Evaluating the Impact of Ecological Growth Strategies of Bacteria on Plasmid Transfer and Function for Bioremediation of Polycyclic Aromatic Hydrocarbons

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Paige Marie Varner

Department of Civil and Environmental Engineering
Duke University

Date:_____

Approved:

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Claudia K. Gunsch, Supervisor

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Helen Hsu-Kim

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Josh Michener

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David Singleton

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

Remediation of contaminated soils and sediments traditionally involves physical methods that are environmentally intrusive and expensive. Bioremediation is a more environmentally friendly, cost-effective treatment strategy that utilizes naturally occurring indigenous and/or exogenous microorganisms in combination with physico-chemical stimulation to promote degradation of contaminants. However, the predominant challenge of bioremediation that involves adding exogenous microorganisms is the lack of long-term survival of augmented organisms due to their lack of adaptation to environmental conditions often found at contaminated sites. Genetic bioaugmentation aims to overcome this challenge by introducing relevant genes from exogenous bacteria into indigenous microbes adapted to the polluted environment utilizing conjugation, or plasmid transfer, to increase overall contaminant biodegradation.

Genetic bioaugmentation relies on many processes yet to be fully understood including how growth strategies of donors and recipients affect plasmid transfer and the functionality of their degradative properties. Additionally, methods for tracking plasmid transfer *in situ* are lacking, which contributes to the lack of knowledge surrounding properties affecting genetic bioaugmentation as a sustainable remediation approach. The overarching goals of this dissertation were to first develop methods for *in*

situ quantification of conjugation of plasmids capable of degrading polycyclic aromatic hydrocarbons (PAHs), which could then be used to investigate the effect of bacterial growth strategies on conjugation and PAH degradation in both simple, synthetic communities and complex, natural sediment communities. Two plasmids, pNL1 and NAH7, were used to track conjugation and PAH degradation due to their ability to degrade naphthalene and other PAHs, their similarity in transfer mechanisms, and the fact that they are well-characterized plasmids.

The first objective of this dissertation was to develop methods for detecting conjugation of PAH-degrading plasmids that could be applied *in situ* without the need for culturing. To achieve this, plasmids and donor chromosomes were genetically modified to develop fluorescence-based methods for conjugation detection. This method was then used to investigate the effect of growth strategies, GC content, and phylogenetic relatedness on conjugation in simple communities of one donor and one recipient. We observed significant correlations between all three parameters and, specifically, more conjugation into slow growing K-strategists compared to their fast-growing r-strategist counterparts. All three parameters affected conjugation of the PAH-degradative plasmids. The work stemming from this first objective led to the development of a novel method for monitoring conjugation, and, more broadly, those results provided insights into biological factors that affect conjugation and therefore, successful genetic bioaugmentation.

The second objective was to assess the impact of growth strategy on conjugation and naphthalene degradation in a simple bacterial community. Conjugation of two PAH-degrading plasmids into recipients with mixed growth strategies and subsequential degradation of naphthalene in respective communities was assessed using methods developed in the first objective. There were clear conjugation preferences into slow-growing K-strategists for the pNL1 plasmid but no clear preferences for the NAH7 plasmid. However, communities of K-strategists consistently had more naphthalene degradation than their r-strategist counterparts. These findings provide insight into K-strategists being ideal targets for genetic bioaugmentation in simple communities, but further needed to be translated to complex communities.

The final objective was to determine if the findings in simple communities translated over to complex, natural sediment communities, thus providing insights into the field implementation of genetic bioaugmentation. Plasmid donors were added to bioreactors with PAH-contaminated sediment containing a natural microbial community. The bioreactors were biostimulated differentially to promote growth of microbes with varying growth strategies and both conjugation and PAH degradation were quantified. Overall, the growth strategies of transconjugants were most closely associated with effects on conjugation and persistence of transconjugants. The highest amount of conjugation occurred during the latter experimental stages when transconjugants were more representative of slow-growing K-strategists. Although the

impact of growth strategy on PAH biodegradation was unclear within the experimental time frame, differential nutrient biostimulation was found to be a successful approach for promoting differences in transconjugant growth strategies, providing some insights for developing a genetic bioaugmentation framework.

Overall, the work in this dissertation suggests that bacterial growth strategy can be stimulated by differential nutrient biostimulation, and that slow growing K-strategists are optimal targets for genetic bioaugmentation. This dissertation provides the beginning of a framework for identifying favorable conditions needed to promote successful implementation of genetic bioaugmentation targeted to the existing recipient community present at a PAH contaminated site. This fills a critical research gap for the translation of genetic bioaugmentation into field application of precision microbiome engineering.

Dedication

To all the women in STEM whose shoulders I have had the privilege to stand on—may we continue to persist with our confidence and inspire with our intelligence.

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Acknowledgements

I'd like to begin by thanking my advisor, Claudia Gunsch, for your unending support in my research endeavors, but primarily your support outside of the lab. I couldn't have gotten through a PhD during a global pandemic without the care and understanding you give all your students and others in the department. I'm incredibly thankful for the support of my other committee members. Helen Hsu-Kim, thank you for taking the first chance on me as an inexperienced lab technician, and for your continued support throughout the years. I am so lucky to have wonderful female role models through you and Claudia. Thank you to David Singleton for being in the trenches of research with me and always giving me such wise guidance. Thank you to Josh Michener who serendipitously became a collaborator that guided me throughout my entire dissertation. I could not have completed any of this research without the help of you and your lab at Oak Ridge. I'd also like to thank my undergraduate mentor, Erik Sotka, who supported me through the biggest ups and downs, trusted me enough to watch his kids for 2 weeks when he was halfway across the world, and didn't laugh (too hard) when I considered being an optometrist.

I couldn't have completed this PhD without the help from the best lab mates and cohort at Duke. First to my cohort—Anna Lewis, Jake Ulrich, Andrew Middleton, Ethan Hicks, and Lucas Rocha-Melogno—I cannot stress enough how this wouldn't be possible without you all. We really struggled through every class and every challenge

together and I'm so thankful I had you all to lean on, suffer/enjoy Campout together, and laugh with at 1 am finishing Transport projects. We did it! To my amazing lab mates: Eva for being the best undergraduate mentee turned lab mate/friend and always reassuring me it's all going to be okay, Alex for always being an equal source of support and laughter, Daniel for being the main reason I was able to analyze any of my data and for always making me feel smarter than I am, Josh for always giving me a laugh and having the hottest takes on reality TV, Savannah for making me laugh and teaching me that Google has all the answers, and Billy and Courtney for the constant laughs and distractions. Thank you also to Maya and Emily for being the best undergrad/rotation mentees and for all your help and hard work. Finally, thanks to all the ITEHP trainees over the years. This program has always been a beacon of support and laughter and I am grateful to have gotten to know all of you.

To my friends across the country who have become family: I would not be the person I am today without your love and support. Thank you to my Triangle friends Brianna and Richard Eisenreich and Chrissy and Justin Crute who have become our closest friends. I couldn't have gotten through graduate school without the wine nights, coffee and writing sessions, camping trips, and countless games of Avalon. I also thank all the instructors and members at Pure Barre Chapel Hill for being a supportive community of women who cheer each other on and support each other at the barre and beyond. Thank you to the two best friends and college roommates a girl can have:

Aubrey Smith and Grace Fronczak. You both accepted me and supported me throughout college and beyond during times I didn't even accept myself. I would not be the person I am today without your unconditional love. I'm grateful for my other college friends (and frat bros): Ben and Kristin Stephens, Nick Taylor, Jamie Claire, Alexis Violette, Elsa Cousins, Colleen Quaas, Nathan Adamson, and Dillon Presto. Some of my best college memories were spent with this crew not only suffering through chemistry courses but laughing over margaritas at Juanitas or beers at Closed for Business (RIP), Catan games at bro house and Ascot Alley, beach days, and now reuniting every year which feels like no time has passed. Lastly, I cherish my childhood friendships with Lauren Mashburn, Katey Bartrom, Katy and Christian Bandy, and Alex Coomler. It's been so special to watch us all grow up together and I know I have your support for a lifetime.

My family has been a constant source of support and love from the very beginning. To my parents, Paul and Therese: your support, tremendous patience, and sacrifice has never gone unnoticed. Thank you for raising me to be independent, brave, and caring, and for cheering me on even when I moved across the country not knowing a single soul. The best parts of myself are characteristics I gained from both of you. Thank you to my brother, Kyle, for always supporting me and growing with me. Thank you for making me an aunt to the best, funniest, cutest nephews ever.

