sRB_{15} was added to wells at a final volume of 425 µL.

The Seahorse Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA, USA) was used to measure basal respiration of bacterial biofilms with results reported as the oxygen consumption rate (OCR), over a 190-minute period. Basal respiration measurements were recorded with 22 cycles of 1 minute mix, 1 minute wait, and 2 minutes of a measurement period. Three replicate plates were run to obtain basal respiration measurements for bacterial biofilms, grown in quadruplicate wells per plate, with a final sample size of 12 wells per bacteria. Basal respiration rates were also collected with planktonic cells for each bacterium using the same protocol and number of replicates. Measurements were recorded as pmol O_2/min.

For assays requiring pharmacological agents, biofilm replicates were prepared as previously described and the cycling conditions[84] included 1 minute mix, 2 minute wait,

and 3 minute measurement period for the following drug applications in a range of concentrations: basal measurements (3 cycles); dicyclohexylcarbodiimide (DCCD) (0-125 µM concentration, 10 cycles) or oligomycin (0-50µM, 10 cycles); carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (0-50 µM concentration, 3 cycles); and a mixture of antimycin and rotenone (0-125 µM each, 10 cycles).

4.2.6 Data Analyses

All data were analyzed using GraphPad Prism, Version 8.1.1 (GraphPad Software, Inc., La Jolla, CA, USA). Experimental replicates of biofilm basal respiration measurements were averaged on a per plate basis and are reported per timepoint as an average of the triplicate plates with propagated error. Area under the curve analyses were performed for the basal biofilm respiration results and compared between treatments using a one-way ANOVA with multiple comparisons and statistical significance was achieved at $p \leq 0.05$. Biofilm development between bacterial isolates was compared using an ANOVA with multiple comparisons. Statistical significance was determined at $p \leq 0.05$.

4.3 Results

4.3.1 Confirming Multiple PAH-Degradation

Originally isolated on a phenanthrene plate, *N. indicum* was able to degrade fluoranthene and showed potential for pyrene degradation. Originally isolated on fluoranthene plates, *N. pentaromativorans* and *A. faecalis* were also able to degrade

phenanthrene, with *A. faecalis* showing potential as a degrader of pyrene. *H. taeniospiralis* and *Sphingobium* RA C03 were originally isolated on phenanthrene plates and did not show degradation capabilities for fluoranthene or pyrene (Table 4).

Table 4: Table indicating the individual PAHs that can be degraded by Elizabeth River bacterial isolates, as determined by the spray-plate method.
NT indicates a treatment that was not tested. Some bacteria isolated appear capable of degrading multiple PAHs, not just the compound on which they were isolated.

Organism Name	Original PAH enrichment	Phenanthrene clearance	Fluoranthene clearance	Pyrene clearance
Hydrogenophaga taeniospiralis	Phenanthrene	Yes	No	No
Novosphingobium indicum	Phenanthrene	Yes	Yes	Maybe
Sphingobium RA C03	Phenanthrene	Yes	No	No
Novosphingobium pentaromativorans	Fluoranthene	Yes	Yes	No
Alcaligenes faecalis	Fluoranthene	Yes	Yes	Maybe
Stenotrophomonas sp.	Fluoranthene	*NT*	Yes	*NT*

Concentrations of measured PAHs were determined by extrapolating from four-point standard curves of each compound, which are reported in the Appendix (Figures 23-25). Given that the initial concentration of PAHs added to reactors was 1000 ng/µL, there was significant loss of the compounds across treatments (Figures 6-8). This loss may be due to the filtering method used. All of the treatments resulted in a significant reduction in phenanthrene compared to controls ($p \leq 0.05$). The reactors inoculated with *Alcaligenes faecalis* and *Stenotrophomonas* sp. resulted in marginal reduction of fluoranthene compared to controls ($p \leq 0.1$). Pyrene was not significantly removed from reactors after the 7 day incubation period.

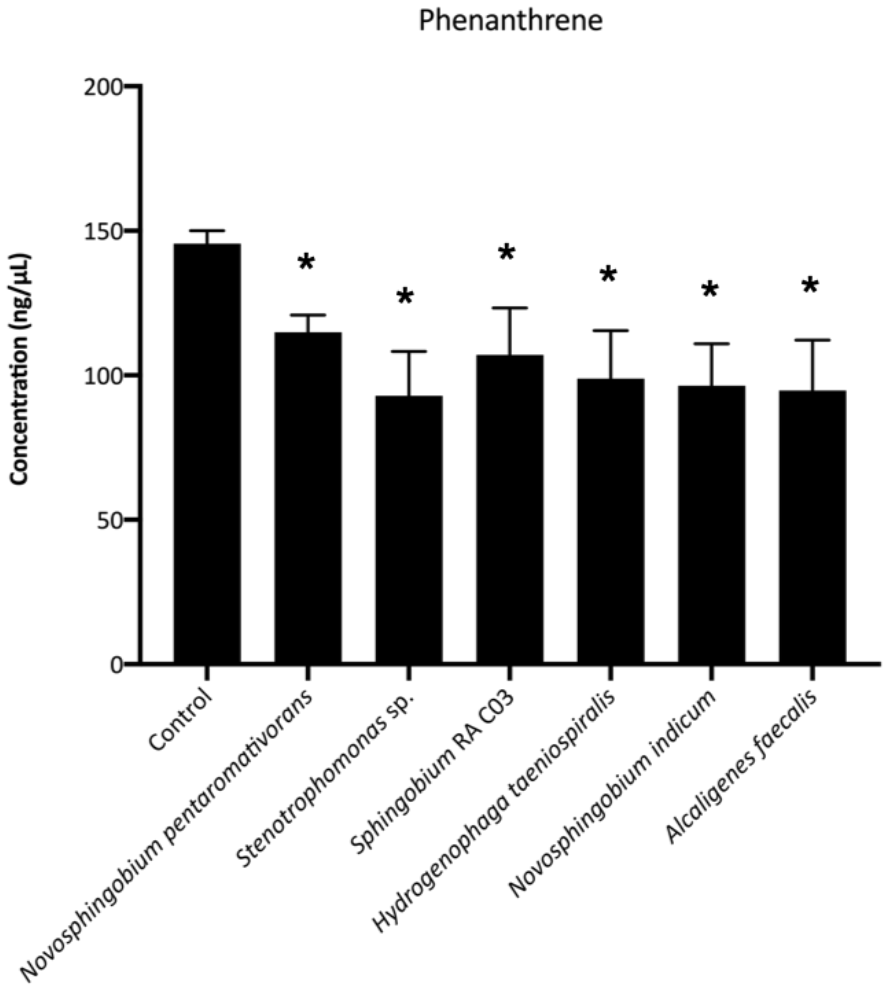

Figure 6: Bar chart showing the change in concentration of phenanthrene in reactors inoculated with PAH-degrading bacteria.

The y-axis represents fluoranthene concentrations and the x-axis denotes which type of bacteria in monoculture was added to reactors. Bacteria were isolated from creosote contaminated sediment in the Elizabeth River, VA and demonstrated PAH-degrading abilities in Aim 1. Significant differences in fluoranthene concentrations between controls and treatments were determined at $p \leq 0.05$.

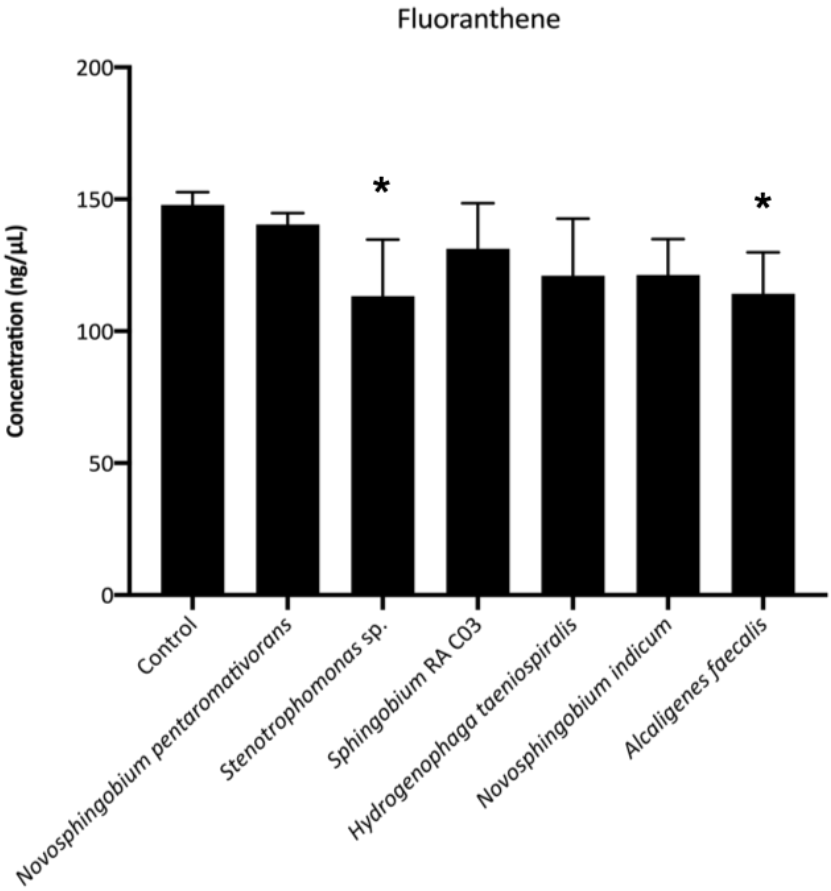

Figure 7: Bar chart showing the change in concentration of fluoranthene in reactors inoculated with PAH-degrading bacteria.

The y-axis represents fluoranthene concentrations and the x-axis denotes which type of bacteria in monoculture was added to reactors. Bacteria were isolated from creosote contaminated sediment in the Elizabeth River, VA and demonstrated PAH-degrading abilities in Aim 1. Marginal differences in fluoranthene concentrations between controls and treatments were determined at $p \leq 0.1$.

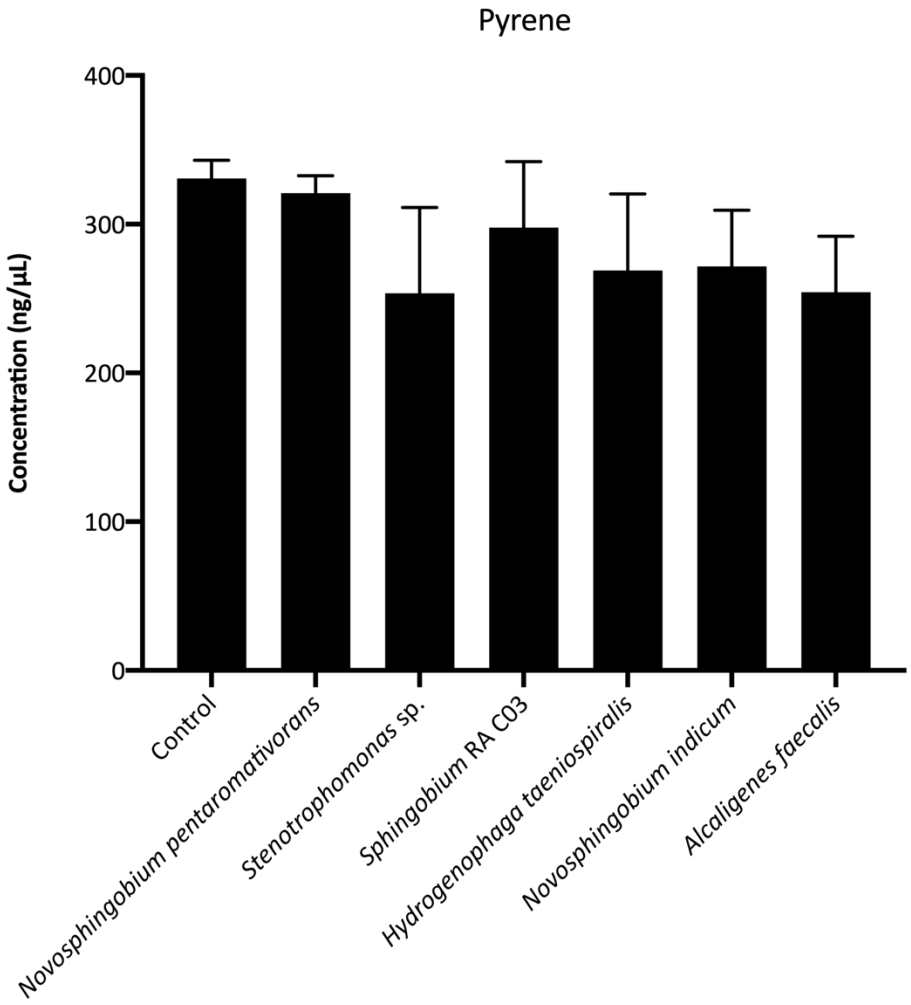

Figure 8: Bar chart showing the change in concentration of pyrene in reactors inoculated with PAH-degrading bacteria.
No significant differences in pyrene concentrations were observed between controls and treatments.

4.3.2 Biofilm Development

Wells containing organisms and either unamended media or water did not significantly differ in the amount of measured biofilm ($p \leq 0.05$), so comparisons to other treatments were performed using only the media-only control. All biofilms derived from a bacterial monoculture (Figure 9) demonstrated significantly greater ($p \leq 0.05$)

formation than the media control, demonstrating that microbial media was uncontaminated and there was minimal adherence of the crystal violent to the microtiter plate. All bacterial biofilms were significantly different from each other except for those produced by the *Stenotrophomonas* sp. and *Hydrogenophaga taeniospiralis* organisms. Minimal variability in growth between replicates suggests that this assay can be utilized for reproducible growth of bacterial biofilms.

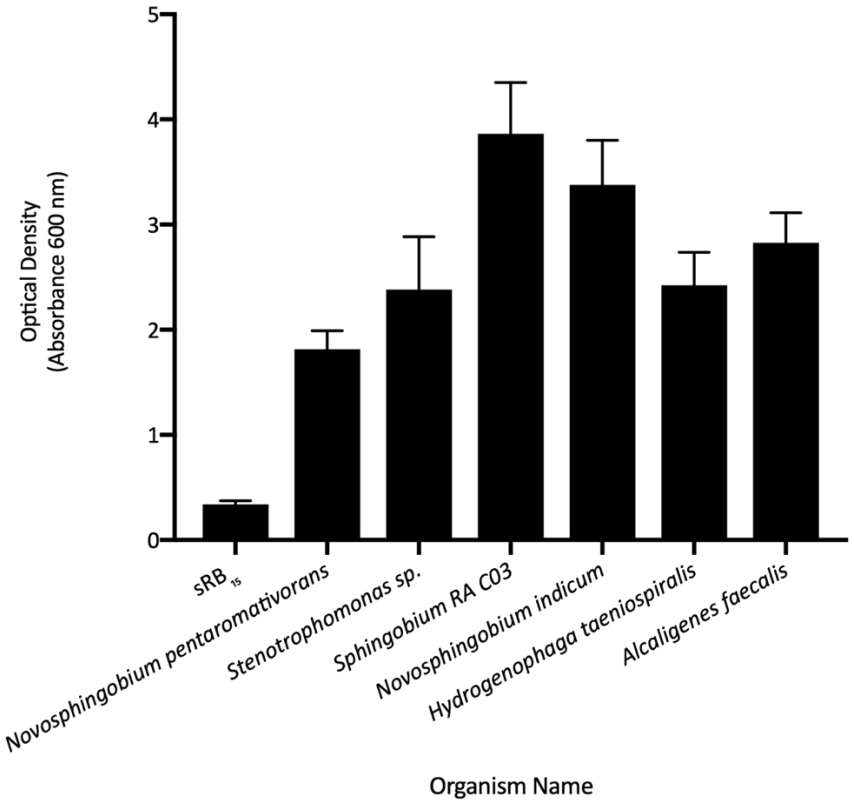

Figure 9: Bar graph indicating relative optical density of abiotic controls and PAH-degrading bacteria from the Republic Creosoting site.
Optical density serves as a proxy for cellular growth. All organisms were added in isolation and equivalent cell concentrations to a nutrient growth medium, sRB_{15}.