Finally, I would like to thank my husband, Travis Varner. It feels like there are not adequate words to thank you for all you've done for me and for your support over the years. I have felt your love and support at the absolute lowest times, which has empowered me to believe the best in myself and accomplish everything that I have. I am so grateful to have you as a partner in this life in both the mountains and the trenches. I look forward to many more adventures with you and Bourbon.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants that accumulate in soils and sediments. PAHs are of environmental and public health concern due to their ubiquity in the environment and carcinogenic and toxic properties (Bamforth & Singleton, 2005; Gilbert & Blossom, 2014; Howard, 1991b; Ronald, 1988; Valko et al., 2005). Remediation of PAH-contaminated soils and sediments typically involves physical methods that are environmentally intrusive and expensive. In contrast, bioremediation utilizes natural occurring indigenous and/or exogenous microbes in combination with physico-chemical stimulation to promote degradation of contaminants making this approach more environmentally friendly and cost-effective. The predominant challenge of bioremediation implementation that relies on adding exogenous organisms is the lack of long-term survival of augmented organisms due to their lack of adaptation to harsh conditions often found at contaminated sites.

Genetic bioaugmentation is a remediation approach that aims to overcome this challenge by introducing relevant genes to microorganisms adapted to the polluted environment using conjugation, or plasmid transfer, to increase contaminant degradation (Ikuma et al., 2012; Top et al., 2002; Top & Springael, 2003). Though promising, genetic bioaugmentation relies on many processes that are yet to be fully understood. In particular, this approach requires the survival of the donor organisms

harboring the degradative plasmids, recipients accepting the plasmid at a significant rate, and long-term expression of degradative genes. However, it is still unclear how certain biological and community properties affect these processes. One factor in particular that is under-explored is bacterial growth strategies which provides a measure of a microorganism's evolutionary trade-off between high reproduction rate or resource utilization (Atlas & Bartha, 1997). Depending on the resource availability and the microbial density, the ecology of an ecosystem may change over time to favor a particular life strategy which can affect the success of genetic bioaugmentation processes.

Another barrier to understanding and investigating the processes underlying genetic bioaugmentation is the lack of methods for detecting plasmid *in-situ* and without the need for culturing of organisms. Common methods for tracking conjugation involve selection of transconjugants with antibiotic selection which has resulted in most conjugation studies focusing on smaller, antibiotic resistance plasmids and not degradative plasmids (Henschke & Schmidt, 1990; Neilson et al., 1994; Sengeløv & Sørensen, 1998; Top et al., 2006). However, recent advances in methods for genetic modification, next generation sequencing, and high-throughput flow cytometry have made way for gathering more information regarding conjugation occurrence and

transfer rate of a broader range of plasmids (Klümper et al., 2015a; Musovic et al., 2010a; Pinilla-Redondo et al., 2018; Shintani et al., 2008).

To that end, this dissertation focuses on first developing methods for *in-situ* quantification of conjugation of PAH-degrading plasmids, which then is used to investigate the effect of bacterial growth strategies on conjugation and PAH degradation in both simple, synthetic communities and complex, natural sediment communities. The overarching goal of this dissertation is to better understand the impact of bacterial growth strategy in the context of genetic bioaugmentation and provide some guidance towards the implementation of a bioremediation framework for the long-term remediation of PAHs in sediments.

1.1 Research Objectives

To date, it is not well understood how bacterial properties such as ecological growth strategies affect the implementation of genetic bioaugmentation as a remediation scheme for PAHs. The research presented here uses novel *in-situ* conjugation detection methods to quantify plasmid transfer and PAH-degrading gene expression in both simple and complex communities to detangle relationships between growth strategies and genetic bioaugmentation.

The specific objectives of this dissertation were to:

1. Develop *in situ* methods for detecting conjugation of PAH-degrading plasmids;

2. Assess the impact of growth strategy on conjugation frequency and naphthalene degradation in a synthetic microbial community; and
3. Investigate the impact of growth strategy on the implementation of genetic bioaugmentation of PAH-degrading plasmids in complex sediments.

Successful completion of these objectives facilitates a better understanding of how growth strategies and growth conditions can impact the success of genetic bioaugmentation. Specifically, investigating plasmid transfer in simple communities and complex communities provides insight into conjugation preferences on a smaller scale as well as long-term plausibility of genetic bioaugmentation as a PAH remediation scheme. Additional long-term outcomes include improved *in-situ* bioremediation techniques and an understanding of plasmid transfer for various biomedical and environmental purposes.

1.2 Research Hypotheses and Approaches

The overall objective of this dissertation was to investigate the effect of ecological growth strategies of bacteria on plasmid transfer and PAH degradation to create a framework for precise genetic bioaugmentation of PAHs. It was hypothesized that ecological growth strategies of both plasmid donors and recipients would impact the rate of transfer and expression of plasmid genes and, therefore, a precise genetic

bioaugmentation scheme for PAH degradation could be devised by promotion of particular growth strategies.

The first dissertation objective was to develop methods for detecting conjugation of PAH-degrading plasmids that could be applied *in-situ* and without the need for culturing of organisms. To achieve this, plasmids and donor chromosomes were genetically modified to develop fluorescence-based methods for conjugation detection. It was hypothesized that when validating this method with a donor and recipient conjugation assay, fast-growing r-strategists would conjugate more efficiently and the addition of naphthalene as a model PAH would create a selective pressure for conjugation and increase conjugation frequencies. The successful completion of this objective led to the development of a novel method for monitoring the success of genetic bioaugmentation and provided insights into conjugation preferences of the selected plasmids.

The second objective was to assess the impact of growth strategy on conjugation and naphthalene degradation in a simple microbial community. It was hypothesized that plasmid transfer and expression of naphthalene-degrading genes was dependent on the ecological role each microbe fills in a mixed synthetic community which would depend on the growth strategies of donor and recipients present in the community. To determine this, conjugation of two PAH-degrading plasmids into recipients with mixed

growth strategies was assessed using methods developed in the first objective of this dissertation. Additionally, naphthalene degradation was quantified to compare the plausibility of long-term remediation with tested communities filling various ecological roles.

The final objective of this work was to investigate growth strategy relationships in complex, natural sediment microbial communities. It was hypothesized that relationships between growth strategy of donors and recipients and conjugation and PAH degradation identified in simple, synthetic communities would translate into complex communities. Additionally, it was hypothesized that an average community growth strategy could be promoted with addition or lack of additional nutrients. To achieve this, plasmid donors were added to bioreactors with PAH-contaminated sediment containing a natural microbial community. Bioreactors were differentially stimulated to promote growth of microbes with varying growth strategies and both conjugation and PAH degradation were quantified to develop a framework for genetic bioaugmentation in complex communities.

2. Background and Literature Review

This chapter presents a brief overview of the current scientific literature that addresses polycyclic aromatic hydrocarbons, bioremediation in soils and sediments, and plasmid conjugation. Specific reviews may be found at the beginning of the remaining objective-based research chapters.

This chapter was published in part under the title "Properties affecting transfer and expression of degradative plasmids for the purpose of bioremediation" in *Biodegradation* in 2021. It is reprinted with permission from Varner, P. M., & Gunsch, C. K. (2021). Properties affecting transfer and expression of degradative plasmids for the purpose of bioremediation. *Biodegradation*, *32*(4), 361–375. https://doi.org/10.1007/s10532-021-09950-1

2.1 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a class of persistent organic pollutants that are widely produced to this day through natural, industrial, and anthropogenic processes. PAHs are primarily characterized by having two or more fused benzene rings (Figure 1). They are components of coal and petroleum but are mainly introduced into the environment as a byproduct of burning wood and fossil fuels (Cerniglia, 1984; Freeman & Cattell, 1990; Lim et al., 1999). Another significant source of PAH contamination is creosote which was commonly used as a wood preservative until

the late 1990s. Because creosote contains approximately 85-90% of PAHs by weight, it a large contributor of PAH pollution particularly in sediments and soils in contact with treated wood such as near docks and railroads (Cerniglia, 1984; Di Giulio & Clark, 2015).

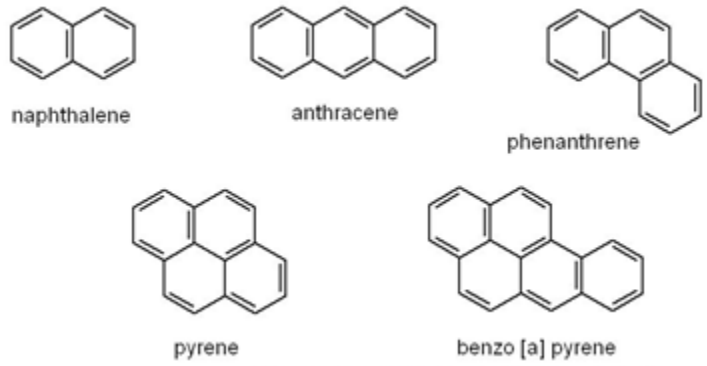

Figure 1: Examples of common PAHs.

These chemicals are of great concern because of their toxic, mutagenic, and carcinogenic properties as well as their rapid uptake into mammalian fat tissue. PAHs are able to form DNA adducts through the reactive diol epoxide metabolite which leads to DNA damage (Delistraty, 1997). Additionally, some aromatic hydrocarbons induce cytochrome P450 proteins via the intracellular aryl hydrocarbon receptor which can lead to production of reactive oxygen species and further DNA damage (Poland & Knutson, 1982). As a result, continuous exposure to PAHs can lead to biomagnification of the chemicals which can increase the possibility of long-term adverse health outcomes in humans and wildlife, such as kidney damage, liver damage, and cancer (Bamforth & Singleton, 2005; Cancer, 2019; Gilbert & Blossom, 2014; Ronald, 1988; Valko et al., 2005).

This class of pollutants is additionally troublesome because of their ubiquitous persistence in the environment. Due to the chemical nature of PAHs, they are extremely hydrophobic and tend to sorb to soils and sediments where they can persist for decades depending on the size of the PAH (Howard, 1991a). Generally, as the number of benzene rings and molecular weight increases in a PAH, the hydrophobicity increases as well as the toxicity (Bastiaens et al., 2000; Cerniglia, 1984; Juhasz & Naidu, 2000a). Because of these cumulative properties, the EPA has listed 16 PAHs as pollutants that are of high priority for remediation—the most toxic being benzo [α] pyrene (Liu et al., 2001).

2.2 Remediation of PAHs in Soils and Sediments

Soils and sediments are large sinks for hydrophobic and recalcitrant compounds such as PAHs. These environmental matrices are the primary focus for remediation of PAHs and similar compounds and will be the main focus of review.

2.2.1 Physical Remediation Methods

Physico-chemical *ex-situ* treatment strategies are commonly used to remediate soils and sediments contaminated with PAHs due to their heavy sorption and low bioavailability (Figure 2). Excavating and/or dredging the contaminated soil is a frequent beginning step in remedial action. This approach can lead to long term ecological impact due to the environmental harm caused by unintentionally removing and disturbing native flora and fauna. In addition to the environmental disturbance, *ex-*

situ treatment of contaminated sediments can be extremely costly (Varjani, 2017). For example, incineration of contaminated sediment done by heating the sediment up to 1200°C is costly and can cause additional contamination via volatilization of PAHs (Y. Chen et al., 2013). Storage of contaminated sediment in landfills can cause additional issues if leakage occurs.

Another common physical remediation strategy utilized in soils and sediments is capping. This is achieved by forming a thick layer on top of the contaminated sediment using materials such as sand and clay to prevent leaching of the contaminant into the surrounding waters. However, similar problems exist as dredging such as causing environmental harm and the costly nature of capping. Additionally, the sediment cap can erode over time and risk leaking contamination back into the surface water or ground water—exposing ecosystems and the public to the contaminant (Payne et al., 2017).

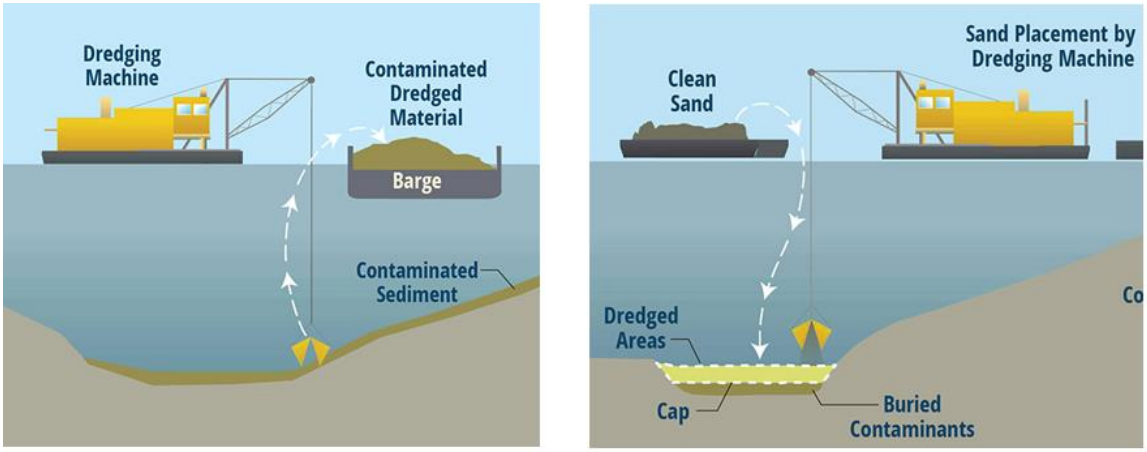

Figure 2: Common sediment remediation strategies which include dredging (left) and capping (right).

2.2.2 Bioremediation

Due to the costly and environmentally intrusive nature of physical remediation methods, there exists a need for an alternative, eco-friendly method. Bioremediation—the use of plants, fungi, and bacteria to naturally degrade xenobiotics—is a more cost-effective and eco-friendly technology for remediation of select contaminants that can be applied *ex-situ* or *in-situ*. A suite of bacteria and fungi have been identified that are able to partially or fully degrade PAHs, thus bioremediation remains a promising technology for removal of PAHs from polluted sediments (Cerniglia, 1984; Goltapeh et al., 2013). Nonetheless, bioremediation tends to be slow and remains challenging to implement due to the heterogeneous nature of PAH contaminated sites.

There are a suite of bioremediation applications that can be applied depending on the contaminats, abiotic conditions at the polluted site, and community profile of

organisms. The most passive bioremediation approach, natural attenuation, involves simple monitoring techniques to ensure contaminant levels are decreasing without altering environmental conditions or the microbial profile. When there are endogenous organisms present in the environment with the capabilities to degrade the contaminant, biostimulation can be applied. Addition of nutrients such as nitrogen, phosphorus, oxygen, or various carbon sources have been added in biostimulation schemes to encourage growth of PAH-degrading organisms (Digregorio et al., 2015; Lladó et al., 2009). When there are low to no concentrations of organisms present in the sediment able to degrade the contaminant, exogenous organisms with these capabilities are augmented in an approached termed bioaugmentation (Digregorio et al., 2015; Kuppusamy et al., 2016). Oftentimes both bioaugmentation and biostimulation are utilized together in remediation schemes for increased and sustained contaminant degradation (Sayara et al., 2011).

Although bioremediation is still a developing remediation technology, it has been successful in contaminant reduction at over 100 Superfund sites with 30% of these sites listing PAHs as the predominant class of chemicals of concern (EPA, 2001). However, there are still challenges associated with the implementation of these bioremediation schemes—especially at sites where complex microbial communities, chemical profiles, and heterogeneous physical conditions exist. Degradation rates are

often dependent on chemical bioavailability, the microbial community, and other abiotic factors.

2.2.3 Genetic Bioaugmentation

One predominant challenge of bioremediation, and bioaugmentation in particular, is the lack of long-term survival of augmented organisms because they are not well-adapted to the harsh conditions often found at a contaminated site (Thompson et al., 2005). A bioremediation approach to alleviate this problem is genetic bioaugmentation which aims to introduce relevant genes to microorganisms adapted to the polluted environment using conjugation, or plasmid transfer, to increase contaminant degradation (Ikuma et al., 2012; Top et al., 2002; Top & Springael, 2003). In this case, a donor bacterium harboring a catabolic plasmid will transfer the plasmid to a recipient cell (transconjugant) which can then express the degradative function (Figure 3).

Catabolic plasmids containing genes for xenobiotic degradation play a large role in the spread of catabolic pathways in bacterial communities (Top & Springael, 2003). Because a handful of catabolic plasmids encode genes for degradation of PAHs, genetic bioaugmentation is a feasible method for long-term bioremediation of PAHs (Duetz & Andel, 1991; C. Thomas, 1987). However, due to the complexity of the biological and chemical environment at a contaminated site and lack of methods for tracking conjugation, this process has not been well-studied and issues still arise when

promoting a successful genetic bioaugmentation scheme. This dissertation work aims to understand what ecological properties and nutrient conditions promote plasmid transfer and PAH degradation gene expression for the successful implementation of a genetic bioaugmentation remediation framework.