Biofilms comprising multiple organisms (Figure 10) displayed higher variability in biomass production compared to the biofilms from monoculture. Regardless of organisms, all biofilms demonstrated significantly greater biomass production than the media-only control. There was no significant correlation between biomass production and the organisms used in the biofilm co-culture as determined by the microtiter assay alone and none of the different combinations of bacteria produced significantly greater biomass than the others ($p \leq 0.05$)

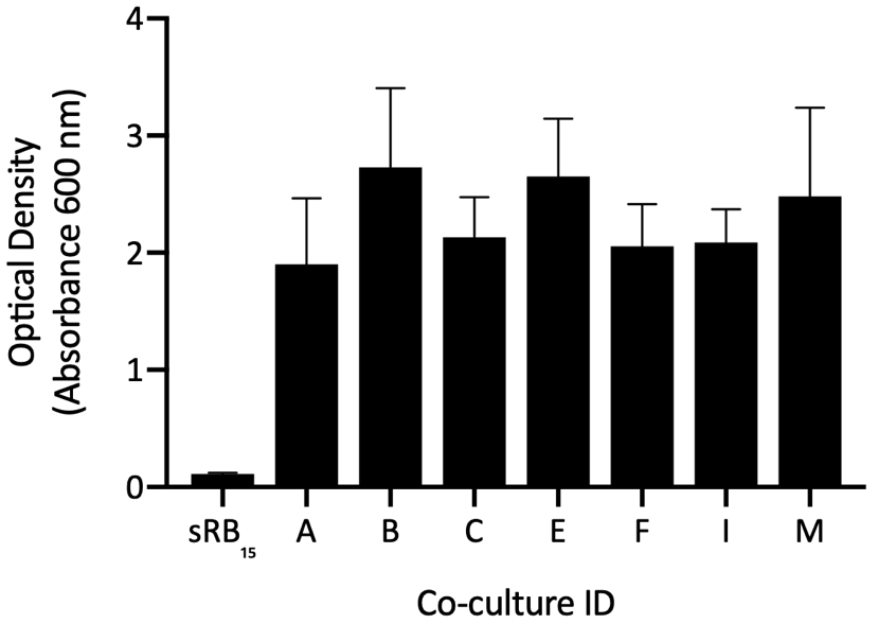

Figure 10: Bar chart representing optical density as a proxy for cell concentration measured via absorbance at a 600nm wavelength. X-axis includes identification codes for co-culture biofilms incubated in a microtiter plates in replicates of 8. ID codes are as follows: A= *Novosphingobium pentaromativorans* and *Stenotrophomonas* sp.; B= *Novosphingobium pentaromativorans* and *Hydrogenophaga taeniospiralis*; C= *Novosphingobium pentaromativorans* and *Alcaligenes faecalis*; E= *Stenotrophomonas* sp. and *Hydrogenophaga taeniospiralis*; F= *Stenotrophomonas* sp. and *Alcaligenes faecalis*; I= *Alcaligenes faecalis* and *Hydrogenophaga taeniospiralis*; M= *Novosphingobium pentaromativorans, Stenotrophomonas* sp., *Hydrogenophaga taeniospiralis*, and

***Alcaligenes faecalis*. All co-culture biofilms demonstrated significantly greater growth than the medium, sRB_{15}, control.**

4.3.3 Biofilm Development in Seahorse Microtiter Plates

All biofilms derived from a bacterial monoculture and plated in the proprietary Seahorse microtiter plates demonstrated significantly greater ($p \leq 0.05$) formation than the media control (Figure 11). This suggests that microbial media was uncontaminated and there was minimal adherence of the crystal violent to the microtiter plate. Further, the measured biofilm growth of organisms in the Seahorse microtiter plates differed from the trends observed within and between organisms plated in the Corning microtiter plates. This suggests that the surface structure or materials used can have an impact on the development and maturation of bacterial biofilms.

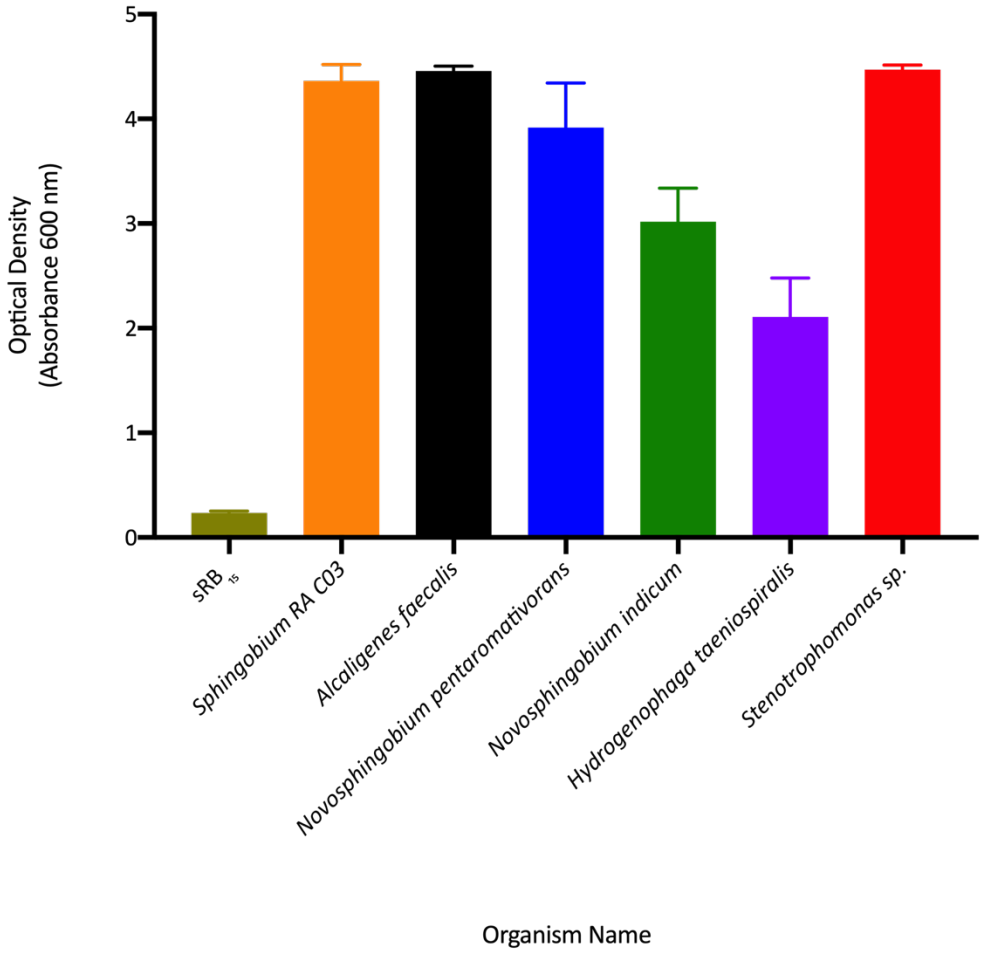

Figure 11: Bar graph indicating biofilm development in the Seahorse XFe Extracellular Flux microplates.

Optical density measurements, as determined by measuring absorbance at a wavelength of 600nm. The control treatment (sRB_{15}) was the growth medium used for cells and was used to measure background levels of the Seahorse XF24 Cell Culture Microplates staining by the crystal violet dye. Experimental treatments included six unique bacteria isolated from RC site sediment grown and measured in monoculture biofilms.

4.3.4 Biofilm Bioenergetic Assessment

All blank wells had negligible values of respiration, indicating that the growth medium utilized was uncontaminated with cellular material. The basal respiration of the biofilms comprised of *Sphingobium* RA C03 and *Stenotrophomonas* sp., were almost half

that of the other organisms, suggesting that these organisms may have a reduced respiration capacity or growth rate (Figure 12). Alternatively, those strains may require less energy to maintain homeostasis or are producing energy through other anaerobic processes. The basal respiration rates for the *Alcaligenes faecalis*, *Novosphingobium pentaromativorans*, *Novosphingobium indicum*, and *Hydrogenophaga taeniospiralis* biofilms were not significantly different ($p \leq 0.05$), largely due to variation in measurements per timepoint. All organisms showed significantly greater metabolic scope compared to the control treatment, demonstrating that growth media was uncontaminated with foreign cells.

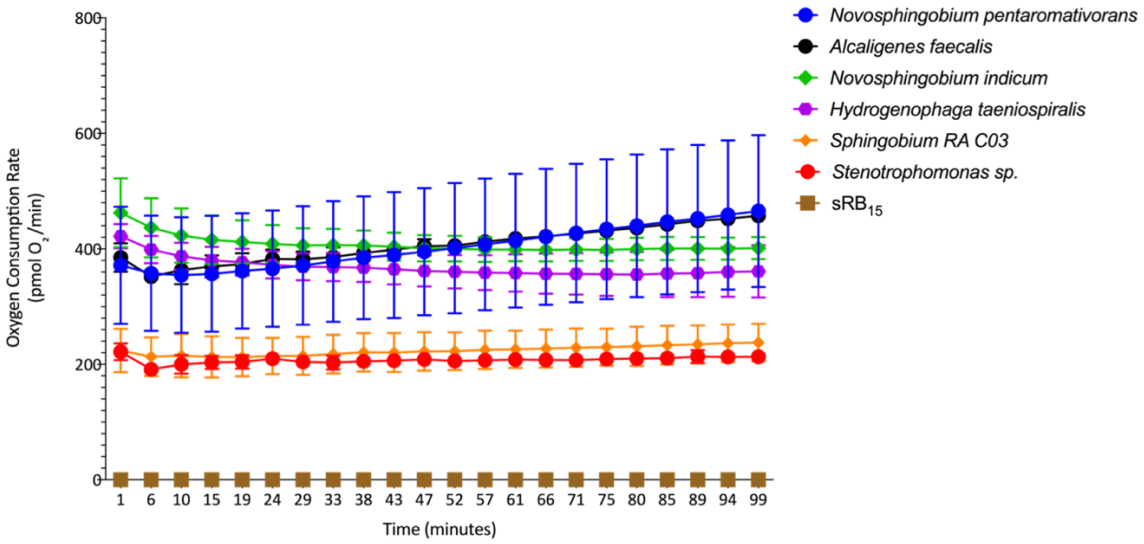

Figure 12: Basal respiration measurements reported as oxygen consumption rate (pmol/min) for monoculture biofilms of bacteria.
Data reflect the average of replicates from 3 plates (n=12) with reported standard error of the mean. All organisms were aliquoted in equal cell concentrations and allowed to form a mature biofilm after a 7 day incubation. Differences in basal respiration suggest varying metabolic scope of organism.

Bacteria in a planktonic cellular state demonstrated less variability in measured respiration rates for replicates within and amongst plates, when compared to the biofilm samples (Figure 13). Area under the curve analyses indicated that all organisms had significantly different ($p \leq 0.01$) respiration rates in their planktonic cellular states, except for *Hydrogenophaga taeniospiralis* and *Stenotrophomonas* sp.

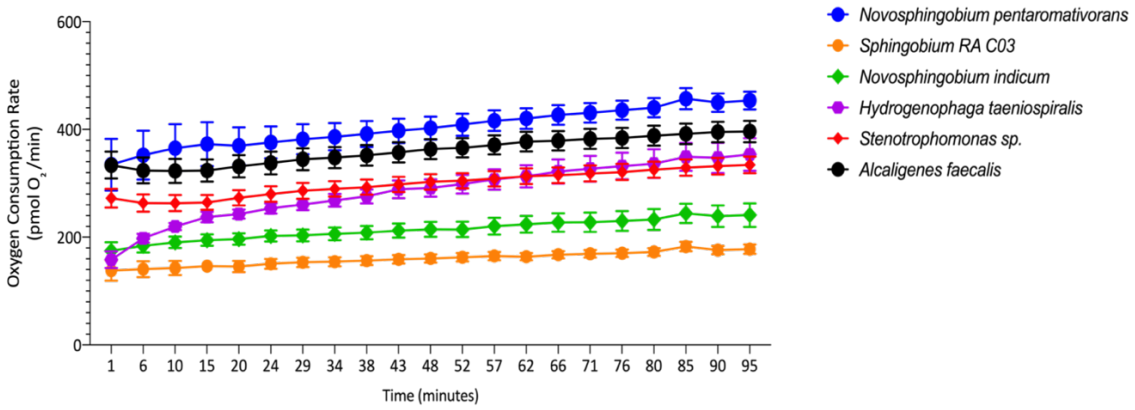

Figure 13: Basal planktonic respiration measurements reported as oxygen consumption rate (pmol/min) for monoculture planktonic bacteria. Data reflect the average of replicates from 3 plates (n=12) with reported standard error of the mean. All organisms were aliquoted in equal cell concentrations. Differences in basal respiration suggest varying metabolic scope of each organism.

The Seahorse XFe Extracellular Flux Assay also allows for users to apply pharmacological agents to inhibit specific facets of cellular respiration and the electron transport chain. While various agents were tested, these drugs did not affect the bacterial biofilms at concentrations at and exceeding those used in previous work[85] or in a dose-response curve, therefore these data were excluded from analyses. Drug agents were intended to determine oxygen consumption during specific stages of cellular respiration, such as basal respiration from the electron transport chain, ATP production,

proton leak, maximal respiration, spare respiratory capacity, and non-cellular respiration.

4.4 Discussion

4.4.1 PAH-Degradation Capability

The PAH-degrading capabilities of six bacterial isolates from creosote contaminated sediments were evaluated using two methods. In the spray plate method, which was utilized to obtain the bacterial isolates, bacteria were grown on a nutrient media and 2% agar plate with either phenanthrene, fluoranthene, or pyrene sprayed on the surface. This method resulted in the visual disappearance of phenanthrene and fluoranthene on plates, suggesting that some of the bacteria isolated from this site can degrade multiple PAHs of varying ring complexity.

Incubations with liquid nutrient media, PAHs, and each type of bacteria in isolation resulted in significant reductions of phenanthrene across treatments, but only two bacteria produced significant reductions in fluoranthene using this method. Pyrene concentrations were not reduced by any of the bacterial treatments.

These contradictory results are likely due to experimental design and may more closely align if both PAH concentration and cell concentrations are optimized. It is possible that the high concentrations of PAHs used in the incubations required longer incubation periods and higher cell concentrations of each bacteria in order to see significant reductions in concentrations.

4.4.2 Biofilm Formation

The Microtiter Plate Assay is a high-throughput, static system that can be used to visualize and quantify biofilm development[83,86]. While the experiments performed in this work were designed to investigate the biofilm formation potential by PAH-degrading bacteria, some organisms demonstrated an ability to grow a more robust biofilm matrix than others. The biofilms grown in monoculture featured little variability and while the distribution of cells within the biofilm matrix may be random and not reproducible, the results in this study suggest that the biomass produced by these organisms under these conditions is a reproducible occurrence. However, it is worth noting that using optical density as a proxy for cell growth may be erroneous due to variation in cell size between different bacteria. Bacterial characterizations, such as cell size, were not completed in this work.

4.4.3 Implications of Biofilm Respiratory Scope

The use of the Seahorse Extracellular Flux Analyzer for the measurement of bacterial biofilm respiration is a novel application of this technology. While an important capability of this technology is to determine how various stages of respiration are affected by user treatments, as determined by the introduction of pharmacological agents, we were unsuccessful in optimizing an efficacious assay to this effect. The drug trials produced highly variable results and required drug concentrations greatly exceeding the manufacturer's protocol and previous reports for evaluating cells. We

interpret these results to likely be due to the inability of the drugs to penetrate the biofilm extracellular matrix and were therefore unable to be exposed to metabolically active cells. These results suggest that this technology may not be suitable for use in biological samples likely to excrete polymeric substances that coat or protect cells.

In this work, we observed that biofilm biomass did not necessarily indicate greater respiration capacity. This is evidenced by less biomass measured within *Hydrogenophaga taeniospiralis* and *Novosphingobium indicum* biofilms, despite competitive respiration capacities. Similarly, *Stenotrophomonas* sp. and *Sphingobium* RA C03 had significantly reduced respiration capacities compared to the other bacteria but had high biomass production within microplates. It is possible that some organisms were utilizing substrates for the production of cellular material, which may explain their higher respiration rates but reduced biofilm production. Conversely, other organisms may have been allocating resources towards producing a robust biofilm matrix, which could explain the lower respiration rates but higher biofilm production.

There are various benefits to forming a robust biofilm matrix including protection and metabolic cooperativity[87]. Organisms with a greater respiratory capacity may require higher concentrations of nutrients, which may be limited in the natural environment. In the context of bioremediation, organisms with a higher metabolic scope may be able to degrade more of the target contaminant, however sediments where contaminants are generally heterogeneously disbursed or not bioavailable may not sustain a large bacterial population with these nutritional requirements. Further, in

environments where contamination is present in extremely high concentrations or exists in mixtures with other classes of contaminants, organisms with a lower respiratory capacity may thrive by using this life strategy to avoid toxic effects.

It is also important to note that the variability in biofilm respiration measurements cannot be explained by cell concentrations or variable biofilm matrix development because microplate growth assay results indicate that biomass production for biofilms does not have significant variability amongst replicates. These results may suggest variability in the data acquisition of the applied technology or that respiration rates amongst individual biofilm structures vary, despite identical organism composition.

Finally, it is important to note that in this work PAHs were not included as a carbon substrate due to the required use of proprietary plastic microplates and the high likelihood of PAH-adsorption to the plates. Therefore, respiration rates measured reflect those of bacteria in the presence of a bioavailable and easily metabolized carbon substrate included in the growth media. Future work could include the addition of a chemical concentration gradient to determine if bacterial respiration is inhibited or promoted by the compound or if nutrient supplementation could be beneficial for degradation rates[81].

5. Developing a Bioamendment for the Field Delivery of a PAH-Degrading Biofilm to Sediments.

5.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are comprised of multiple, fused benzene rings-a structure which lends to their hydrophobic behavior and a high sorption potential[88]. PAHs enter aquatic environments via atmospheric deposition of compounds adsorbed to particulate matter, with subsequent sedimentation. As a result, sediments can accumulate large concentrations of recalcitrant contaminants[89] that generally exist as chemical mixtures. While some abiotic processes may affect PAH transformation, microbial metabolism is the primary process by which PAHs are degraded in subaquatic environments[1,9,90].

In situ remediation of sediment is an ideal treatment method over other common approaches for sediment remediation including removal processes such as dredging and the implementation of physical barriers, often referred to as caps[89]. These physical treatment options tend to be cost prohibitive and can result in deleterious ecological effects due to habitat destruction and the resuspension of sequestered contaminants[91]. Furthermore, over time caps become compromised[15,92] by the flux of contaminated porewater, thus failing to reduce chemical risk[14].

Bioaugmentation is an in situ biological remediation strategy that can be applied to increase PAH degradation rates and efficacy by increasing cell concentrations of bacteria with PAH-degradation capabilities. While bioaugmentation has been shown to

reduce pollutant concentrations, the use of this technology in sediment environments can be challenging due to the difficulty of delivering the organisms to sediment[14] and maintaining an active population of augmented microorganisms. Various materials including clay, nylon, and chitin[20] have been used as biocarriers to deliver microbes to sediments and soils. However, these materials serve no other purpose in the remediation effort, which may not make them an economically competitive remediation technology.

Composite amendments that include activated carbon (AC) have been demonstrated to serve as an effective remediation strategy for PAHs and other hydrophobic organic contaminants. The strong binding potential of black carbon significantly reduces the bioavailable fraction of these compounds. In fact, a composite amendment that contains AC, sand, and clay for weight, has recently been validated for the use of PCB remediation in sediments[14,91,93]. This product, known as SediMite™, has been utilized for PCBs, which have similar physicochemical properties to PAHs in terms of hydrophobicity and high sorption[94].