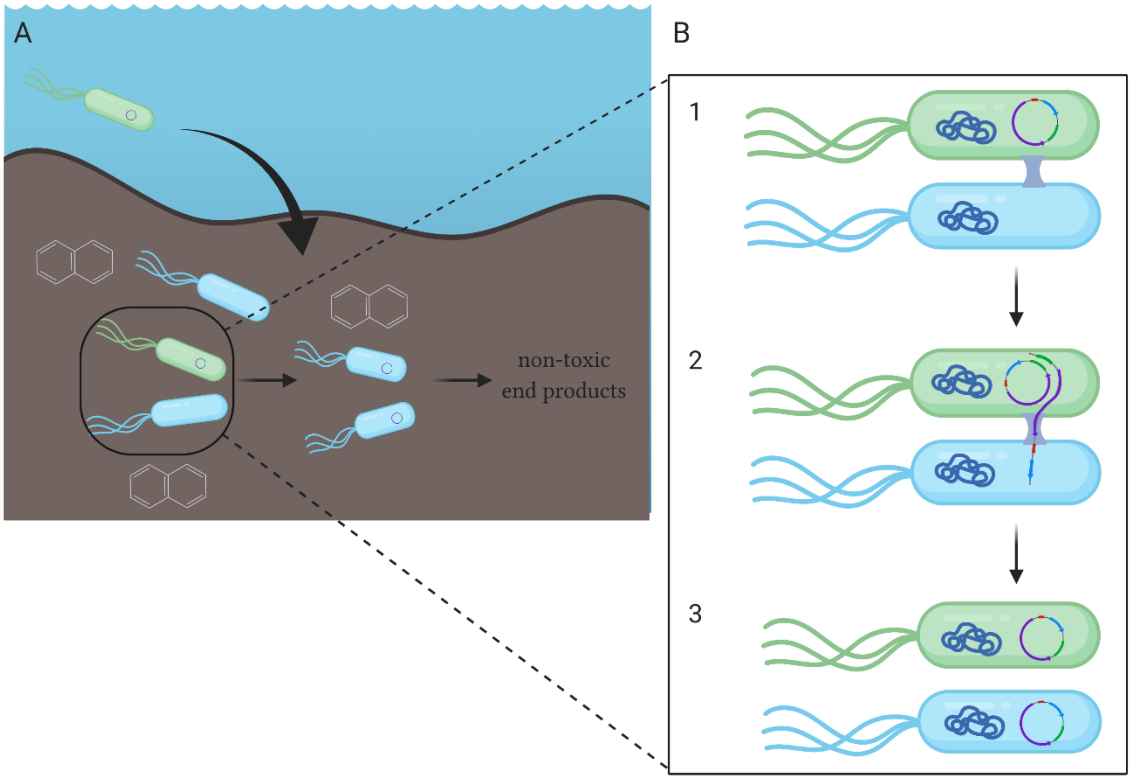

Figure 3: Schematic representing a genetic bioaugmentation remediation scheme. A) Bacteria harboring degradative plasmids are augmented into the environment where they can transfer plasmids to well-adapted native bacteria for contaminant degradation. B) Conjugation of a degradative plasmid occurs via transfer through the pilus of a replicated plasmid.

2.2.4 Bacteria and Plasmids Used in Bioremediation

Researchers have identified many genera of fungi and bacteria capable of catabolically transforming PAHs and other xenobiotics. However, bacteria are particularly useful because of their ability to transform complex mixtures of PAHs to non-toxic byproducts such as carbon dioxide and water. In this process, PAHs act as an electron donor and source of carbon for the production of energy and microbial biomass. PAHs are catabolized first by the cleavage of aromatic rings by dioxygenases (Cerniglia, 1984; Johnsen & Karlson, 2004). Common intermediates from this process include *cis*-dihydrodiol and catechol.

Typically gram-negative bacteria are responsible for aromatic degradation. PAH-degrading microorganisms often belong to the genera *Pseudomonas, Flavobacterium, Sphingobium, and Alcaligenes* (Mrozik & Piotrowska-Seget, 2010). These bacterial genera are also found to harbor catabolic plasmids responsible for PAH degradation in the host bacterium. These bacteria are typically able to degrade lower molecular weight PAHs ranging from two- to four-ringed PAHs. There are no reported cases of bacteria utilizing high molecular weight PAHs, such as benzo[a]pyrene, as their sole carbon and energy source. However, bacterial degradation of high molecular weight PAHs has been demonstrated in the presence of another carbon source through co-metabolic processes (Haritash & Kaushik, 2009).

Catabolic plasmids play a large role in the dissemination of catabolic pathways in bacterial communities. Many catabolic plasmids belong to the IncP incompatibility group (Top & Springael, 2003). These plasmids have been found to degrade a wide variety of xenobiotics including, but not limited to: toluene, PAHs, dioxin, chlorpyrifos, oil-based hydrocarbons, and pesticides (Garbisu et al., 2017a; Top et al., 2002). One well-characterized PAH-degrading plasmid is the NAH7 plasmid which originates in *Pseudomonas putida* G7 (Figure 4). The NAH7 plasmid is an 83-kilobase, IncP-9, self-transmissible plasmid capable of completely degrading naphthalene and has been known to aid in the breakdown of phenanthrene and anthracene (Sanseverino et al., 1993; Sota et al., 2006). Naphthalene is degraded to catechol by the *nah* genes located on two operons on the NAH7 plasmid. The upper operon, nah1, converts naphthalene to salicylate and the lower operon, nah2, converts salicylate to acetaldehyde and pyruvate. Additionally, the NAH7 plasmid contains genes enabling chemotaxis to naphthalene (Grimm & Harwood, 1999; Sota et al., 2006).

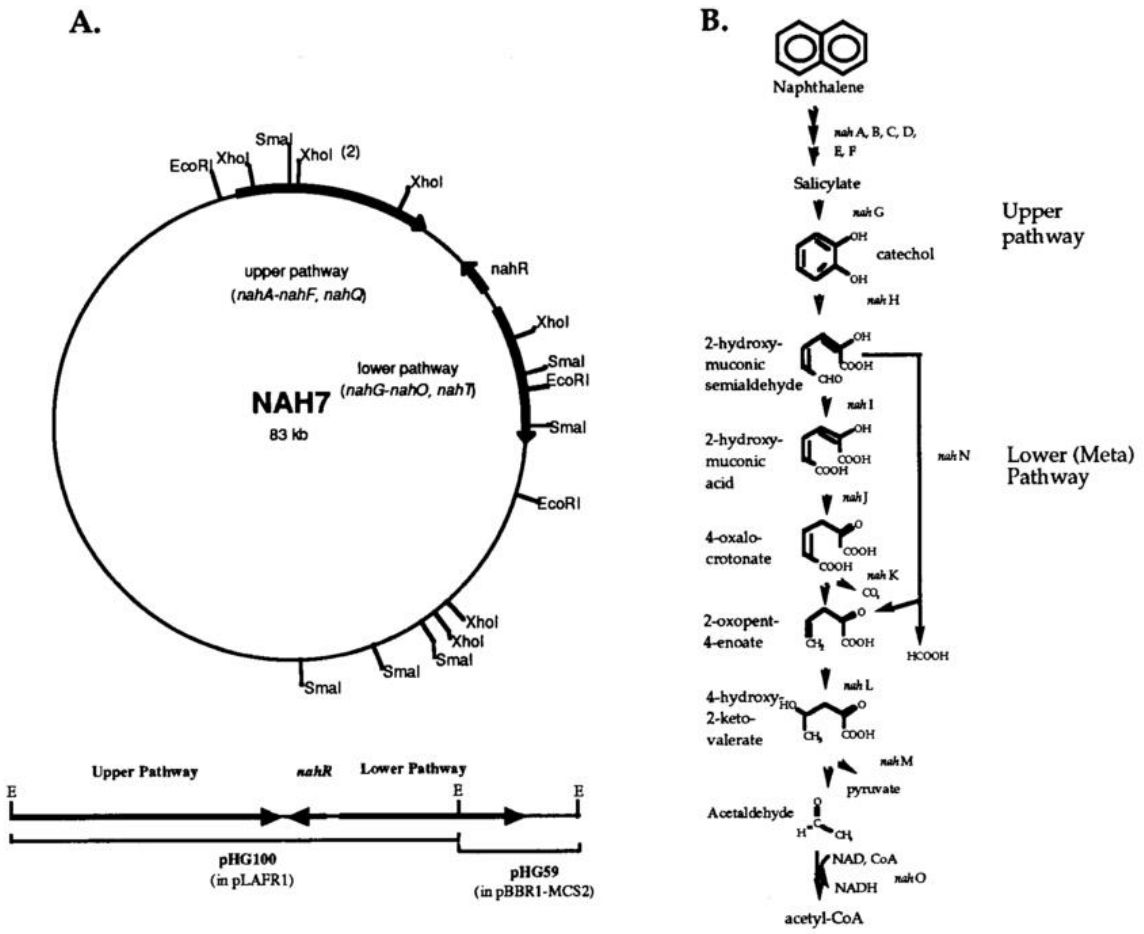

Figure 4: Map of the NAH7 plasmid of *Pseudomonas putida* G7 and the naphthalene degradation pathway. A) Map of NAH7 with degradation pathways indicated by arrows. B) Naphthalene catabolic pathway encoded by NAH7 (adapted from Grimm and Harwood 1999).

Another notable plasmid responsible for PAH degradation is the pNL1 plasmid originating in *Novosphingobium aromaticivorans* F199. The pNL1 plasmid is a 184-kilobase, IncP-9, self-transmissible plasmid capable of degrading not only naphthalene and fluorene but other xenobiotics such as biphenyl, xylene, and cresol. The first steps in the degradation of naphthalene and similar structures are catalyzed by a single set of

enzymes encoded by genes on 4 *bph* operons. These operons encode enzymes to degrade naphthalene to salicylate. However, unlike many other PAH degradation pathways, it is not clear whether degradation occurs through a catechol intermediate (Romine et al., 1999).

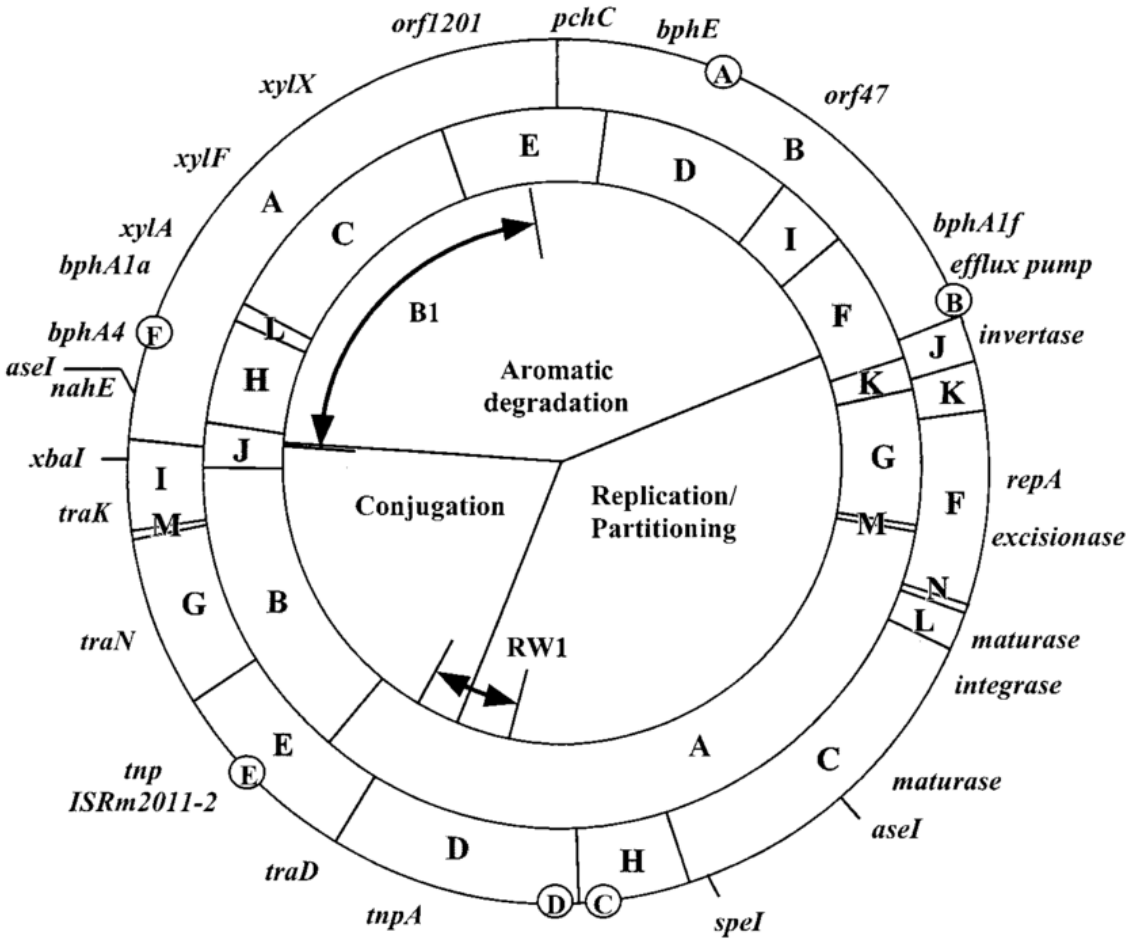

Figure 5: Map of the pNL1 plasmid of *Novosphingobium aromaticivorans* F199. The positions of selected genes are shown outside the rings and the regions encoding genes associated with aromatic degradation, conjugation, and plasmid partitioning and replication are noted (adapted from Romine et al. 1999).

2.3 Conjugation

Successful genetic bioaugmentation relies on a fundamental understanding of conjugation of degradative plasmids. Plasmids have played a key role in the rapid evolution and gene sharing of microorganisms because of the ability to horizontally transfer plasmids into organisms of various species (Frost et al., 2005; Gogarten & Townsend, 2005). Plasmids are circular genetic elements made up of deoxyribose nucleic acid (DNA) that can replicate independently from the chromosome of the host cell. The F plasmid from *Escherichia coli* K-12 was the first described plasmid responsible for genetic transfer. The fertility (F) factor encodes a sex pilus responsible for connecting the donor and recipient species to allow for transfer of plasmid DNA (Cavalli et al., 1953). All F-like plasmids carrying this unique fertility factor, though functionally diverse in replication or partition systems, have been classified into the Incompatibility Group F (IncF). Plasmids in unique incompatibility groups are unable to stably coexist in a cell due to sharing elements of the same replication system. Competition of replication factors leads to the ability of one plasmid to solely exist in the cell (Novick & Hoppensteadt, 1978).

In addition to IncF plasmids, there are multiple other incompatibility groups that contain self-conjugatable plasmids. Plasmids in incompatibility groups P, N, W, and X form a short, rigid pilus compared to the more ancestral long and flexible pilus formed by F-like plasmids (Fernandez-Lopez et al., 2016). This type of sex pilus is more relevant

compared to the F-type sex pilus because most xenobiotic-degrading plasmids that are self-conjugatable belong to these incompatibility groups (Fernandez-Lopez et al., 2017). The genetic region involved in incompatibility groups P, N, W, and X is shorter than its ancestral relative's transfer region and is controlled by multiple promoters (Frost et al., 1994; Kennedy et al., 1977; Lawley et al., 2003). This region is constituted as a Type IV secretion system (T4SS). The T4SS is initiated by the binding of a relaxase and accessory factors to the origin of transfer (*oriT*), resulting in a complex called the relaxosome. The relaxosome is responsible for translocating the DNA through a channel as well as catalyzing recirculation of the DNA in the recipient cell (César et al., 2006; Draper et al., 2005; Garcillán-Barcia et al., 2007). The channel used in translocation of the DNA is formed by the mating-pair formation (mpf). The mpf is comprised of an ATPase and a cross-membrane protein subunit—together which are responsible for the translocation channel as well as the pilus filament (Christie & Cascales, 2009; Lawley et al., 2003).

Genes responsible for the relaxosome and mpf complexes are located on a 34 kb transfer region consisting of *tra* and *trb* genes and two regulatory genes, *fin*P and *fin*O (Figure 6). The relaxosome consists of proteins both chromosomally and plasmid encoded, particularly from the *tra* genes (Furste et al., 1989; Lanka & Wilkins, 1995). The mpf complex is primarily formed by eleven components encoded by genes *trb*B to *trb*L, as well as *tra*F (Haase et al., 1995). Outside of these two complexes, accessory genes in the transfer region aid in regulation and incompatibility. Regulation is carried out by

*fin*P and *fin*O which repress the positive regulatory product of *tra*J in which transfer is dependent upon. Surface exclusion, carried out by the gene products of *tra*S and *tra*T, is a method of plasmid incompatibility in which they block the entry of a similar T4SS plasmid into a cell that already contains one. This T4SS transfer region consisting of *tra*, *trb*, and *fin* genes are regulated by promotors P_m, P_i, and P_y (Grohmann et al., 2003). Many genes present in this region, however, still have unknown function and importance in conjugation.

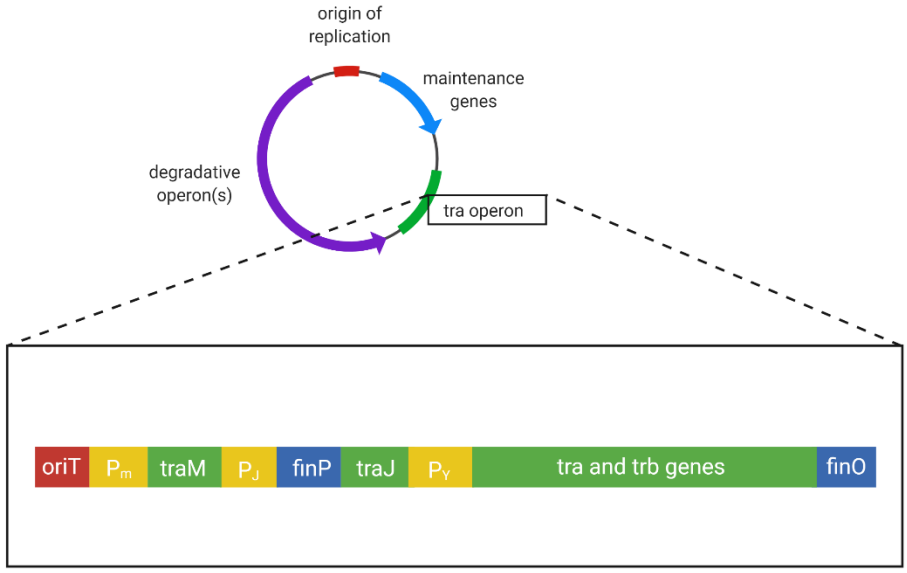

Figure 6: A generalized map of a mobilizable catabolic plasmid zoomed in to reveal the tra operon. Represented within the operon are the origin of transfer (red), promotors (yellow), regulatory genes (blue) and genes responsible for transfer (green).