The purpose of this work was to validate the use of SediMite™ as a bioamendment for both the delivery of a PAH-degrading bacterial consortium and the reduction of PAH concentrations in sediment and porewater. High and low concentrations of PAHs were tested in this work to determine if the SediMite™ technology was protective against potentially toxic levels PAHs even if those same compounds are easily biodegradable at low concentrations[95].

5.2 Material and Methods

5.2.1 Chemicals

Phenanthrene (98%), pyrene (98%), and fluoranthene (98%) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Performance reference compounds (PRCs) for polyethylene (PE) passive samplers included pyrene-D10 (98%) chrysene-D12 (98%), fluorene-D10 (98%), and benz(a,h)anthracene-D12 (98%) and were acquired from Cambridge Isotope Laboratory (Tewksbury, MA).

5.2.2 Bacterial Consortium

Bacterial isolates obtained from Aim 1 and validated for PAH-degradation, biofilm formation potential, and respiration capacity (Aim 2) were used in this study. The bacteria selected for this study included *Alcaligenes faecalis*, *Hydrogenophaga taeniospiralis*, *Novosphingobium pentaromativorans,* and *Stenotrophomonas* sp. Bacteria were added to reactors at a cell concentration of 10^5, as determined by colony forming unit counting on LB agar plates. This cell concentration was selected based on previous work[14] using a similar experimental design outlined in the present study.

5.2.3 Sediment Collection and Spiking

Sediment was collected from the Mains Creek (MC) site (Elizabeth River, VA, USA), which has been previously used in scientific investigations as a reference site hosting low concentrations of PAHs[11,53]. Approximately 1 kg of sediment from the top 7 cm of material at the site was collected in amber glass jars and transported to the

laboratory on ice where it was stored at 4˚C until processing. To obtain size distribution uniformity, sediment was sieved at a 2 mm grain size, which removed large debris such as shells, glass, and organic matter.

For use in experiments, MC sediment was spiked with mixtures of phenanthrene, fluoranthene, and pyrene dissolved in acetone at concentrations of 2000 mg/kg and 100 mg/kg sediment dry weight for each compound. The PAH solution was manually distributed within 1L glass jars until the solvent completely volatilized and solid PAHs remained on the jar interior walls. Jar exteriors were wrapped in aluminum foil to prevent photodegradation of PAHs. Sediment was added to jars at 80% capacity and homogenized using a toxicity characteristic leaching procedure (TCLP) rotary agitator for 2 weeks and stored at 4˚C prior to subsequent analyses.

5.2.4 Preparation of the Polyethylene Passive Sampler

Polyethylene (PE) was used as a passive sampler to measure freely dissolved aqueous concentrations of PAHs in each reactor. Pieces of clear 2mil. PE (Husky, Bolton, Ontario), were cut to a mass of 30mg and a 1 cm stainless-steel hook was added in order to aid in the recovery of the sampler from reactors. PE was first cleaned by soaking in a 1:1 ratio of hexane:acetone for 24hrs. Excess cleaning solution was removed and the PE air dried in a fume hood. To evaluate the recovery of target compounds on the PE, three performance reference compounds (PRCs), representing the expected octanol:water partitioning coefficient of the spiked PAHs, were selected to impregnate PEs. PRCs were

dissolved in either dichloromethane or hexane, then spiked in an impregnation solution of methanol:water (80:20, v:v) for a duration of 2wks on an orbital shaker . Spiking concentrations of PRCs in PE were calculated utilizing methods previously described[96] to obtain a target concentration on the order of ng/g.

5.2.5 Reactor Preparation

MC sediment and PE were added to 50mL Erlenmeyer flasks and brought to 20mL volume with sRB_{15} medium without additional carbon and 15 mL sterile water at 15 ppt salinity (Instant Ocean, Cincinnati, OH). SediMite™ (Sediment Solutions, Ellicott City, MD) was added to 10mL sRB_{15} growth medium and a bacterial consortium at a concentration of 10^5 cells/g dry weight SediMite™. The consortium and SediMite™ were incubated for 3d at 25˚C prior to sediment addition at a 3% sediment dry weight ratio. Reactors were maintained in a slurry phase on a horizontal shaker table at 80 RPM. Flasks were protected from ultraviolet light to prevent algal growth and covered with Parafilm (Bemis Company, Inc.; Neenah, WI) to prevent debris contamination. Flask headspace was recharged every 2 weeks by removing the Parafilm for 10 minutes.

Controls were used to evaluate the influence of the SediMite™ technology, the bacterial consortium, and the combination of the SediMite™ and bacterial consortium. Control reactors included either the 100 mg/kg or 2,000 mg/kg spiked sediment and sterile water at 15 ppt salinity (Instant Ocean, Cincinnati, OH). Where denoted, control reactors included the addition of SediMite™.

5.2.6 Quantification of Polycyclic Aromatic Hydrocarbons in Sediment and Polyethylene

Sediment and PE samples were stored at -20°C until analytical work could be completed. Sediment slurries were centrifuged at 500 RPM for 10 minutes (AxySpinR, Corning; Corning, NY) and approximately 0.5 g of sediment (wet weight) was removed using a stainless steel scupula. This sediment was then dried with anhydrous sodium sulfate by grinding in a mortar and pestle. Each sample was then spiked with surrogate standard mix (200 μL of 2000 ng/mL spiking solution). Each sample was then extracted in a 50:50 acetone:hexane mixture (4mL) via ultrasonication (5 min). Upon sonication, the sample was again centrifuged (500 RPM, 10 min) and the supernatant was collected. This process was repeated for a total of three times, and supernatant extracts were combined. Sulfur was removed from the extract by adding cleaned copper turnings during concentration (SpeedVac concentration, reduced pressure, 35°C to approximately 0.5 to 1 mL), and solvent was exchanged to hexane. Once concentrated in hexane, the sample was further cleaned by solid phase extraction (SPE) through 0.5g of clean silica. The silica was conditioned with 6 mL hexane, and the sample was loaded, and eluted with 6mL hexane. Samples were once again concentrated to approximately 0.5 mL with SpeedVac concentrator (Thermo Scientific). Samples were spiked with internal standards (100 μL, final concentration 200 ng/mL), transferred to muffled autosampler vials, and final volumes were brought to 1 mL. Samples were then capped

and stored at -20°C until analysis by GC/MS. At least one procedural blank and one matrix spike was extracted with each batch of sediment.

Polyethylene passive samplers were individually cleaned and dried using lint-free tissue. Once dry, the sampler was weighed and spiked with surrogate standards (100 μL of 2000 ng/mL spiking solution). Each sampler was then extracted with hexanes (5mL) via bath sonication for 30 min. This extraction was repeated three times and extracts were combined for each sample. Combined extracts were concentrated via SpeedVac concentrator and transferred to an autosampler vial before being spiked with internal standards (100 μL, final concentration 200 ng/mL) and brought to 1 mL with hexane. Vials were then capped and stored until analysis by GC/MS. Lab blanks were analyzed with each batch.

PAH samples were analyzed using an Agilent 7890A digital gas chromatograph coupled to a 5975C mass selective detector (Santa Clara, CA) operating in the electron ionization (70 eV) and selected ion-monitoring mode (GC-EI/MS). Two μL of each extract was injected in the splitless mode with a 7693 series autosampler, and target analytes were separated using a HP-5MS column (Agilent J&W Scientific; 30 m × 0.25 mm × 0.25 μm) with ultra-high purity helium as the carrier gas at a flow rate of 1.3 mL/min. The GC oven was programed as follows:45 °C (1 min hold); ramp at 7.5 °C/min to 300 °C (18 min hold); for a total run time 51 min.

Internal standards used included: d_8-Naphthalene, d_{10}-Anthracene, d_{10}-fluoranthene, d_{12}-benz(a)anthracene, d_{12}-benzo(a)pyrene, and d_{14}-dibenzo(a)pyrene.

Surrogate standards included: d_{10}-2-methylnaphthalene, d_{10}-phenanthrene, d_{-12}-perylene, and d_{12}-indeno(1,2,3-c,d)pyrene. All isotopically labelled standards were purchased from Cambridge Isotopes (Tewskbury, MA) and used without further purification.

5.2.7 Enumeration of PAH-Degrading Organisms

Extraction of total DNA was performed on sediment from reactors harvested after 1 month and 2 month sampling timepoints using the PowerSoil DNA isolation kit (QIAGEN; Valencia, CA). Manufacturer's instructions were followed except that 0.3g of sediment was used and the final DNA elution was performed with 30 µL of Solution C6 after a 5 minute incubation at room temperature to increase DNA yield.

Target organisms were enumerated using quantitative PCR (qPCR). Briefly, each reaction on the BioRad CFX96™ Real-Time System (BioRad; Hercules, CA) consisted of 1 µL of template DNA, 10 µL of 2X iTaq™ Universal SYBR® Green Supermix (Qiagen; Valencia, CA), forward and reverse primers (final concentration 0.2 µM each), and water to a final volume of 20 µL. The program consisted of an initial denaturation of 2 min at 95˚C, followed by 40 cycles of 15 sec at 95˚C, 30 sec at 56˚C, and 30 sec at 72˚C. Data were collected during the extension cycle and a melt curve obtained from 65 to 95˚C. Samples were run in duplicate to account for technical or template variability. Unique primer sets were designed for use with the SYBR® chemistry to target the *Alcaligenes faecalis* and *Stenotrophomonas* sp. bacteria (Table 5). Values were compared to a 6-

point standard curve of gene copy numbers ranging from 10^1 to 10^8. Gene copy numbers were reported as number of genes per 0.3g sediment and log transformed. Gene copy numbers were further normalized to total 16S rRNA gene copies per 0.3g sediment, according to previously described methods[97].

Table 5: Quantitative PCR primers developed to amplify clones evaluated in this study.
[1]Number of sequences with matching hits outside of target, as determined by the Ribosomal Database Project II, Release 11.5.

Primer Target	Primer direction	Primer sequence (5' -> 3')	Amplicon length	RDP Alignment
Alcaligenes faecalis	Forward Reverse	TACGGGATACTGCTGACGGT TGCACCCTACGTATTACCGC	80	444
Stenotrophomonas sp.	Forward Reverse	GACCTACGGGTGAAAGCAGG GCATCGGCTCATTCAATCGC	56	412

5.2.8 Data Analyses

For statistical analyses, data were analyzed using GraphPad Prism, Version 8.1.1 (GraphPad Software, Inc., La Jolla, CA, USA). Effects of experimental treatments were determined using one-way ANOVA with multiple comparisons. Statistical significance is noted in figure captions depending on experimental treatment. Chemical data reflects individual technical samples from 4 replicates per experimental treatment. Biological data reflects 2 technical replicates from 3 sediment subsamples of 4 experimental replicates. Individual data points are reported to show variance.

5.3 Results

5.3.1 Whole Sediment Concentrations

Figure 14 shows the phenanthrene, fluoranthene, and pyrene concentrations in sediments that were originally spiked with 100 mg/kg of mixed PAHs after one month of incubation. Concentrations of these compounds measured in the original spiked sediment are reported in the Appendix (Table 8). Less than 50% of each of the spiked compounds remained in reactors after a 1 month period of time. However, there was no statistical significance between the control reactors with SediMite™ (SM) or reactors without the AC ($p<0.05$), likely due to the inability to separate these two media and analytically differentiate their chemical concentrations. Because of this, only data from the control reactors without SediMite™ are represented in figures. There was no statistical significance between the experimental treatments and the control for any of the PAHs measured ($p<0.05$).

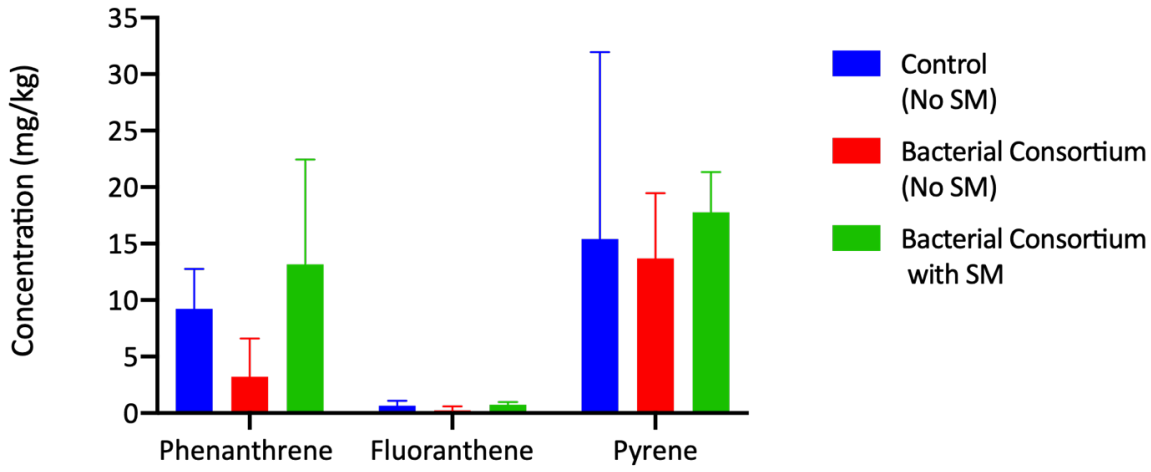

Figure 14: Bar graph representing PAH concentrations after a 1 month incubation period with sediments spiked at 100 mg/kg. Bars reflect averages of experimental replicates (n=4).

Figure 15 represents phenanthrene, fluoranthene, and pyrene concentrations in sediments originally spiked at 2000 mg/kg after 1 month of incubation. There was no significant difference between the control reactors with SediMite™ or without the AC ($p<0.05$). For these experimental conditions, there was no statistical significance between the control reactors or those with an added bacterial consortium for any of the PAHs ($p<0.05$). However, there was a significant difference ($p<0.01$) in concentrations of all three PAHs added between the controls and reactors amended with both the bacterial consortium and SediMite™.

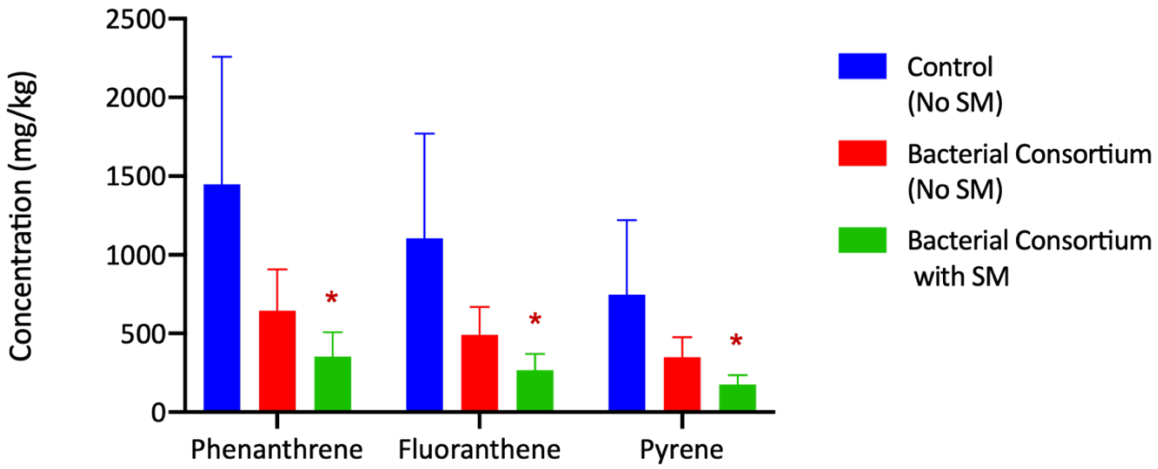

Figure 15: Bar graph representing PAH concentrations after a 1 month incubation period with sediments spiked at 2000 mg/kg.
Bars reflect averages of experimental replicates (n=4). Asterisks denote statistically significant differences ($p<0.1$) from "Control (no SM)" reactors that contained sediment spiked at 2000 mg/kg, a polyethylene sampling device, and sterile water (15ppt salinity).

5.3.2 Aqueous Concentrations

Freely dissolved aqueous concentrations of individual PAHs were determined by measuring chemical concentrations of chemicals adsorbed to PE sampling devices. Figure 16 represents phenanthrene, fluoranthene, and pyrene concentrations in PE after one month of incubation in reactors with sediments originally spiked at 100 mg/kg. Phenanthrene concentrations were too low to be represented on this graph but are included in the Appendix (Figure 32). Fluoranthene was present at a significantly lower freely dissolved concentration in the treatments including a bacterial consortium both with and without SediMite™ ($p<0.05$). Treatments with just sediment and SediMite™ did not significantly reduce aqueous concentrations of fluoranthene ($p<0.05$). Pyrene was

significantly removed from the aqueous phase in reactors treated with just SediMite™, as well as those including SediMite™ plus the bacterial consortium.