2.3.1 Properties that Affect Conjugation

2.3.1.1 Abiotic Properties

Many abiotic and biotic properties have been proven to affect conjugation and plasmid gene expression of catabolic plasmids which, in turn, alter the success of genetic bioaugmentation. First, moisture content affects contact between donor and recipient cells which will decrease the physical contact that is needed for conjugation to occur (Aminov, 2011; Miller et al., 2004). The optimal soil moisture content for conjugation and contaminant degradation is likely between 60-100%, and the optimal temperature around 30°C (Gao et al., 2015; Johnsen & Kroer, 2007; Shintani et al., 2008; Zhang et al., 2012).

Additionally, temperature is thought to be a key factor in conjugation possibly due to its role in the regulation of proteins encoded in the transfer (*tra*) regions of mobilizable plasmids (Forns et al., 2005). An increase in conjugation is generally observed with increasing temperatures with peak conjugation frequency occurring between 30-37°C (Fernandez-Astorga et al., 1992; Headd & Bradford, 2018; Lafuente et al., 1996). In few studies performed with degradative plasmids the optimal temperature for conjugation and degradation was between 29-35°C (Gao et al., 2015; Johnsen & Kroer, 2007; Wang et al., 2014; Zhang et al., 2012). Similarly, pH is known to affect the stability of proteins depending on the acceptable range of the donors and recipients and therefore is another important abiotic factor to consider. In general, the range of pH that

confers the highest amount of plasmid transfer is between 6 and 8 (Fernandez-Astorga et al., 1992; Lafuente et al., 1996).

As can be expected from the success of biostimulation schemes, the addition of nutrients such as oxygen, nitrogen, and phosphorus increase degradation and possibly conjugation (Seoane et al., 2010; Wang et al., 2014). The first step in most aerobic xenobiotic degradation involves the addition of oxygen to the molecule. Therefore, most degradative plasmids are only useful to aerobic organisms that already require oxygen as an electron acceptor with an abundance oxygen present in the environment. To our knowledge, no studies have looked a catabolic plasmid transfer under anaerobic conditions. Krol et al. (2011) found that an increase in oxygen concentration can increase plasmid transfer through an oxygen-related mechanism or indirectly due to its impact on normal cell functionality. For inorganic nutrients such as nitrogen and phosphorus, results remain unclear whether or not the addition of nutrients stimulates conjugation—particularly in cases where addition of nitrogen and phosphorus happens less frequently than every day (Elsas & Bailey, 2006; Fox et al., 2008; Shintani et al., 2008). However, there is evidence that an increase in nutrients increases degradative plasmid transfer and degradation of contaminants (Seoane et al., 2010; Wang et al., 2014). It is likely, therefore, that the diversity of microorganisms and complexity of the environment plays a large role in determining whether the increased availability of nutrients is necessary for an increase in conjugation frequency.

For catabolic plasmids, the presence of the substrate in high enough concentrations is often necessary as a selective pressure for a cell to host the plasmid and encourage conjugation. DiGiovanni et al. (1996) observed that 2,4-D present exhibited enough selective pressure for the transfer of the relative degradative plasmid. In many cases for conjugation of degradative plasmids, higher concentrations of the substrate will increase conjugation and degradation (Wang et al., 2014; Zhang et al., 2012). Contrarily, however, Ikuma and Gunsch (2012) found that environmentally relevant concentrations of toluene might not exhibit a selective pressure high enough to promote transfer of the toluene-degrading TOL plasmid. Oftentimes the substrate is not bioavailable because of its chemical nature or interaction with the organism. For example, hydrophobic contaminants, such as PAHs and PCBs, readily sorb to soils and sediments and avoid the aqueous phase, making it harder for bacteria to uptake (Megharaj et al., 2011). Without the selective pressure of the contaminant of interest, the metabolic burden of large catabolic plasmids might be too great to encourage production of conjugation machinery or even continue to host the plasmid. Organic matter or carbon sources other than the contaminant of interest has also been shown to increase conjugation of catabolic plasmids indirectly. For example, adding organic growth substrates to bioreactors has been shown to increase conjugation and plasmid expression of antibiotic plasmids (Bleakley & Crawford, 1989). Another example of growth substrate added to increase conjugation is glucose. Studies such as Pearce *et al*.

(Pearce et al. 2008) have described an increase in conjugation with the addition of glucose in soils. Similarly, Johnsen and Kroer (Johnsen and Kroer 2007) found an increase in transfer of the 2,4-D degradative plasmid pRO103 with addition of maltose. Furthermore, many plasmid-encoded degradation phenotypes are co-metabolic and necessitate an additional carbon source to carry out the function of interest.

2.3.1.2 Biotic Properties

The biotic and community properties affecting conjugation and plasmid gene expression are less understood due to the complexities of bacterial communities. However, there are few factors that have been shown to be important. For example, the ability of both the donor and recipient in conjugation to form biofilms also has a great effect on competition and conjugation. Biofilms not only protect bacteria against predation or toxins, but also provide environments where conjugation can happen more frequently due to the close nature of cells in the biofilm, making biofilms 'hot spots' for horizontal gene transfer (Stalder & Top, 2016). Similarly, cell motility is a property of both donor and recipient strains that can directly affect the occurrence and rates of conjugation or indirectly affect these rates by encouraging biofilm formation (Garbisu et al., 2017b). Interestingly, many study designs involve homogenous liquid cultures of bacteria which is suboptimal for biofilm formation which, in turn, could result in lower conjugation rates due to the lower likelihood of contact between cells. Pinedo and Smets (Pinedo and Smets 2005) determined the most effective ratio of donor to recipient to be

1:10^3 in liquid cultures due to the requirement of cell-to-cell contact. More studies in environments promoting biofilm formation are needed to identify how biofilms increase conjugation events of catabolic plasmids and help differentiate the effects of other properties on plasmid transfer (Stalder & Top, 2016).

Although some degradative plasmids have a broad-host range, phylogenetic relatedness, guanine-cytosine (G+C) content, codon usage biases, and restriction proficiency of the recipient have been shown to be important properties affecting conjugation efficiency and phenotypic expression (Ikuma & Gunsch, 2012; Pinedo & Smets, 2005; C. M. Thomas & Nielsen, 2005). Phylogenetic relatedness of donor and recipient strains is a property that encompasses a combination of genetic properties of both bacteria. However, many studies indicate an increase in transfer of degradative plasmids with closer species relatedness. Through a meta-analysis of thirty-two plasmid conjugation studies, Alderliesten et al. (Alderliesten et al. 2020) reported a lower conjugation frequency in liquid matings with an increase in phylogenetic distance. For filter matings, the influence of relatedness on conjugation is still unclear. Interestingly, Hall et al. (Hall et al. 2016) describes that single-species transfer might limit sustained plasmid transfer compared to two-species communities in both models and experiments. This phenomenon, termed 'source-sink plasmid dynamics', is where the source of a plasmid (donor) transfers the plasmid to recipient which is a sink for further conjugation. The two-species communities are able to better maintain access to plasmid

accessory genes compared to single-species communities due to the segregation and purifying selection that occurs in conjugation between a singular species. The impact of phylogenetic relatedness on degradative plasmid transfer is a multi-level, intricate relationship that still needs to be further investigated.

The influence phylogenetic relatedness has on conjugation occurrence and frequency is likely linked to genetic factors such as G+C content and codon usage bias (Santos & Ochman, 2004). Popa et al. (Popa et al. 2011) demonstrated that donors and recipients that underwent successful conjugation had less than a 5% deviation in G+C content Additionally, genomic G+C content may be an indicator of codon usage biases which could create differences between codon usage in the donor and recipient. Codon usage is especially important in transcription and translation which particularly will affect the expression of the plasmids more than transfer (Garbisu et al., 2017b).