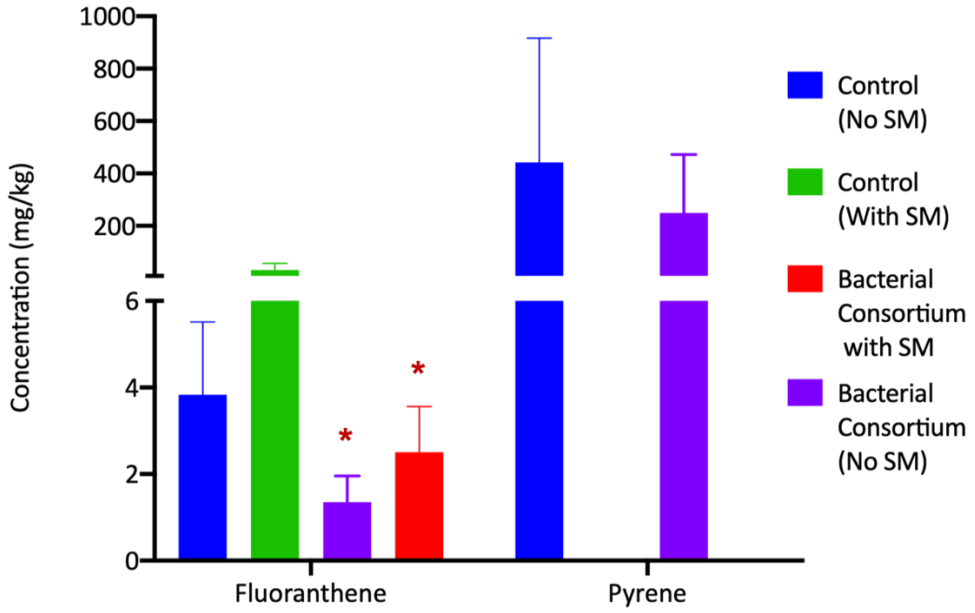

Figure 16: Bar graphs representing concentrations of phenanthrene, fluoranthene, and pyrene in polyethylene passive samplers after a 1 month incubation period with sediments spiked at 100 mg/kg. Bars reflect experimental replicates (n=4). Asterisks denote statistically significant differences ($p<0.05$) from "Control (with SM)" reactors that contained sediment spiked at 100 mg/kg, a polyethylene sampler, and sterile water (15ppt salinity).

Figure 17 represents phenanthrene, fluoranthene, and pyrene concentrations in PE after one month of incubation in reactors with sediments originally spiked at 2000 mg/kg. Phenanthrene was not detected in PE samples. Concentrations of PAHs captured on PE disks were comparable to or, in some cases, even exceeded concentrations of compounds detected in whole sediment samples. Fluoranthene was present at a significantly lower aqueous concentration in the treatments with a bacterial consortium

both with and without SediMite™. Pyrene was significantly removed from the aqueous phase in reactors treated with just SediMite™, as well as those including SediMite™ plus the bacterial consortium or the bacterial consortium exclusively. Furthermore, the treatment combination of SediMite™ and the engineered consortium significantly reduced aqueous concentrations of pyrene.

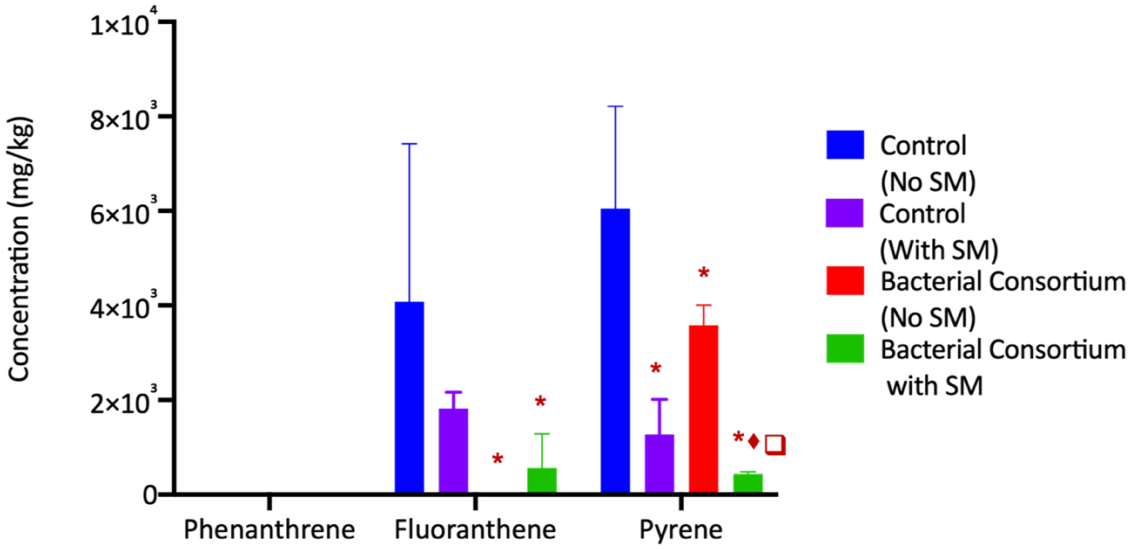

Figure 17: Bar graphs representing concentrations of phenanthrene, fluoranthene, and pyrene in polyethylene passive samplers after a 1 month incubation period with sediments spiked at 2000 mg/kg. Bars reflect experimental replicates (n=4). Asterisks denote marginal differences (p<0.1) from "Control (no SM)", which included reactors that contained sediment spiked at 2000 mg/kg, a polyethylene sampler, and sterile water (15ppt salinity). Diamonds denote marginal differences (p<0.1) from "Control (no SM)", which included reactors that contained sediment spiked at 2000 mg/kg, a polyethylene sampler, and sterile water (15ppt salinity) and "Control (with SM)" which were prepared similarly to the other control reactors, but with the addition of SediMite™. Squares denote statistically significant differences (p<0.05) between the reactors amended with a bacterial consortium and those amended with both a bacterial consortium and the SediMite™.

5.3.3 Prevalence of PAH-degrading organisms

Quantitative PCR was used to enumerate specific bacteria within the consortium inoculated into select reactors. Gene copy numbers were log transformed and significant differences were determined by comparing the control without the inclusion of SediMite™ to the other experimental treatments. Figure 18 indicates gene copy numbers for *Alcaligenes faecalis* after a 1 month incubation with both the low (100 mg/kg) and high (2000 mg/kg) spiked sediments. There was an order of magnitude lower numbers of between *Alcaligenes faecalis* 16S rRNA genes after 1 month in both the low and high concentration control sediments than were present in reactors spiked with the bacterial consortium and the SediMite™. For the low concentration sediments, the reactors with the SediMite™ and consortium included significantly more copies of 16S rRNA genes from *Alcaligenes faecalis* than the consortium without the SediMite™ addition.

For the high concentration sediments, there was not a significant difference in *Alcaligenes faecalis* prevalence in the SediMite™ control reactors compared to the reactors inoculated with the consortium alone ($p<0.05$). However, there was a significant difference between the SediMite™ treated controls and the reactors with the SediMite™ plus the bacterial consortium ($p<0.05$). There were also significant differences in the copies detected in the reactors with the bacterial consortium alone and the consortium plus the SediMite™ ($p < 0.05$). Finally, there were significantly more copies of *Alcaligenes*

faecalis 16S rRNA genes in both of the bacterial consortium treatments at the high concentration than at the low concentration.

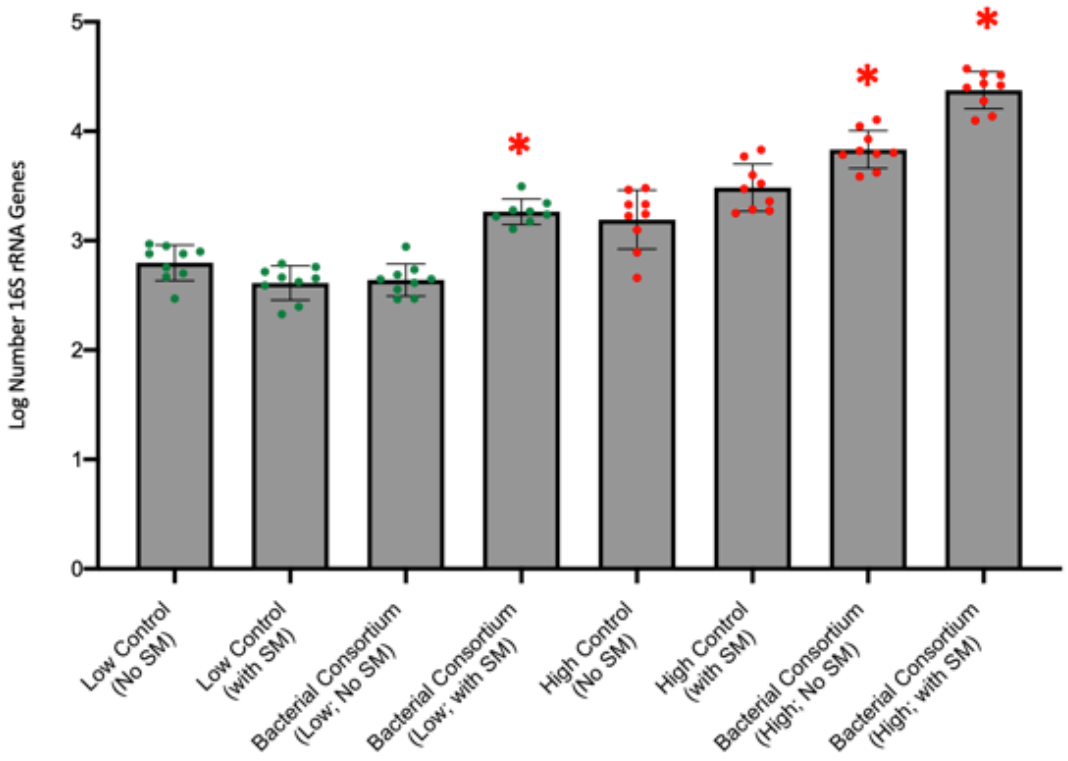

Figure 18: Bar graph indicating 16s rRNA gene copy numbers detected for *Alcaligenes faecalis* in reactors incubated for 1 month.
Bar graph indicating 16S rRNA gene copy numbers detected for *Alcaligenes faecalis* in reactors incubated for 1 month. Y-axis denotes log transformed gene copy numbers and x-axis denotes experimental treatment. The "Low" series represents reactors containing sediment spiked at 100 mg/kg. The "High" series represents reactors containing sediment spiked at 2000 mg/kg. Significance is indicated by a red asterisk ($p \leq 0.05$). Significance was determined by comparing experimental treatments to the Control without SediMite™ ("Low Control, No SM" or "High Control, No SM").

Figure 19 indicates 16S rRNA gene copy numbers for *Stenotrophomonas* sp. after a 1 month incubation with both the low (100 mg/kg) and high (2000 mg/kg) spiked sediments. Overall, there were no significant differences in the gene copy numbers of

Stenotrophomonas sp. in any of the experimental treatments for the low concentration sediments ($p<0.05$). The only experimental treatment that contained significantly greater copies in the high concentration sediments was the one that included the bacterial consortium and the SediMite™ ($p<0.05$). This treatment also contained significantly more *Stenotrophomonas* sp. than the bacterial consortium alone ($p<0.05$). Further, there were significantly more *Stenotrophomonas* sp. in high concentration reactors (except for the high control without SediMite™) compared to the low control without SediMite™.

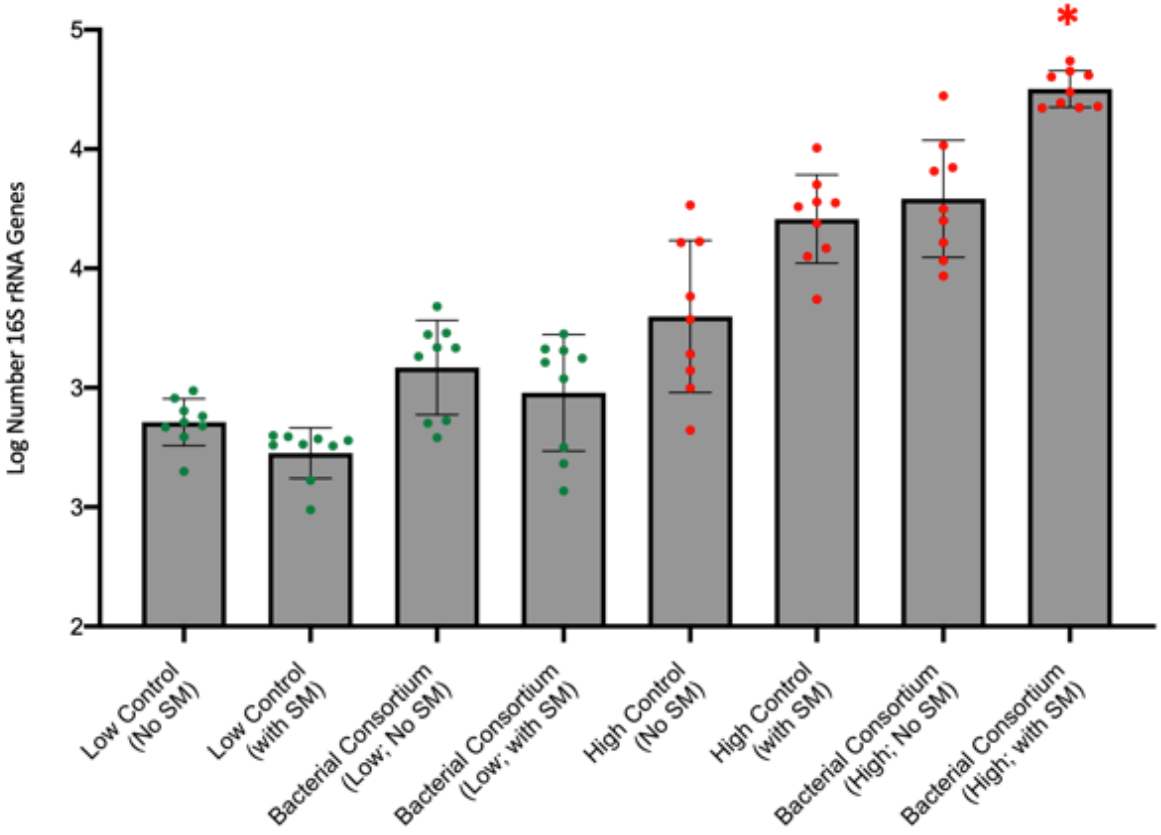

Figure 19: Bar graph indicating gene copy numbers detected for *Stenotrophomonas* sp. in reactors incubated for 1 month. Y-axis denotes log transformed gene copy numbers and x-axis denotes experimental treatment. The "Low" series represents reactors containing sediment

spiked at 100 mg/kg. The "High" series represents reactors containing sediment spiked at 2000 mg/kg. Statistical significance is denoted by a red asterisk (p≤0.05). Significance was determined by comparing experimental treatments to the Control without SediMite™ ("Low Control, No SM" or "High Control, No SM").

Figure 20 shows gene copy numbers for *Alcaligenes faecalis* after 2 months incubation with both the low (100 mg/kg) and high (2000 mg/kg) spiked sediments. For both the low and high concentration sediments, there were significantly (p≤0.05) reduced numbers of *Alcaligenes faecalis* 16S rRNA genes after 2 months for the reactors containing the bacterial consortium with and without the SediMite™ amendment. There was also a significant difference in the low controls that contained SediMite™ versus the controls without the amendment (p<0.05). Gene copy numbers from the 1 month timepoint and 2 month timepoint in the low concentration reactors were generally increased or remained unchanged, except for in the reactors containing SediMite™. In the high concentration series, there were significant reductions in overall *Alcaligenes faecalis* prevalence in the consortium-inoculated reactors from the 1 to 2 months.

Furthermore, the reactors with the inoculated consortium *Alcaligenes faecalis* comprised 2 to 4% of the total bacterial population, as determined by 16S gene copy number quantification (Figures 28 & 30). These results may suggest that this particular organism is either not competitive within the community over time or is highly selective with the PAHs it degrades.

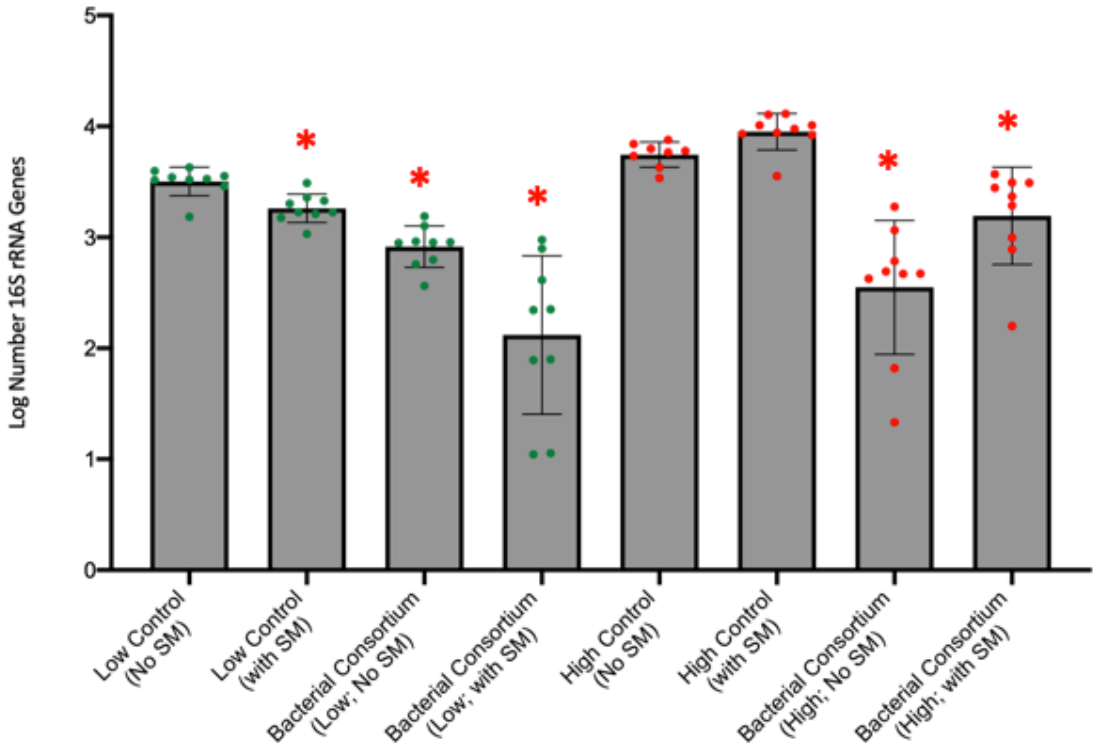

Figure 20: Bar graph indicating gene copy numbers detected for *Alcaligenes faecalis* in reactors incubated for 2 months.
Y-axis denotes log transformed 16S rRNA gene copy numbers and x-axis denotes experimental treatment. The "Low" series represents reactors containing sediment spiked at 100 mg/kg. The "High" series represents reactors containing sediment spiked at 2000 mg/kg. Statistical significance is denoted by a red asterisk ($p \leq 0.05$). Significance was determined by comparing experimental treatments to the Control without SediMite™ ("Low Control, No SM" or "High Control, No SM").

Figure 21 shows gene copy numbers for *Stenotrophomonas* sp. after 2 months incubation with both the low (100 mg/kg) and high (2000 mg/kg) spiked sediments. There were no significant differences ($p \leq 0.05$) between the abundance of *Stenotrophomonas* sp. in any of the experimental treatments at the low concentration. At the high sediment concentration, there were significantly more *Stenotrophomonas* sp. in the reactor

inoculated with the bacterial consortium plus the addition of SediMite™ ($p<0.05$). There was a slight increase in the abundance of these organisms in the low concentration reactors after 2 months compared with the 1 month community, however for the reactors spiked with a higher concentration of PAHs, there was an overall decrease in the prevalence of quantified *Stenotrophomonas* genes.

The overall abundance of *Stenotrophomonas* sp. relative to the entire bacterial community (16S gene quantification) generally remained the same between the 1 month and 2 month timepoints (Figures 29 & 31). Despite a decrease in the copy numbers of this organism, specifically, relative to the entire community, *Stenotrophomonas* sp. abundance appeared to be fairly stable.

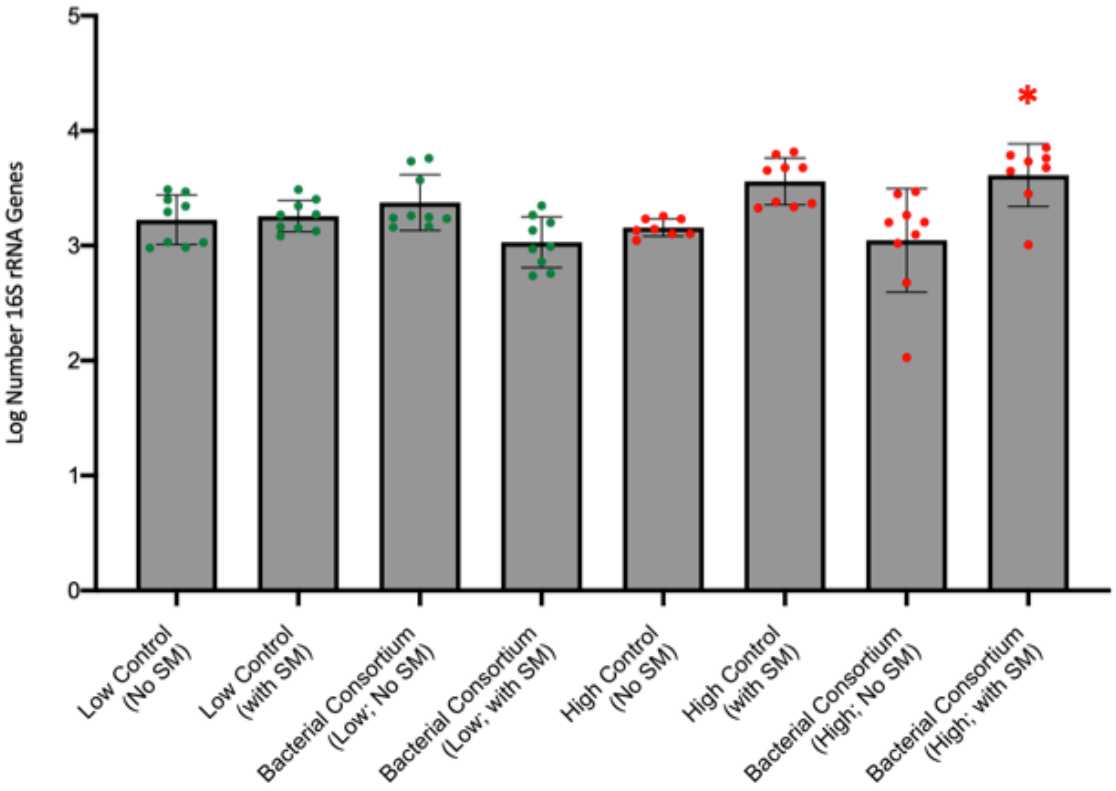

Figure 21: Bar graph indicating gene copy numbers detected for *Stenotrophomonas* sp. in reactors incubated for 2 months. Y-axis denotes log transformed gene copy numbers and x-axis denotes experimental treatment. The "Low" series represents reactors containing sediment spiked at 100 mg/kg. The "High" series represents reactors containing sediment spiked at 2000 mg/kg. Statistical significance is denoted by a red asterisk ($p \leq 0.05$). Significance was determined by comparing experimental treatments to the Control without SediMite ("Low Control, No SM" or "High Control, No SM").

5.4 Discussion

5.4.1 SediMite™ Technology

While bioaugmentation is a promising strategy for the long-term removal of PAHs, delivery of organisms to sediments remains challenging[14]. One possible solution is

to deliver immobilized organisms on particles, which can offer cells a protective growth support structure for colonization[98]. Such delivery mechanisms can either be nutritive (i.e. chitin[87]) or non-nutritive (i.e. biochar[98] or nylon[20]), but should generally made from environmentally friendly materials due to the irretrievability of the application[98].

At sites heavily-contaminated with PAHs, such as the RC site from this study, using a substrate that can also serve as a remediation technology may be beneficial for reaching regulation standards within a reasonable period of time. Advancing remediation rates and enhancing overall contaminant removal and degradation can be achieved by combining physical and biological remediation approaches. Developed originally as an activated-carbon based, composite amendment for the rapid absorption of hydrophobic organic compounds, with this study the use of the SediMite™ amendment has been expanded to serve as a growth support structure for the delivery of microorganisms[14,99]. The weight and slow-decomposition of the amendment allow for the product to be more easily and efficiently introduced to sediments, with reduced product loss and more precise application. Further, these characteristics may improve the administration of an engineered bioaugmentation strategy such that the amendment and adhered community can remain stable while passing through the water column to sediments.

While these data indicate a positive correlation between bioaugmentation with amendments and removal of PAHs, future refinement of the method is required to deploy the bioamendment technology proposed in this chapter most efficiently. These

steps include optimizing cell concentrations and biofilm attachment, determining an appropriate application method for the site, and scaling up production from the bench. Typically, amendments are delivered to sediment either through layering over the surface, tilling, mixing, or injection into the sediment, or by including the amendment in a mixture with a traditional cap a mixture with a traditional cap[89] – however the optimal delivery strategy was not explicitly tested in this study and cannot be determined from our limited data.

5.4.2 PAH Remediation

The work presented in this chapter features phenanthrene, fluoranthene, and pyrene concentrations in sediment and PE after a 1 month incubation with sediments spiked at either 100 mg/kg or 2000 mg/kg. Because the fraction of PAHs bound to the SediMite™ amendment cannot be analytically measured independent from whole sediment concentrations, the sediment concentrations between controls with and without SediMite™ were not expected to and did not show significant PAH reduction, and indeed this was the observed result. However, it had been previously demonstrated that the addition of the SediMite™ amendment to sediment contaminated with hydrophobic organic compounds could significantly reduce porewater concentrations, which likely represent the largest fraction of bioavailable PAHs[14], therefore reducing the overall risk to the benthic and aquatic ecosystem. In this study the concentration of PAHs in porewater followed expected trends with the addition of SediMite™ and the

bacterial consortium for fluoranthene and pyrene added at 2000 mg/kg. An opposite effect was observed at the 100 mg/kg concentration of fluoranthene in which the concentrations of porewater from reactors with SediMite™ were higher than controls without the AC.

Further, the PE data for both the low and high concentrations sometimes exceeded the PAH concentrations measured in the sediment fraction. This finding suggests that the PE sampler may have been serving as a sink and concentrating the target chemicals. Those measurements also featured large variations between experimental replicates, suggesting that technical replication may be required. This was further validated by the lack of parallel variance between the biological replicates measured for the same reactors and treatments.

For the reactors containing a lower concentration of spiked PAHs the data obtained in this work do not show a clear impact of any of the selected remediation strategies, either the SediMite™ alone or when paired with the bacterial consortium. In contrast, the high-concentration reactors demonstrated the expected trend of reduced concentrations of all three PAHs after incubation with the bacterial consortium, with even more pronounced and significant reductions with the consortium plus the SediMite™. However, concentrations from whole sediments were markedly lower from concentrations measured in originally spiked sediments (Table 8) for all compounds measured. This may have been due to volatilization of the compound during the sediment spiking process or due to variability with chemical extractions[7] and

measurements, which may be resolved by running replicate analytical samples of originally spiked sediments.

5.4.3 Bioaugmentation Efficacy

PAHs can have deleterious effects on organisms at high concentrations, including microorganisms. In this work, the use of a low and high concentration chemical mixture was to determine if there were negative, or toxic, effects on the bacterial community at higher concentrations, and whether AC amendment could protect introduced bacteria from these effects. In addition, we speculated that the AC amendment might be beneficial in circumstances where metabolites of PAHs produced through microbial biotransformation induce greater toxic effects than parent compounds, a phenomenon which is not well understood[100]. AC amendments could serve to sequester and remove these compounds, thereby reducing their bioavailability and potentially toxic effects to the aquatic ecosystem[101].

While the direct production of toxic metabolites was not investigated in this work, it appeared that in reactors with the higher concentration of PAHs, the inclusion of SediMite™ led to increased cellular abundance of both introduced *Alcaligenes faecalis* and *Stenotrophomonas* sp. bacteria at the 1 month timepoint. This suggests that the SediMite™ may have created environmental conditions for the successful growth of this organism, perhaps due to the amendment is absorbing and sequestering bioavailable PAHs, as reflected by significant reductions in fluoranthene and pyrene

porewater concentrations. However, we began to observe a downward trend in the prevalence of these bacteria for the 2 month samples at the lower concentrations of PAHs, suggesting that at such concentrations the bioavailablility of the carbon substrates may be too low to sustain the nutritional demands of the bioaugmented community. This could be optimized in future experiments by altering the percent of SediMite™ added to sediment and changing the concentration of bacterial cells inoculated.

We observed a decrease in the relative bacterial abundance of both *Alcaligenes faecalis* and *Stenotrophomonas* sp. in the bacterial communities within high-concentration PAH reactors, as observed by the gene copy numbers normalized to total 16S rRNA gene copy number (Figures 28-31). This trend was not consistently correlated to the absolute gene copy numbers for these organisms, suggesting that as the microbial community equilibrates, these organisms may not have retained their competitive edge within the community. This trend might be further evaluated with the addition of enumerating the other organisms inoculated within the consortium, or through microbiome analyses of the total community structure to determine major shifts in the microbial communities..

An additional explanation for the change in prevalence of *Alcaligenes faecalis* and *Stenotrophomonas* sp. in the high concentration reactors with SediMite™ from the 1 month timepoint and the 2 month timepoint may be provided by the inoculation method. The addition of the bacterial consortium with the SediMite™ amendment may

have provided these organisms with a growth support structure that allowed them rapid integration with the sediments where substrates were prevalent due to solubility limitations. However, as the substrate became more limited (due to both degradation and strong sorption to the SediMite™), bacterial growth rates began to stagnate or even decrease. This hypothesis is supported by the relative abundance of target organisms to total 16S rRNA gene normalized data (Figures 28-31).

6. Conclusion

6.1 Conclusions from Chapter 3

The goal of this work was to identify and isolate PAH-degrading organisms from creosote-contaminated sediment in the RC site. We used multiple methods to characterize and isolate functional PAH-degrading bacteria in creosote contaminated sediments from a site in the Elizabeth River, VA. We further analyzed a next-generation sequencing amplicon library to determine dominant members of the bacterial community and the prevalence of these specific PAH-degrading bacteria within PAH contaminated sediment. The low relative abundance of isolated or SIP-detected PAH-degrading bacteria in the community suggests that a bioaugmentation strategy could be a good approach to remediating these polluted sediments.

The findings from this work will inform a bioaugmentation strategy utilizing these indigenous strains of bacteria to enhance rates of PAH biodegradation at the RC site. The focus of using environmentally-relevant conditions to isolate active and prominent PAH-degrading bacteria may be utilized to increase rates of contaminant degradation, which could result in reduced technology failure and decrease the financial burden of remediation costs to responsible parties.

6.2 Conclusions from Chapter 4

In this work, we aim to improve extrinsic bioremediation strategies by developing a systematic method for selecting autochthonous bacteria to participate in an optimized

biofilm for PAH-degradation. We designed experiments to confirm that the biofilm structure could be a potentially successful unit for the bioaugmentation of a creosote-contaminated sediment site in the Elizabeth River, VA by confirming the organisms could degrade multiple contaminants of varying molecular weight, assemble in a cooperative biofilm unit, and demonstrate a sufficient respiration capacity.

Future work should evaluate the potential for these organisms to degrade wider variety of PAHs when assembled in the biofilm structure. Additional work is necessary to confirm if biofilm respiration rates are affected by the introduction of PAHs as a substrate to the media. This particular experiment could be difficult due to the likely adsorption of PAHs to the propriety and required plastic microtiter plates used in the Seahorse XFe system. More work is required to evaluate the community dynamics of the assembled consortium and if there are synergistic or competitive interactions between the bacteria selected for use in the consortium.

6.3 Conclusions from Chapter 5

This work aimed to validate the use of an activated-carbon based technology (SediMite™) for the remediation of PAHs and the delivery of an optimized bacterial consortium to creosote contaminated sediments. Reactors were assembled in a factorial design to test the influence of the amendment and the bacterial consortium on phenanthrene, fluoranthene, and pyrene concentrations in both sediment and porewater. This work was the first to explore the use of the activated carbon-based

remediation technology, SediMite™ amendment for the remediation PAHs. Here we tested two concentrations of a PAH mixture to determine how bacterial activity may be affected by extremely high concentrations of PAHs and if the SediMite™ amendment may be able to rescue potentially toxic effects.

Chemical biodegradation in whole sediments was only reliably observed at the high concentrations and followed expected trends with respect to the experimental parameters of SediMite™ and bacterial consortium inclusion. Aqueous concentrations measured exceeded expected solubility and calculated partitioning and will require the evaluation of technical replicates in future studies of this type. The addition of the biological consortium did not affect bacterial gene copy numbers in the low concentration reactors but remained prominent in the high concentration reactors during the 1 month timepoint. Future work should evaluate the appropriate cell concentrations for reactor inoculation, as well as the potential for stages of inoculation to promote chemical degradation.

6.4 Engineering Significance

This dissertation aimed to characterize Elizabeth River sediment bacterial communities and develop a bioaugmentation strategy for Republic Creosoting site sediments using a biofilm comprised of PAH-degrading bacteria. This work evaluates the combined efficacy of physical and biological remediation technologies for the reduction of PAH contaminants in environmentally relevant polluted sediment. The work

presented in this dissertation utilizes strategies that can be employed at other sites hosting various classes of chemical contaminants. Specifically, the utilization of both molecular and culture-based methods to identify and isolate contaminant-degrading bacteria may be employed using other chemical selection pressures to characterize functional and viable microorganisms for bioaugmentation. Further, using the bioamendment technology described within this dissertation may be a promising method for both absorbing hydrophobic compounds, as well as for deploying microorganisms to difficult to access matrices, such as sediment.

Appendix A: Supplementary Information for Chapter 3

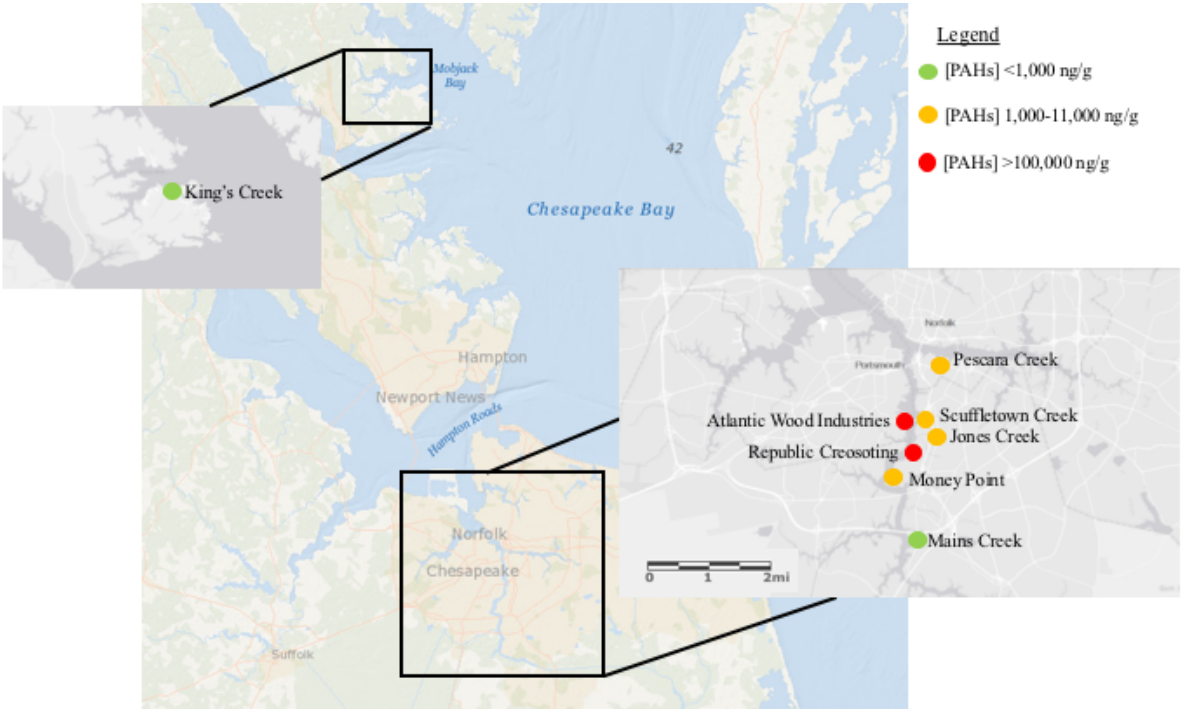

Figure 22: Map of study sites located in the Elizabeth River, Virginia. Total PAH concentrations reported in ng/g (ppb[53]).