2.3.2 Plasmid Expression and Stability

Successful bioaugmentation relies not only on fast and sustained plasmid transfer but also expression of degradation genes and stability of the plasmid in individual bacteria and throughout the environment long-term. First, expression of a plasmid phenotype may be associated with the G+C content of the recipient cell. The basis of this concept is that the expression of transferred genes depends most importantly on the recognition of the promoter sequence by the transcription machinery. This promoter recognition is dependent on the G+C content of the sequence which may

be deduced from the overall genomic G+C content and, subsequently, phylogenetic relatedness (Mulligan & Mcclure, 1986). Two studies from Navarre et al. (Navarre et al. 2006, 2007) found transferred DNA that is G+C-poor compared to the recipient genome may experience silencing and therefore will not be expressed. Furthermore, studies by Ikuma and Gunsch (Ikuma and Gunsch 2012, 2013) demonstrated that expression of the catabolic pWW0 TOL plasmid was higher in transconjugants with a similar genomic G+C content and phylogenetic relatedness to the donor. They also found an increase in expression of transconjugants with the addition of glucose as an alternative carbon source to the toluene. Some additional evidence has reached contradictory conclusions regarding the more successful plasmid expression in phylogenetically related organisms. Between members of the same genus and even the same species, De Gelder et al. (De Gelder et al. 2007) found that the stability of an IncP antibiotic resistance plasmid had large variation in the stability of the plasmid. Variation seemed to be due to segregational loss of the plasmid and high plasmid cost rather than phylogenetic relatedness. The stability of plasmids, particularly larger plasmids, appears to be dependent on specific genetic interaction between the plasmid and the host chromosome which is increasingly complex in diverse environments (Hall et al., 2016; Kottara et al., 2018). Some of the genetic interactions can be described by direct factors such as G+C content and general phylogenetic relatedness, however these relationships are often complex and other factors likely also contribute to gene expression patterns.

The complexity of a plasmid stably being maintained and expressed within a host often is associated with the metabolic burden a plasmid exerts on a host. The host bacterium may not express or keep a plasmid if the metabolic burden becomes too large so that the bacterium's fitness is decreased in particular environments. The reduced fitness effects for the host include increased biosynthetic burden, reduced translational efficiency, impaired chromosomal replication, and other energetic costs (Baltrus, 2013; San Millan et al., 2018; Shachrai et al., 2010; Vogwill & Maclean, 2015). These fitness effects can be especially problematic for larger plasmids. However, plasmid expression and survival is possible if other factors are able to overcome the negative fitness effects of the plasmid. One of the most common explanations for the survival and expression of a plasmid is in an environment with enough selective pressure to give the host an advantage (Bergstrom et al., 2000; Simonsen, 2018). Selective pressures are often essential for plasmid transfer as well because of their necessity in long-term survival of the plasmid; however, the type and amount of pressure required depends on the plasmid. For example, DiGiovanni et al. (DiGiovanni et al. 1996) found that the contaminant 2,4-D, in which the harbored plasmid could degrade, was sufficient pressure to promote conjugal transfer. Contrarily, theoretical analysis has often suggested that fast transfer rate could possibly compensate for the fitness cost of a plasmid and prevent its loss (Andreoni & Gianfreda, 2007; De Gelder et al., 2007). Recently, studies have experimentally shown that high transfer rates are able to

overcome the metabolic burden and fitness costs of antibiotic resistance plasmids (Lopatkin et al., 2017; Svara & Rankin, 2011). However, because large catabolic plasmids exhibit a greater burden on the host cell than smaller, antibiotic resistance plasmids, it is yet to be determined if fast transfer rates can overcome these costs to benefit genetic bioaugmentation.

Plasmids can benefit the host in multiple other ways besides the direct effect of giving the host a competitive advantage in a selective environment which can lead to the expression and survival of plasmids without these selective pressures. Plasmids can co-evolve with hosts in as little as fifty generations due to mutations in regulatory genes. This plasmid-host co-evolution is likely why some plasmids persist in particular hosts for many years, yet the mechanisms and mutations contributing to this are poorly understood (Harrison et al., 2015; MacLean & San Millan, 2015). Plasmid persistence can also be explained by "plasmid hitch-hiking" which is a phenomenon where plasmids transfer into already fit organisms (Bergstrom et al., 2000). These fitness benefits of an antibiotic resistance IncP plasmid have been confirmed in complex communities (L. Li et al., 2020). However, not one model has been identified that universally describes these associations likely due to variations between phylotypes.

2.3.3 Detecting Conjugation

A fuller understanding of plasmid transfer to promote successful genetic bioaugmentation is limited by the lack of methods available to accurately quantify

conjugation occurrence, transfer rates, and particularly *in-situ* transfer of degradative plasmids. Most studies aimed at tracking conjugation have been focused on antibiotic resistance plasmids due to the public health concern of antibiotic spread and the simplicity selecting for transconjugants. However, recent advances in methods for genetic modification, next-generation sequencing, and high-throughput flow cytometry have made way for gathering more information regarding conjugation occurrence and transfer rate of a broader range of plasmids—including degradative plasmids.

Many early approaches for conjugation detection focus on utilizing plasmid-specific phenotypes for selection of transconjugants such as antibiotic resistance, heavy metal resistance, or catabolic pathways (Henschke & Schmidt, 1990; Neilson et al., 1994; Sengeløv & Sørensen, 1998; Top et al., 2006). Although efficient, these approaches have an extreme downfall in the fact that they are cultivation-dependent. Considering that the estimated fraction of culturable microorganisms is around 1%, these approaches are unable to detect a wide range of organisms present in complex environments as possible recipients of plasmid transfer (Torsvik et al., 1990). Furthermore, many of these studies require artificial lab conditions and well-characterized donors and recipients which differ from the complex communities undergoing plasmid transfer in natural environments.

Fluorescent reporter genes have enabled the detection of conjugation in both culturable and non-culturable microorganisms. However, only a few fluorescent

conjugation systems have been constructed with most of them being for antibiotic resistance plasmids. The first systems created were engineered for green fluorescent protein (GFP) to be expressed on the plasmid but repressed when the plasmid resides in the donor cell (Dahlberg et al., 1998). With this system, many studies detected conjugation often by microscopy or flow cytometry (Geisenberger et al., 1999; Hausner & Wuertz, 1999; Mølbak et al., 2003; Normander et al., 1998; Sørensen et al., 2003, 2005). Another fluorescence system developed involves labeling a plasmid with a GFP gene but also labeling the donor chromosome with a red fluorescence protein gene, such as mCherry or dsRed (Klümper et al., 2015a; Musovic et al., 2010c). This duel-fluorescent system is extremely beneficial in tracking conjugation due to the ability to separate donors from transconjugants via fluorescence-activated cell sorting (FACS). This high-throughput approach has been utilized to track conjugation of pesticide-degrading plasmids to gain insight into the possible functions and properties affecting transfer (Pinilla-Redondo et al., 2020; Shintani et al., 2014). It is therefore a promising method for tracking the transfer of many other PAH-degrading plasmids *in-situ* to better understand and promote genetic bioaugmentation for remediation of PAHs.

2.4 Ecological Growth Strategies

One factor affecting the success of genetic bioaugmentation that has received little attention is the ecological growth strategies of bacteria. Historically, bacterial evolution has favored two general ecological growth strategies: high reproduction rate

(r-strategists) or optimal resource utilization (K-strategists) (Figure 7). This designation stems from survival curves where r-strategists maximize their intrinsic rate of growth (r) and K-strategists are well-adapted when populations are near carrying capacity (K) (Atlas & Bartha, 1997). Depending on the resource availability and the microbial density, the ecology of an ecosystem may change over time to favor a particular life strategy which can change the functional dynamics of the microbial community and the gene pool. Fierer et al. (Fierer et al. 2007; Fierer 2017) attempted an ecological classification of bacterial phyla to better understand the structure and function of bacterial communities based on a phylum's growth response to carbon amendments. They found a negative relationship between carbon amendment level, nutrient level and Acidobacteria abundance, and a positive relationship for Bacteriodetes and β-Proteobacteria suggesting those phyla were K- and r-strategists, respectively.

Though helpful in a larger context, these designations are not narrow enough for use in the implementation of genetic bioaugmentation. Bacterial strains residing in the same phylum or even in the same genus can adapt widely different ecological growth strategies. In addition, characterizing these ecological strategies solely on growth could be problematic as organismal growth is dependent on environmental conditions and community dynamics. Therefore, genetic relationships such as the copy number of 16S rRNA genes have been used to denote ecological growth strategies. A higher copy number of 16S rRNA genes in the genome is associated with a higher specific growth

rate and lower carbon use efficiency (Klappenbach et al., 2000; Ortiz-Álvarez et al., 2018; Roller et al., 2016; Wu et al., 2017). This finding supports the notion that ecological growth strategies have been inherently adapted by bacterial species and the adaptation has been imprinted in the genome on a spectrum—r-strategists on one side and K-strategists on the other.