Table 6: Relative Abundances of ASVs in 16S amplicon libraries for DNA-SIP clones.

Sample Name	Croceicoccus sp.	Azoarcus sp.	Hydrogenophaga sp.
SC 1	0	0	0
SC 2	0	0	0
SC 3	0	0	0
SC 4	0.001119169	0.010027755	0.006715015
SC 5	0	0	0
SC 6	0	0	0
SC 7	0	0	0.018994152
SC 8	0.001015203	0.007329273	0.007948299
SC 9	0.000719205	0.001590969	0.012695057
KC 1	0	0.001159306	0.015382005
KC 2	0	0	0
KC 3	0	0	0
KC 4	0	0	0
KC 5	0	0	0
KC 6	0	0	0
KC 7	0.001553115	0.020364824	0.016133378
KC 8	0	0	0
KC 9	0	0.000757621	0.005153817
REP 1	0	0	0.001626293
REP 2	0.000282282	0	0.003757873
REP 3	0.00025377	0.002088167	0.009918794
REP 4	0	0	0.001576271
REP 5	0	0	0
REP 6	0	0	0.001374138
REP 7	0	0	0
REP 8	0	0	0
REP 9	0	0.020593763	0.003615456
MC 1	0.001394203	0.009990041	0.011777347
MC 2	0	0	0
MC 3	0	0	0
MC 4	0	0	0.000664912
MC 5	0.000452882	0.001726611	0.00932181
MC 6	0.002015312	0.011528773	0.004524574
MC 7	0	0	0.00100854
MC 8	0	0	0.000766019
MC 9	0	0	0

Table 7: Percent identity matrix of *Hydrogenophaga taeniospiralis* identified through DNA-SIP incubations and isolations.

Cells highlighted in green represent isolated *H.taeniospiralis* and blue cells represent those identified in DNA-SIP incubations. Numbers are reported as percentages.

Rep38	100	92.42	92.42	91.21	91.69	91.73	91.43	91.49	92.25	92.19	92.13	91.71
Rep61	92.42	100	100	99.23	99.63	99.63	99.44	99.44	100	100	99.82	99.63
Rep62	92.42	100	100	99.23	99.63	99.63	99.44	99.45	100	100	99.82	99.63
P2_38	91.21	99.23	99.23	100	99.22	99.23	99.03	99.03	99.23	99.22	99.42	99.22
P2_45	91.69	99.63	99.63	99.22	100	99.67	99.48	99.48	99.63	99.65	99.83	99.66
P1_26	91.73	99.63	99.63	99.23	99.67	100	99.48	99.49	99.63	99.65	99.83	99.66
P1_47	91.43	99.44	99.44	99.03	99.48	99.48	100	99.31	99.45	99.47	99.66	99.48
P2_36	91.49	99.44	99.45	99.03	99.48	99.49	99.31	100	99.45	99.47	99.66	99.49
Rep59	92.25	100	100	99.23	99.63	99.63	99.45	99.45	100	100	99.82	99.63
Rep60	92.19	100	100	99.22	99.65	99.65	99.47	99.47	100	100	99.82	99.65
P2_33	92.13	99.82	99.82	99.42	99.83	99.83	99.66	99.66	99.82	99.82	100	99.83
P2_35	91.71	99.63	99.63	99.22	99.66	99.66	99.48	99.49	99.63	99.65	99.83	100
Rep59	92.25	100	100	99.23	99.63	99.63	99.45	99.45	100	100	99.82	99.63
Rep60	92.19	100	100	99.22	99.65	99.65	99.47	99.47	100	100	99.82	99.65
P2_33	92.13	99.82	99.82	99.42	99.83	99.83	99.66	99.66	99.82	99.82	100	99.83
P2_35	91.71	99.63	99.63	99.22	99.66	99.66	99.48	99.49	99.63	99.65	99.83	100

Appendix B: Supplementary Information for Chapter 4

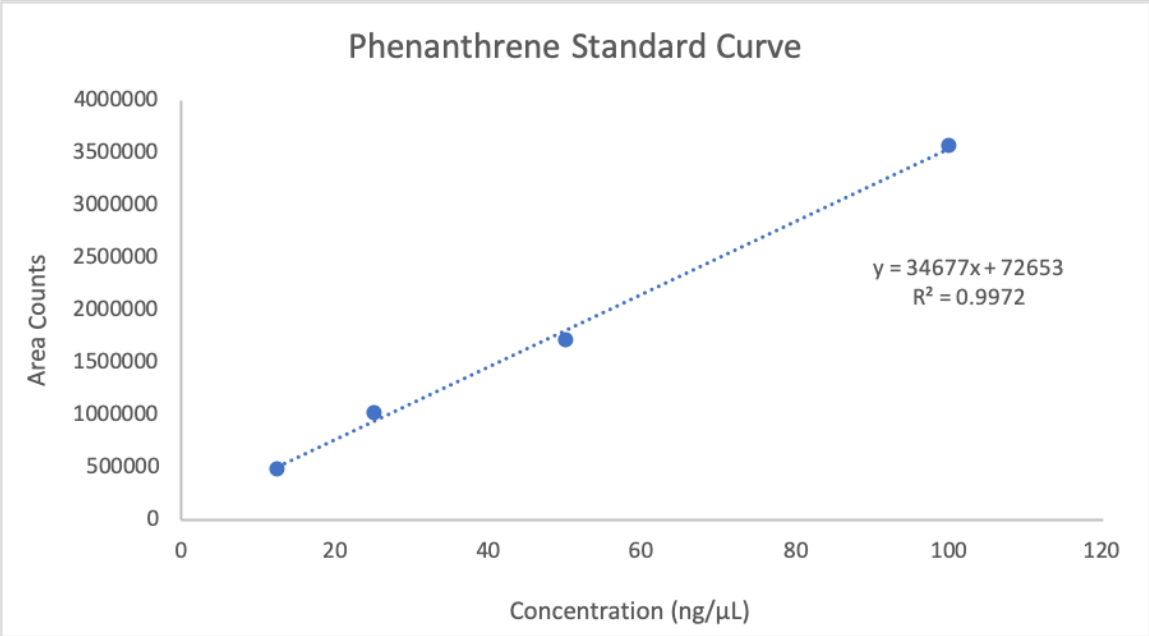

Figure 23: Standard Curve for phenanthrene concentrations as measured using HPLC.
Equation for trendline was used to extrapolate measured values in experimental samples.

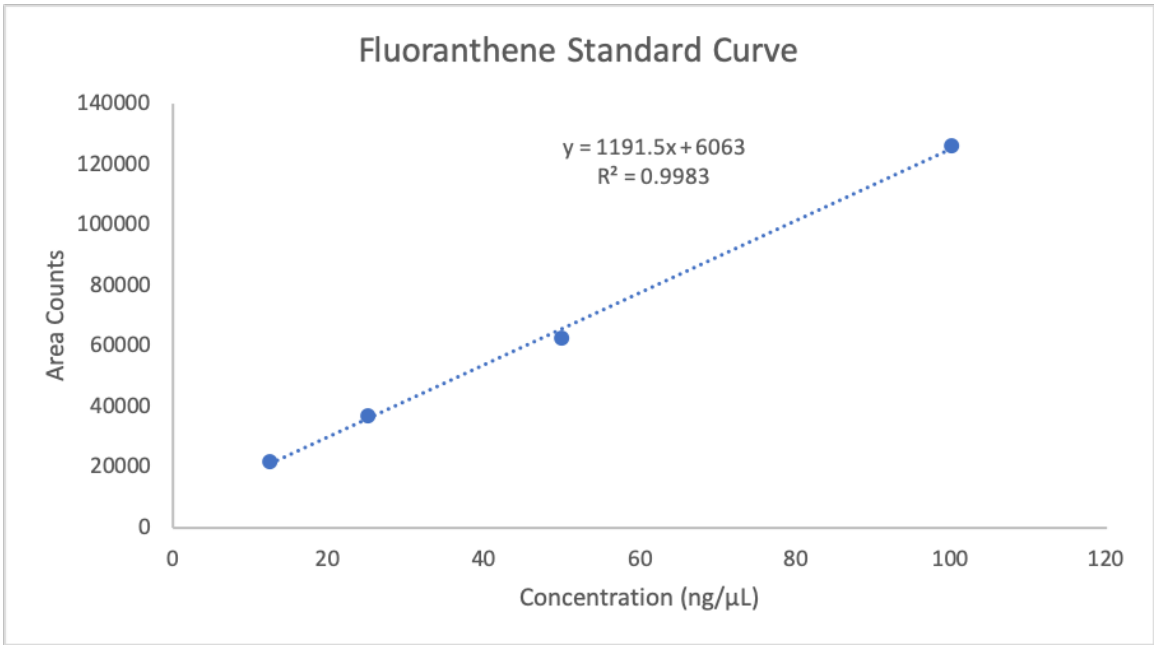

Figure 24: Standard Curve for fluoranthene concentrations as measured using HPLC.
Equation for trendline was used to extrapolate measured values in experimental samples.

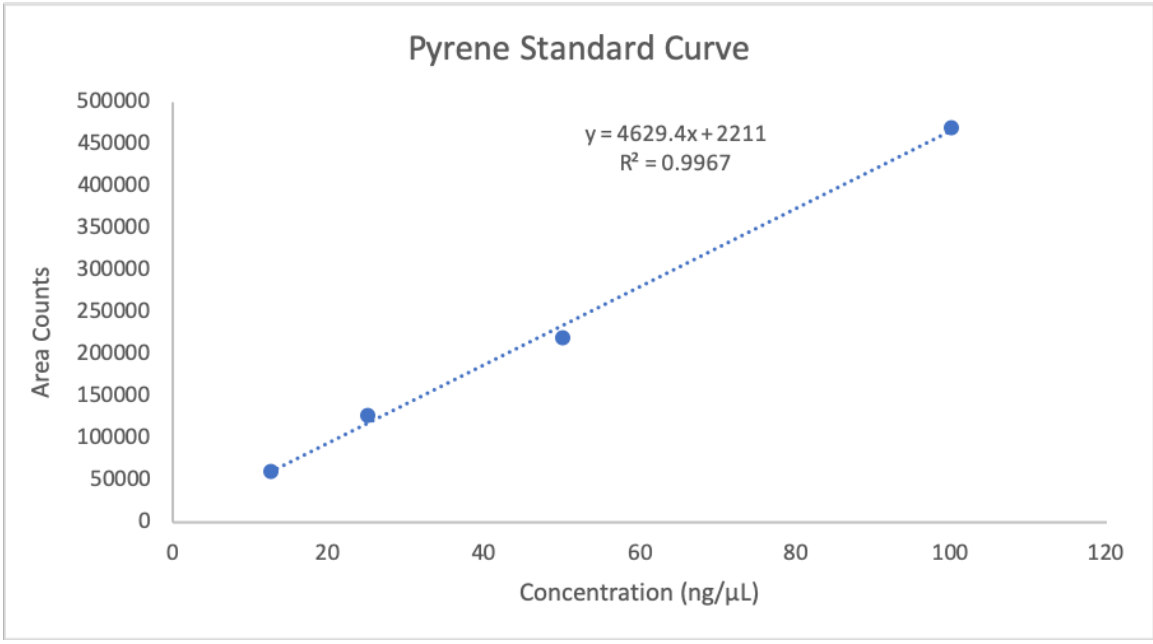

Figure 25: Standard Curve for pyrene concentrations as measured using HPLC. Equation for trendline was used to extrapolate measured values in experimental samples.

Appendix C: Supplementary Information for Chapter 5

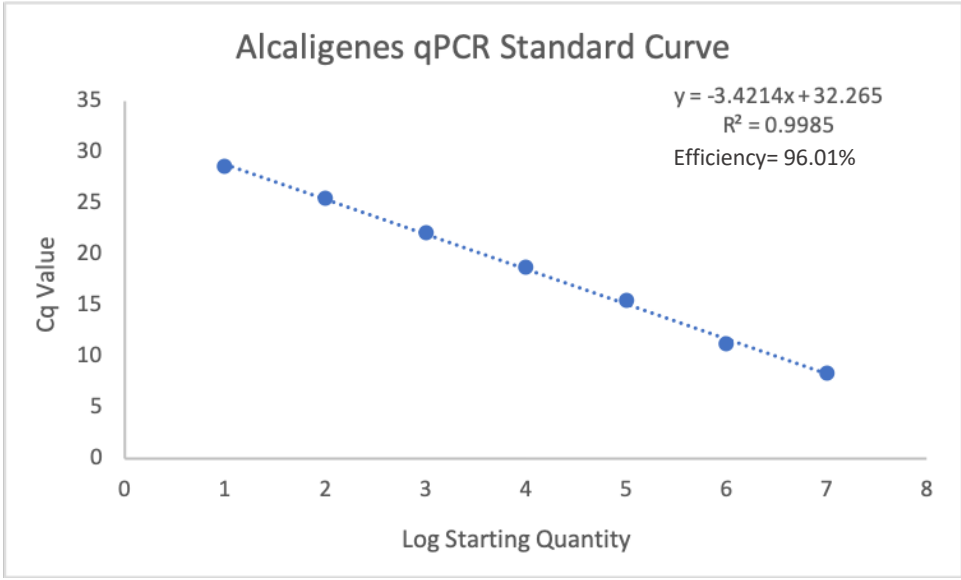

Figure 26: Quantitative PCR standard curve for novel primers designed to amplify the 16S rRNA region of *Alcaligenes faecalis*.
gBlocks (Integrated DNA Technologies, Inc.; Coralville, IA) were designed for use as the template to generate the standard curve.

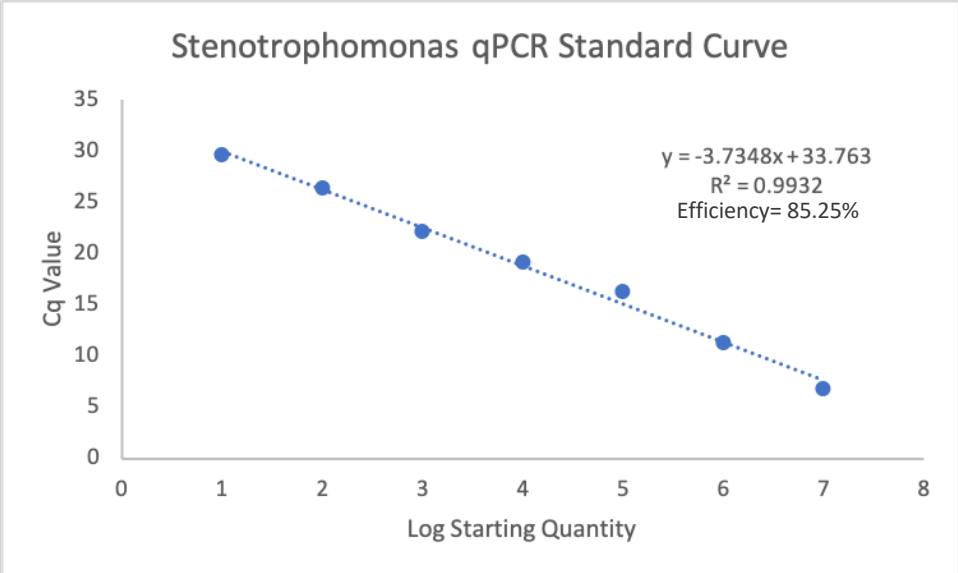

Figure 27: Quantitative PCR standard curve for novel primers designed to amplify the 16S rRNA region of *Stenotrophomonas* sp.
gBlocks (Integrated DNA Technologies, Inc.; Coralville, IA) were designed for use as the template to generate the standard curve.

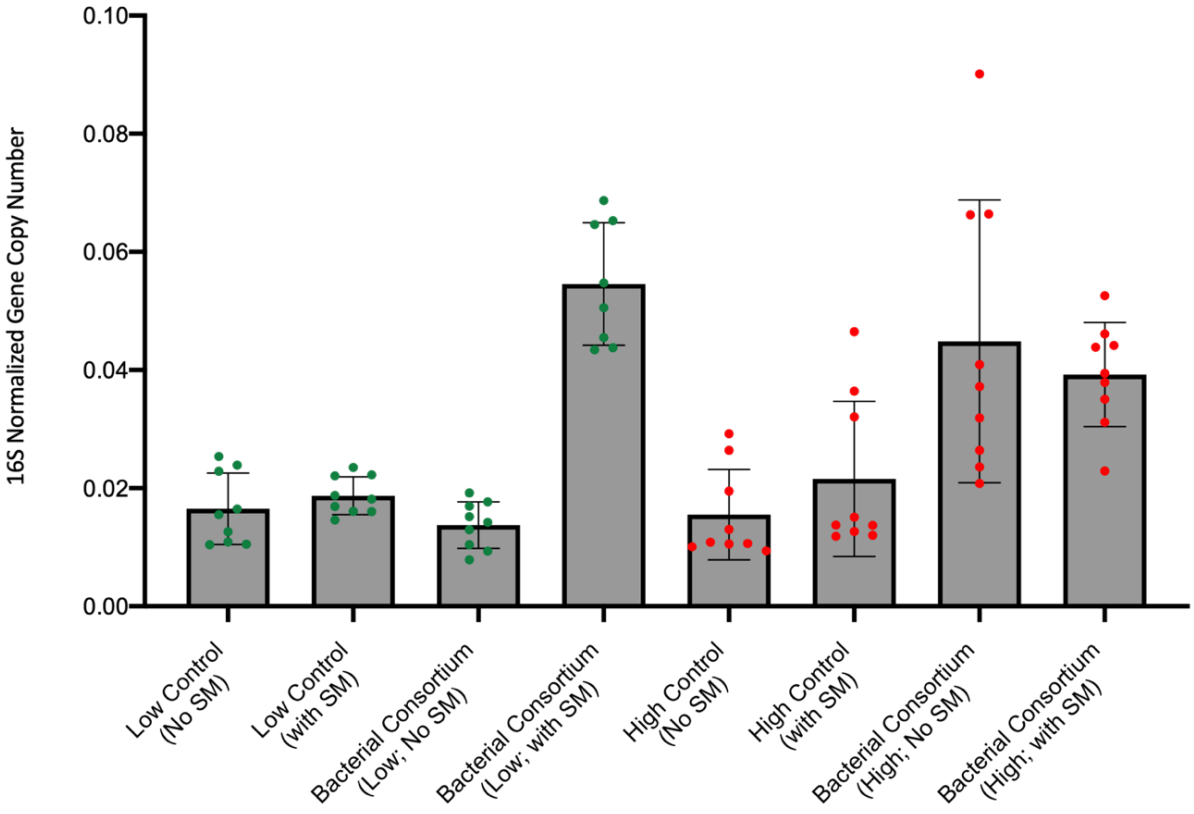

Figure 28: Bar graph indicating gene copy numbers detected for *Alcaligenes faecalis* in reactors incubated for 1 month.

Y-axis denotes *Alcaligenes faecalis* 16S rRNA gene copy numbers normalized to total 16S rRNA gene copy numbers. X-axis denotes experimental treatment. The "Low" series represents reactors containing sediment spiked at 100 mg/kg. The "High" series represents reactors containing sediment spiked at 2000 mg/kg.

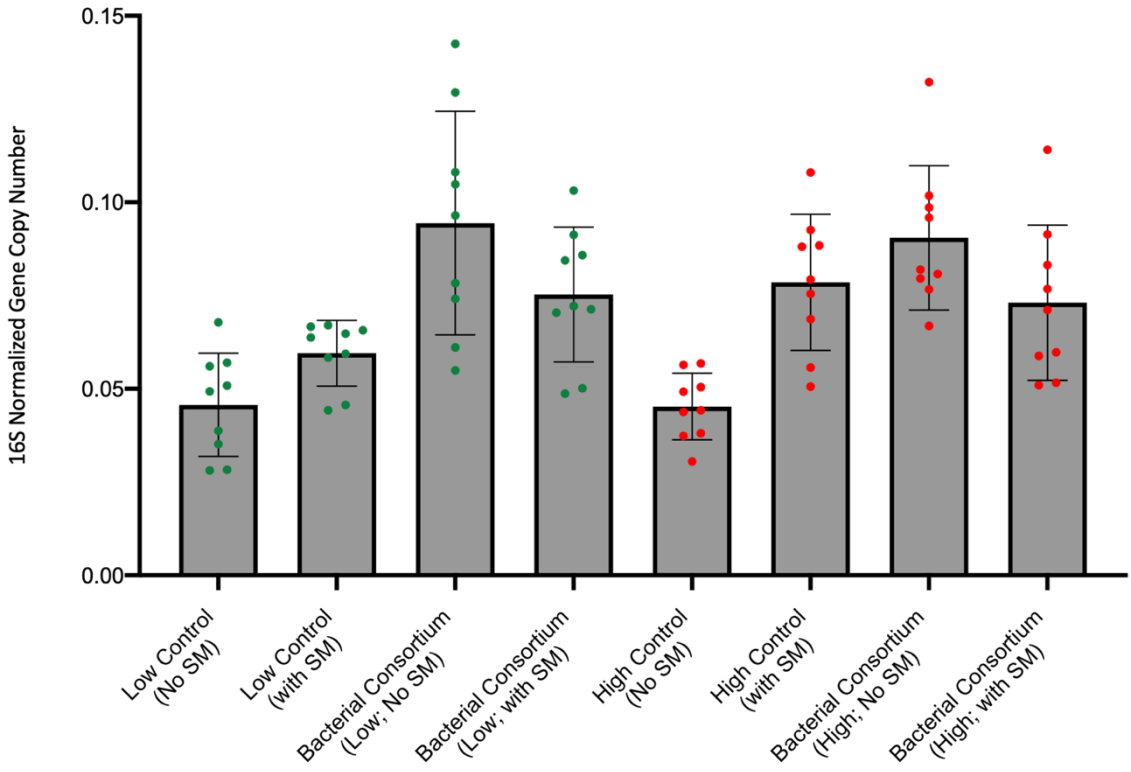

Figure 29: Bar graph indicating gene copy numbers detected for *Stenotrophomonas* sp. in reactors incubated for 1 month. Y-axis denotes *Stenotrophomonas* sp. 16S rRNA gene copy numbers normalized to total 16S rRNA gene copy numbers. X-axis denotes experimental treatment. The "Low" series represents reactors containing sediment spiked at 100 mg/kg. The "High" series represents reactors containing sediment spiked at 2000 mg/kg.

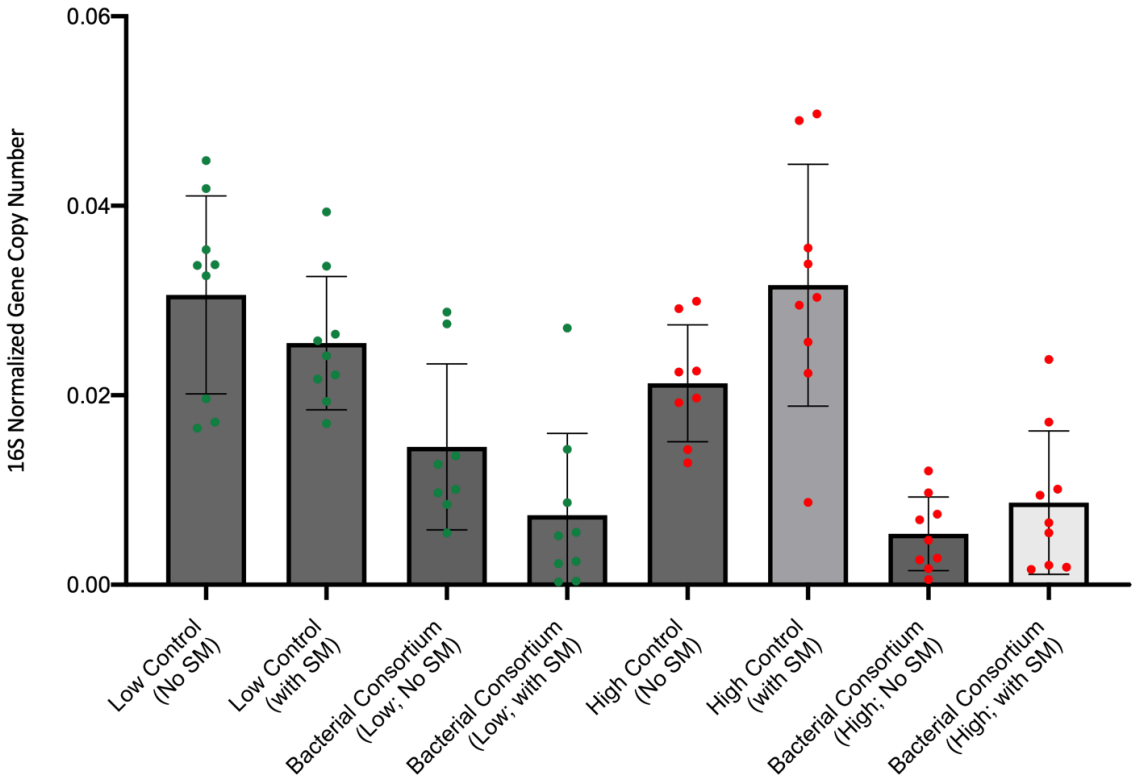

Figure 30: Bar graph indicating gene copy numbers detected for *Alcaligenes faecalis* in reactors incubated for 2 months.

Y-axis denotes *Alcaligenes faecalis* 16S rRNA gene copy numbers normalized to total 16S rRNA gene copy numbers. X-axis denotes experimental treatment. The "Low" series represents reactors containing sediment spiked at 100 mg/kg. The "High" series represents reactors containing sediment spiked at 2000 mg/kg.

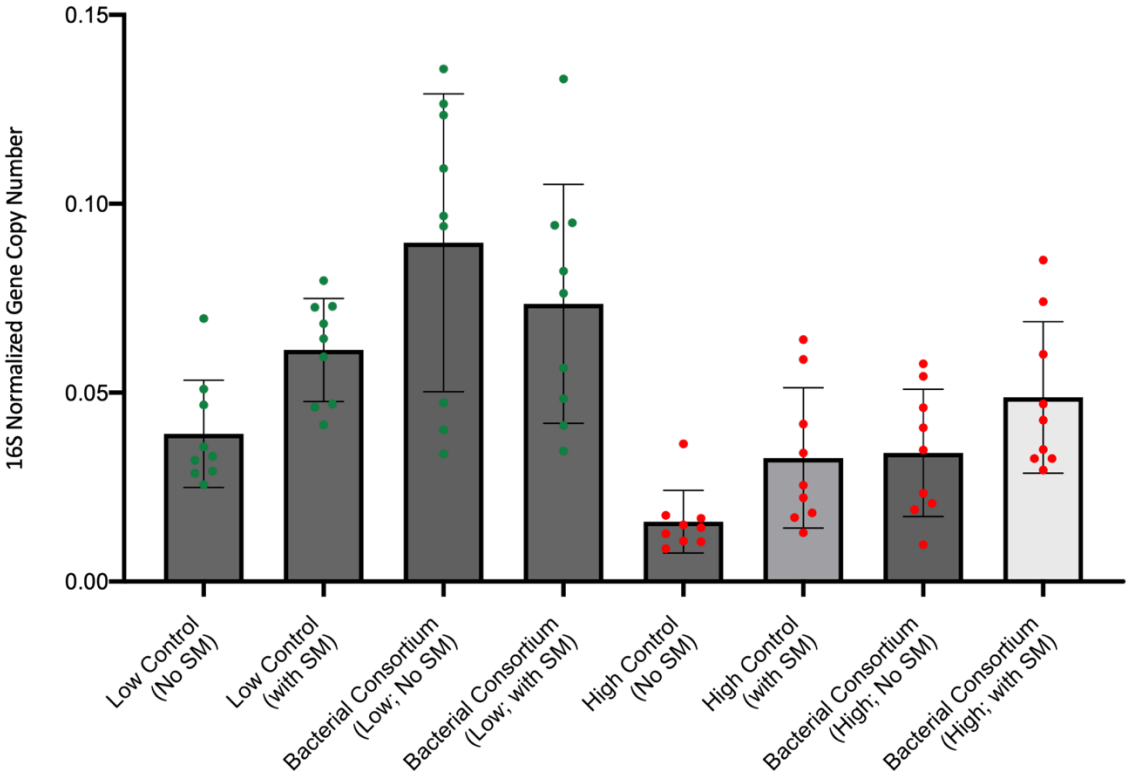

Figure 31: Bar graph indicating gene copy numbers detected for *Stenotrophomonas* sp. in reactors incubated for 2 months. Y-axis denotes *Stenotrophomonas* sp. 16S rRNA gene copy numbers normalized to total 16S rRNA gene copy numbers. X-axis denotes experimental treatment. The "Low" series represents reactors containing sediment spiked at 100 mg/kg. The "High" series represents reactors containing sediment spiked at 2000 mg/kg.

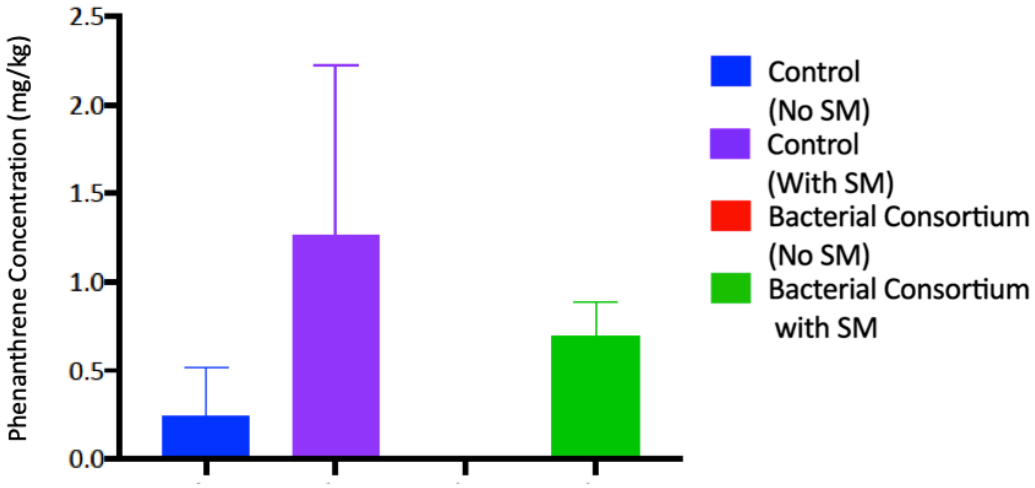

Figure 32: Bar graph representing the concentration of phenanthrene in polyethylene passive samplers after a 1 month incubation period with sediments spiked at 100 mg/kg. Bars reflect experimental replicates (n=4).

Table 8: Concentrations of PAHs measured in time zero spiked sediment and PE.

Values are reported in mg/kg and time zero sediments were run in triplicate with standard deviation reported below. PE was not run with triplicate analytical samples.

PAH	Measured Sediment Concentration for Time 0, 100 mg/kg (mg/kg)	Standard Deviation of Sediment Concentration	Measured Sediment Concentration for Time 0, 2,000 mg/kg (mg/kg)	Standard Deviation of Sediment Concentration	PE Concentration (mg/kg)
Phenanthrene	32.30	3	830	290	393.68
Fluoranthene	56.8	5.1	1430	440	473.55
Pyrene	36.9	3.4	960	270	264.56

References

1. Cerniglia, C. E. Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation* **3**, 351–368 (1992).

2. Zhang, X.-X., Cheng, S.-P., Zhu, C.-J. & Sun, S.-L. Microbial PAH-Degradation in Soil: Degradation Pathways and Contributing Factors. *Pedosphere* **16**, 555–565 (2006).

3. Johnsen, A. R., Wick, L. Y. & Harms, H. Principles of microbial PAH-degradation in soil. *Environ. Pollut.* **133**, 71–84 (2005).

4. White, D. C. Environmental Biotechnology:Principles and Applications. *Curr. Opin. Biotechnol.* **7**, 357–365 (1996).

5. DiGiulio, R. T. *et al.* The Elizabeth River Story: A Case Study in Evolutionary Toxicology. *J. Toxicol. Environ. Heal. Part B* **18**, 259–298 (2015).

6. Juhasz, A. L. & Naidu, R. Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: A review of the microbial degradation of benzo[a]pyrene. *Int. Biodeterior. Biodegrad.* **45**, 57–88 (2000).

7. Edwards, N. T. Polycyclic Aromatic Hydrocarbons (PAHs) in the Terrestrial Environment-A Review. *J. Environ. Qual.* **12**, 427–441 (1983).

8. Nikolaou, A. D., Kostopoulou, M., Lofrano, G. & Meric, S. Determination of PAHs in marine sediments: analytical methods and environmental concerns. *Glob. NEST J.* **11**, 391–405 (2009).

9. Morelli, I. *et al. Fungi as Bioremediators*. **32**, (Springer Berlin Heidelberg, 2013).

10. Bastiaens, L. *et al.* Isolation of adherent polycyclic aromatic hydrocarbon (PAH) - degrading bacteria using PAH-sorbing carriers. *Appl. Environ. Microbiol.* **66**, 1834–1843 (2000).

11. Clark, B. W., Cooper, E. M., Stapleton, H. M. & Giulio, R. T. Di. Compound and Mixture-Specific Differences in Resistance to Polycyclic Aromatic Hydrocarbons and PCB-126 among Fundulus heteroclitus Subpopulations throughout the Elizabeth River Estuary (Virginia, USA). *Environ. Sci. Technol.* **47**, 10556–10566 (2013).

12. Boonchan, S., Britz, M. L. & Stanley, G. a. Degradation and mineralization of high

molecular weight polycyclic armoatic hydrocarbons by defined fungal bacterial cocultures. *Appl. Environ. Microbiol.* **66**, 1007–1019 (2000).

13. Billiard, S. M., Meyer, J. N., Wassenberg, D. M., Hodson, P. V. & Di Giulio, R. T. Nonadditive effects of PAHs on early vertebrate development: Mechanisms and implications for risk assessment. *Toxicol. Sci.* **105**, 5–23 (2008).

14. Payne, R. B., Ghosh, U., May, H. D., Marshall, C. W. & Sowers, K. R. Mesocosm Studies on the Efficacy of Bioamended Activated Carbon for Treating PCB-Impacted Sediment. *Environ. Sci. Technol.* **51**, 10691–10699 (2017).

15. Sass, B. M., Fimmen, R. L., Foote, E. A., Magar, V. S. & Ghosh, U. *Characterization of Contaminant Migration Potential Through In-Place Sediment Caps (ER-1370)*. (2009).

16. Varjani, S. J. Microbial degradation of petroleum hydrocarbons. *Bioresour. Technol.* **223**, 277–286 (2017).

17. U.S. EPA. *Use of Bioremediation at Superfund Sites*. (2001).

18. Aranda, E. Promising approaches towards biotransformation of polycyclic aromatic hydrocarbons with Ascomycota fungi. *Curr. Opin. Biotechnol.* **38**, 1–8 (2016).

19. Silva, I. S., Grossman, M. & Durrant, L. R. Degradation of polycyclic aromatic hydrocarbons (2-7 rings) under microaerobic and very-low-oxygen conditions by soil fungi. *Int. Biodeterior. Biodegrad.* **63**, 224–229 (2009).

20. Mrozik, A. & Piotrowska-Seget, Z. Bioaugmentation as a strategy for cleaning up of soils contaminated with aromatic compounds. *Microbiol. Res.* **165**, 363–375 (2010).

21. Ikuma, K. & Gunsch, C. K. Genetic bioaugmentation as an effective method for in situ bioremediation: Functionality of catabolic plasmids following conjugal transfers. *Bioengineered* **3**, (2012).

22. Ikuma, K. & Gunsch, C. K. Successful genetic bioaugmentation with Pseudomonas putida for toluene degradation in soil columns. *Environ. Chem. Lett.* **11**, 365–370 (2013).

23. Redfern, L. K. Microbial Communities and Polycyclic Aromatic Hydrocarbons:

Exposure Related Adaptations in Environmental Microbiomes and Their Potential ofr Bioremediation. (Duke University, 2017).

24. Johnsen, A. R. & Karlson, U. Evaluation of bacterial strategies to promote the bioavailability of polycyclic aromatic hydrocarbons. *Appl. Microbiol. Biotechnol.* **63**, 452–459 (2004).
25. Gkorezis, P. *et al.* The interaction between plants and bacteria in the remediation of petroleum hydrocarbons: An environmental perspective. *Front. Microbiol.* **7**, 1–27 (2016).

26. Ghosal, D., Ghosh, S., Dutta, T. K. & Ahn, Y. Current state of knowledge in microbial degradation of polycyclic aromatic hydrocarbons (PAHs): A review. *Front. Microbiol.* **7**, (2016).

27. Daane, L. L., Harjono, I., Zylstra, G. J. & Häggblom, M. M. Isolation and Characterization of Polycyclic Aromatic Hydrocarbon-Degrading Bacteria Associated with the Rhizosphere of Salt Marsh Plants Isolation and Characterization of Polycyclic Aromatic Hydrocarbon-Degrading Bacteria Associated with the Rhizosphere . *Appl. Environ. Microbiol.* **67**, 2683–2691 (2001).

28. Festa, S., Coppotelli, B. M. & Morelli, I. S. Bacterial diversity and functional interactions between bacterial strains from a phenanthrene-degrading consortium obtained from a chronically contaminated-soil. *Int. Biodeterior. Biodegrad.* **85**, 42–51 (2013).

29. Hilyard, E. J., Jones-Meehan, J. M., Spargo, B. J. & Hill, R. T. Enrichment, Isolation, and Phylogenetic Identification of Polycyclic Aromatic Hydrocarbon-Degrading Bacteria from Elizabeth River Sediments. *Appl. Environ. Microbiol.* **74**, 1176–1182 (2008).

30. Andreoni, V. *et al.* Bacterial communities and enzyme activities of PAHs polluted soils. *Chemosphere* **57**, 401–412 (2004).

31. Møller, J. & Ingvorsen, H. Biodegradation of phenanthrene in soil microcosms stimulated by an introduced Alcaligenes sp. *FEMS Microbiol. Lett.* **102**, 271–278 (1993).

32. Haritash, A. K. & Kaushik, C. P. Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. *J. Hazard. Mater.* **169**, 1–15 (2009).

33. Berlanga, M. & Guerrero, R. Living together in biofilms: The microbial cell factory

and its biotechnological implications. *Microb. Cell Fact.* **15**, 1–11 (2016).

34. Stoodley, P., Sauer, K., Davies, D. G. & Costerton, J. W. Biofilms as complex differentiated communities. *Annu. Rev. Microbiol.* **56**, 187–209 (2002).

35. Xavier, J. B. & Foster, K. R. Cooperation and conflict in microbial biofilms. *Proc. Natl. Acad. Sci. USA* **104**, 876–881 (2007).

36. Costerton, J. W. Introduction to biofilm. *Int. J. Antimicrob. Agents* **11**, 217–221 (1999).

37. Stach, J. E. M. & Burns, R. G. Enrichment versus biofilm culture: A functional and phylogenetic comparison of polycyclic aromatic hydrocarbon-degrading microbial communities. *Environ. Microbiol.* **4**, 169–182 (2002).

38. Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R. & Lappin-Scott, H. M. Microbial biofilms. *Annu. Rev. Microbiol.* **49**, 711–745 (1995).

39. Edwards, S. J. & Kjellerup, B. V. Applications of biofilms in bioremediation and biotransformation of persistent organic pollutants, pharmaceuticals/personal care products, and heavy metals. *Appl. Microbiol. Biotechnol.* **97**, 9909–9921 (2013).

40. Singh, R., Paul, D. & Jain, R. K. Biofilms: implications in bioremediation. *Trends Microbiol.* **14**, 389–397 (2006).

41. Blankenship, J. R. & Mitchell, A. P. How to build a biofilm: a fungal perspective. *Curr. Opin. Microbiol.* **9**, 588–594 (2006).

42. Ghazali, F. M., Rahman, R. N. Z. A., Salleh, A. B. & Basri, M. Biodegradation of hydrocarbons in soil by microbial consortium. *Int. Biodeterior. Biodegrad.* **54**, 61–67 (2004).

43. Festa, S., Coppotelli, B. M. & Morelli, I. S. Comparative bioaugmentation with a consortium and a single strain in a phenanthrene-contaminated soil: Impact on the bacterial community and biodegradation. *Appl. Soil Ecol.* **98**, 8–19 (2016).

44. Gallego, S., Vila, J., Tauler, M., Springael, D. & Grifoll, M. Community structure and PAH ring-hydroxylating dioxygenase genes of a marine pyrene-degrading microbial consortium. 543–556 (2014). doi:10.1007/s10532-013-9680-z

45. Zafra, G., Absalón, Á. E., Cuevas, M. D. C. & Cortés-Espinosa, D. V. Isolation and selection of a highly tolerant microbial consortium with potential for PAH biodegradation from heavy crude oil-contaminated soils. *Water. Air. Soil Pollut.* **225**, (2014).

46. Wargo, M. J. & Hogan, D. A. Fungal-bacterial interactions: a mixed bag of mingling microbes. *Curr. Opin. Microbiol.* **9**, 359–364 (2006).

47. Ghigo, J. M. Natural conjugative plasmids induce bacterial biofilm development. *Nature* **412**, 442–445 (2001).

48. Cerniglia, C. E. Oxidation of Benzo[a]pyrene by the Filamentous Fungus Cunninghamella elegans. *J. Biol. Chem.* **254**, 12174–12180 (1979).

49. Walker, S. E. & Dickhut, R. M. Sources of PAHs to Sediments of the Elizabeth River , VA. *Soil Sediment Contam.* **10**, 611–632 (2001).

50. Romantschuk, M. *et al.* Means to improve the effect of in situ bioremediation of contaminated soil: An overview of novel approaches. *Environ. Pollut.* **107**, 179–185 (2000).

51. Zhang, Z., Sangaiah, R., Gold, A. & Ball, L. M. Synthesis of uniformly 13C-labeled polycyclic aromatic hydrocarbons. *Org. Biomol. Chem.* **9**, 5431–5435 (2011).

52. Singleton, D. R. *et al.* Stable-isotope probing of bacteria capable of degrading salicylate, naphthalene, or phenanthrene in a bioreactor treating contaminated soil. *Appl. Environ. Microbiol.* **71**, 1202–1209 (2005).

53. Volkoff, S. J. *et al.* Embryonic Fundulus heteroclitus responses to sediment extracts from differentially contaminated sites in the Elizabeth River, VA. *Ecotoxicology*.

54. Corteselli, E. M., Aitken, M. D. & Singleton, D. R. Description of Immundisolibacter cernigliae gen. nov., sp. nov., a high-molecular-weight polycyclic aromatic hydrocarbon-degrading bacterium within the class Gammaproteobacteria, and proposal of Immundisolibacterales ord. nov. and Immundisolibacteraceae f. *Int. J. Syst. Evol. Microbiol.* **67**, 925–931 (2017).

55. Kiyohara, H., Nagao, K. & Yana, K. Rapid Screen for Bacteria Degrading Water-Insoluble, Solid Hydrocarbons on Agar Plates. *Appl. Environ. Microbiol.* **43**, 454–457 (1982).

56. Turner, S., Pryer, K. M., Miao, V. P. W. & Palmer, J. D. Investigating Deep Phylogenetic Relationships among Cyanobacteria and Plastids by Small Subunit rRNA Sequence Analysis. *J. Eukaryot. Microbiol.* **46**, 327–338 (1999).

57. Lane, D. J. 16S/23S rRNA Sequencing. *Nucleic Acid Tech. Bact. Syst.* 1150175 (1991).

58. Altschul, S. F., Gish, W., Miller, W., Myers, E. E. W. W. & Lipman, D. J. Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403–10 (1990).

59. Lane, J. *et al.* Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. **83**, (1986).

60. Schloss, P.D., et al. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* **75**, 7537–7541 (2009).

61. Callahan, B. J. *et al.* DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **13**, 581–583 (2016).

62. Quast, C. *et al.* The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* **41**, 590–596 (2013).

63. McMurdie, P. J. & Holmes, S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS One* **8**, (2013).

64. Sohn, J. H., Kwon, K. K., Kang, J. H., Jung, H. B. & Kim, S. J. Novosphingobium pentaromativorans sp. nov., a high-molecular-mass polycyclic aromatic hydrocarbon-degrading bacterium isolated from estuarine sediment. *Int. J. Syst. Evol. Microbiol.* **54**, 1483–1487 (2004).

65. Luo, Y. R. *et al.* Genome sequence of benzo(a)pyrene-degrading bacterium Novosphingobium pentaromativorans US6-1. *J. Bacteriol.* **194**, 907 (2012).

66. Yuan, J., Lai, Q., Zheng, T. & Shao, Z. Novosphingobium indicum sp. nov., a polycyclic aromatic hydrocarbon-degrading bacterium isolated from a deep-sea environment. *Int. J. Syst. Evol. Microbiol.* **59**, 2084–2088 (2009).

67. Pinyakong, O., Habe, H. & Omori, T. The unique aromatic catabolic genes in sphingomonads degrading polycyclic aromatic hydrocarbons (PAHs). *J. Gen. Appl. Microbiol.* **49**, 1–19 (2003).

68. Kertesz, M. A. & Kawasaki, A. Hydrocarbon-Degrading Sphingomonads: Sphingomonas, Sphingobium, Novosphingobium, and Sphingopyxis. in *Handbook of Hydrocarbon and Lipid Microbiology* 1693–1706 (2010). doi:10.1007/978-3-540-77587-4

69. Samanta, S. K., Singh, O. V. & Jain, R. K. Polycyclic aromatic hydrocarbons: Environmental pollution and bioremediation. *Trends Biotechnol.* **20**, 243–248 (2002).

70. Toledo, F. L., Calvo, C., Rodelas, B. & González-López, J. Selection and identification of bacteria isolated from waste crude oil with polycyclic aromatic hydrocarbons removal capacities. *Syst. Appl. Microbiol.* **29**, 244–252 (2006).

71. Fahy, A., Ball, A. S., Lethbridge, G., Timmis, K. N. & McGenity, T. J. Isolation of alkali-tolerant benzene-degrading bacteria from a contaminated aquifer. *Lett. Appl. Microbiol.* **47**, 60–66 (2008).

72. Sul, W. J. *et al.* DNA-stable isotope probing integrated with metagenomics for retrieval of biphenyl dioxygenase genes from polychlorinated biphenyl-contaminated river sediment. *Appl. Environ. Microbiol.* **75**, 5501–5506 (2009).

73. Lambo, A. J. & Patel, T. R. Isolation and characterization of a biphenyl-utilizing psychrotrophic bacterium, Hydrogenophaga taeniospiralis IA3-A, that cometabolize dichlorobiphenyls and polychlorinated biphenyl congeners in Aroclor 1221. *J. Basic Microbiol.* **46**, 94–107 (2006).

74. Boonchan, S., Britz, M. L. & Stanley, G. A. Surfactant-Enhanced Biodegradation of High Molecular Weight Polycyclic Aromatic Hydrocarbons by Stenotrophomonas maltophilia. *Biotechnol Bioeng* **59**, 482–494 (1998).

75. Tiwari, B., Manickam, N., Kumari, S. & Tiwari, A. Biodegradation and dissolution of polyaromatic hydrocarbons by Stenotrophomonas sp. *Bioresour. Technol.* **216**, 1102–1105 (2016).

76. Gao, S. *et al.* Multiple degradation pathways of phenanthrene by Stenotrophomonas maltophilia C6. *Int. Biodeterior. Biodegrad.* **79**, 98–104 (2013).

77. Kohlmeier, S. *et al.* Taking the fungal highway: Mobilization of pollutant-degrading bacteria by fungi. *Environ. Sci. Technol.* **39**, 4640–4646 (2005).

78. Peacock, A. D. *et al.* Utilization of Microbial Biofilms as Monitors of Bioremediation. *Microb. Ecol.* **47**, 1–9 (2004).

79. Jahnke, R. & Craven, D. Quantifying the role of heterotrophic bacteria in the carbon cycle : A need for respiration rate measurements ’. *Limnol. Oceanogr.* **40**, 436–441 (1995).

80. Briand, E., Pringault, O., Jacquet, S. & Torreton, J.-P. The use of oxygen microprobes to measure bacterial respiration for determining bacterioplankton growth efficiency. *Limnol. Oceanogr. Methods* **2**, 406–416 (2004).

81. Hollender, J., Althoff, K., Mundt, M. & Dott, W. Assessing the microbial activity of soil samples , its nutrient limitation and toxic effects of contaminants using a simple respiration test. **53**, 269–275 (2003).

82. Kiyohara, H., Nagao, K., Kouno, K. & Yano, K. Phenanthrene-degrading phenotype of Alcaligenes faecalis AFK2. *Appl. Environ. Microbiol.* **43**, 458–461 (1982).

83. Merritt, J. H., Kadouri, D. E. & O’Toole, G. A. Growing and Analyzing Static Biofilms. *Curr. Protoc. Microbiol.* 1–29 (2005). doi:10.1002/9780471729259.mc01b01s00.Growing

84. Luz, A. L., Smith, L. L., Rooney, J. P. & Meyer, J. N. Seahorse XFe24 Extracellular Flux Analyzer-based analysis of cellular respiration in Caenorhabditis elegans. *Curr. Protoc. Toxicol.* **66**, 1–20 (2016).

85. Luz, A. L., Kassotis, C. D., Stapleton, H. M. & Meyer, J. N. The high-production volume fungicide pyraclostrobin includes triglyceride accumulation associated with mitochondrial dysfunction, and promotes adipocyte differentiation independent of PPAR activation, in 3T3-L1 cells. 150–159 (2019). doi:10.1016/j.tox.2017.11.010.The

86. O’Toole, G. A. Microtiter Dish Biofilm Formation Assay. *J. Vis. Exp.* 10–11 (2011). doi:10.3791/2437

87. Davey, M. E. & O’toole, G. A. Microbial biofilms: from ecology to molecular genetics. *Microbiol. Mol. Biol. Rev.* **64**, 847–67 (2000).

88. Katarzyna, Irena & Teresa. Polycyclic Aromatic Hydrocarbons physicochemical properties, environmental appearance.pdf. **61**, (2004).

89. Patmont, C. R. *et al.* In situ sediment treatment using activated carbon: A demonstrated sediment cleanup technology. *Integr. Environ. Assess. Manag.* **11**, 195–207 (2015).

90. Weissenfels, W. D., Klewer, H. J. & Langhoff, J. Adsorption of polycyclic aromatic hydrocarbons (PAHs) by soil particles: influence on biodegradability and biotoxicity. *Appl. Microbiol. Biotechnol.* **36**, 689–696 (1992).

91. Ghosh, U., Luthy, R. G., Cornelissen, G., Werner, D. & Menzie, C. A. In-situ Sorbent Amendments: A New Direction in Contaminated Sediment Management. *Environ. Sci. Technol.* **45**, 1163–1168 (2011).

92. Gidley, P. T., Kwon, S., Yakirevich, A., Magar, V. S. & Ghosh, U. Advection dominated transport of polycyclic aromatic hydrocarbons in amended sediment caps. *Environ. Sci. Technol.* **46**, 5032–5039 (2012).

93. Marshall, C. W., Ghosh, U., May, H. D., Sowers, K. R. & Payne, R. B. A Pilot-Scale Field Study: In Situ Treatment of PCB-Impacted Sediments with Bioamended Activated Carbon. *Environ. Sci. Technol.* (2019). doi:10.1021/acs.est.8b05019

94. Lohmann, R. & Macfarlane, J. K. Importance of black carbon to sorption of native PAHs , PCBs , and PCDDs in Boston and New York , Harbor sediments Importance of Black Carbon to Sorption of Native PAHs , PCBs , and PCDDs in Boston and New York Harbor Sediments. **39**, 141–148 (2017).

95. National Research Council. Principles of Bioremediation. in *In situ bioremediation: When does it work?* (National Academies Press, 1993).

96. Booij, K., Smedes, F. & van Weerlee, E. M. Spiking of performance reference compounds in low density polyethylene and silicone passive water samplers. *Chemosphere* **46**, 1157–1161 (2002).

97. Taylor, S. C. *et al.* The Ultimate qPCR Experiment: Producing Publication Quality, Reproducible Data the First Time. *Trends Biotechnol.* **37**, 761–774 (2019).

98. Chen, B., Yuan, M. & Qian, L. Enhanced bioremediation of PAH-contaminated soil by immobilized bacteria with plant residue and biochar as carriers. *J. Soils Sediments* **12**, 1350–1359 (2012).

99. Payne, R. B., Ghosh, U., May, H. D., Marshall, C. W. & Sowers, K. R. A Pilot-Scale Field Study: In Situ Treatment of PCB-Impacted Sediments with Bioamended

Activated Carbon. *Environ. Sci. Technol.* **53**, 2626–2634 (2019).

100. Cerniglia, C. E. & Heitkamp, M. A. Microbial degradation of polycyclic aromatic hydrocarbons (PAH) in the aquatic environment. in *Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment* 41–68 (1989).

101. Meynet, P. *et al.* Effect of activated carbon amendment on bacterial community structure and functions in a PAH impacted urban soil. *Environ. Sci. Technol.* **46**, 5057–5066 (2012).

Biography

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