Although there is substantial theoretical evidence for ecological growth strategies of bacteria influencing conjugation, this area of research is vastly under-studied with only a few studies relating to this topic. Conjugation of the toluene-degrading TOL plasmid has been found to increase with the specific growth rate of both the donor and recipient (Seoane et al., 2010; Smets et al., 1993a). This relationship suggests a likely influence of faster-growing bacteria (r-strategists) being able to transfer and receive degradative plasmids better than the slower-growing K-strategists, especially in environments with ample nutrient availability. Sysoeva et al. (Sysoeva et al. 2019) furthered this research with antibiotic resistance plasmids. They discovered that plasmids exhibit three types of transfer regimens: 1) the growth stage of an organism had no effect on conjugation, 2) plasmid transfer occurred more often with donors in exponential phase of growth, and 3) plasmid transfer occurred more often with donors in stationary phase of growth. Not only was the donor's growth phase important, the researchers found that the growth stage of the recipient had a significant effect on the rate of plasmid transfer. This work further supports the rationale that conjugation is

affected by the growth strategies of not only the donor but also the recipient. Brzeszcz et al. (Brzeszcz et al. 2016) was the first study to explicitly assess the relationship between ecological growth strategies and hydrocarbon degradation. They found that *Mycobacterium fredericksbergense* IN53, a K-strategist, was a much more efficient hydrocarbon degrader than *Acinetobacter* sp. IN47, an r-strategist, in an environment with low moisture content and high hydrocarbon load. This suggests that a K-strategist might be more adept at maintaining or transferring a catabolic plasmid in limiting conditions compared to an r-strategist. Although these studies point to an influence of ecological growth strategies on conjugation and expression, much more research needs to be done to disentangle these relationships to understand and promote successful genetic bioaugmentation.

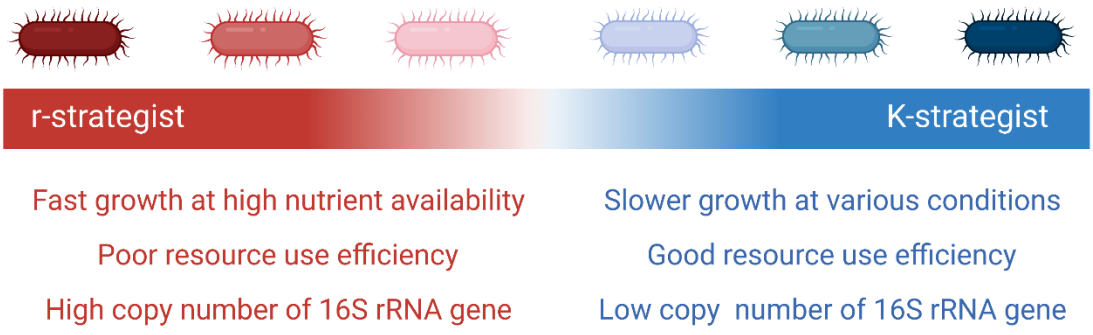

Figure 7: A spectrum of growth strategies of microorganisms exist with r-strategists (red) on one end and K-strategists (blue) on the other. Properties associated with each extreme are listed.

3. Developing *in-situ* methods for detecting conjugation of PAH-degrading plasmids

This chapter was published in part under the title "Complete genome sequence of NAH7-harboring *Pseudomonas putida* strain G7" in *Microbiology Resource Announcements* in 2022. It is reprinted with permission from Varner, P.M. & Gunsch, C.K. (2022). Complete Genome Sequence of NAH7-Harboring Pseudomonas putida Strain G7. *Microbiology Resource Announcements*. https://doi.org/10.1128/MRA.00394-22

This chapter was also published in part under the title "The effect of bacterial growth strategies on plasmid transfer and naphthalene degradation for bioremediation" in *Environmental Technology & Innovation* in 2022. It is reprinted with permission from Varner, P.M.; Allemann, M.N.; Michener, J.K.; Gunsch, C.K. (2022). The effect of bacterial growth strategies on plasmid transfer and naphthalene degradation for bioremediation. *Environmental Technology & Innovation*, *28*, 102910. https://doi.org/10.1016/J.ETI.2022.102910

3.1 Introduction

Physico-chemical treatment strategies commonly used to remediate soils and sediments contaminated with PAHs, such as dredging and capping, can lead to environmental harm and are often extremely costly (Payne et al., 2017; Varjani, 2017). Bioremediation is a more cost-effective and eco-friendly technology for remediation of select contaminants that can be applied *ex-situ* or *in-situ*. One bioremediation approach

that utilizes the conjugative capabilities of plasmids to widely disseminate pollutant degradation genes into the environment is genetic bioaugmentation. Here, the addition of mobilizable degradative plasmids to a polluted environment can increase the abundance of biodegradative genes in indigenous, well-adapted bacteria and stimulate their expression (Ikuma et al., 2012; Top et al., 2002; Top & Springael, 2003). In this case, a donor bacterium harboring a catabolic plasmid will transfer the plasmid to a recipient cell (transconjugant) which can then express the degradative function.

Though promising, genetic bioaugmentation relies on many processes that are yet to be fully understood. In particular, successful genetic bioaugmentation requires the survival of the donor organisms harboring the degradative plasmids, recipients accepting the plasmid at a significant rate, and long-term expression of degradative genes. Abiotic properties such as temperature, moisture, nutrient levels, and substrate availability have been shown to affect the successful implementation of genetic bioaugmentation (Garbisu et al., 2017b). Biotic properties of both donor and recipient, and their effects on conjugation and plasmid gene expression, have been less studied. Many of these knowledge gaps exist due to the lack of methods accessible for quantifying transfer frequencies of degradative plasmids and identification of transconjugants (Varner & Gunsch, 2021).

Early approaches for conjugation detection focused on utilizing plasmid-specific phenotypes for selection of transconjugants, such as antibiotic resistance, heavy metal

resistance, or catabolic pathways (Henschke & Schmidt, 1990; Neilson et al., 1994; Sengeløv & Sørensen, 1998; Top et al., 2006). However, these cultivation-dependent approaches were unable to detect the full range of organisms present in complex environments as possible plasmid recipients. By contrast, fluorescent reporter genes have enabled the detection of conjugation in both culturable and non-culturable microorganisms. However, only a few fluorescent conjugation systems have been constructed with most of them being for antibiotic resistance plasmids (Geisenberger et al., 1999; Hausner & Wuertz, 1999; Klümper et al., 2015a; Mølbak et al., 2003; Musovic et al., 2010a; Normander et al., 1998; Sørensen et al., 2003, 2005). Culture independent methods fluorescence reporters may also be promising for investigating the transfer of PAH-degrading plasmid *in-situ* to better understand and promote genetic bioaugmentation for remediation of PAHs.

The development of *in-situ* conjugation quantification methods would also open up opportunities to investigate properties that affect transfer and expression of PAH-degrading plasmids but have been difficult to study due to the lack of screening tools. An particular under-explored property is ecological growth strategies of bacteria, which are described along a spectrum of strategies favoring high reproduction rate on one end (r-strategists or copiotrophs), or optimal resource utilization on the other end (K-strategists or oligotrophs) (Atlas & Bartha, 1997). These designations have also been described genetically based on the copy number of 16S rRNA genes in the chromosome

of the bacteria. A high copy number of 16S rRNA genes is associated with higher specific growth rate and lower carbon use efficiency (Klappenbach et al., 2000; Ortiz-Álvarez et al., 2018; Roller et al., 2016; Wu et al., 2017).

To elucidate growth strategy relationships and their effect on conjugation, methods are needed that do not rely on culturing microorganisms. Thus, in this study, fluorescence-based methods of tracking conjugation were developed to quantify *in-situ* plasmid transfer of two PAH-degradative plasmids. These methods were then used to investigate conjugation preferences of these two plasmids in simple, 1:1 conjugation assays with recipients that have a range of ecological growth strategies. The methods developed in this study and the investigation into the effect of ecological growth strategies give insight into developing a genetic bioaugmentation remediation scheme for long-term and sustainable bioremediation of PAHs.

3.2 Materials and Methods

3.2.1 Strains and Media

The two donor strains harboring PAH-degrading plasmids used in this work were selected because of their complementary donor and plasmid properties. First, *Pseudomonas putida* G7 (G7) has a high 16S rRNA copy number compared to *Novosphingobium aromaticivorans* F199 (F199) (Stoddard et al., 2015). Second, both plasmids are well-characterized, IncP-9 mobilizable plasmids that degrade naphthalene (Romine et al., 1999; Sota et al., 2006). Inclusion in the same incompatibility group (IncP-

9) indicates the transfer system expressed in both plasmids is the same. Plasmid NAH7 harbored in G7 is significantly smaller at 83 kbp compared to plasmid pNL1 harbored in F199 which is 184 kbp. Naphthalene was selected as the model carbon source for this study as it is the simplest PAH with two fused benzene rings, is found in abundance in creosote, is a relevant PAH for bioremediation as it is a known carcinogen (Abdo et al., 2001).

All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). The selected recipients were *Sphingopyxis lindanitolerans* WS5A3p, *Caulobacter vibrioides* CB15, *Cupriavidus metallidurans* CH34, *Acinetobacter venetianus*, *Serratia marcescens*, and *Enterobacter cloacae*. The recipients were chosen based on multiple factors: 1) They are all Gram-negative Proteobacteria which are known to readily accept IncP plasmids via conjugation; 2) They have a range of ecological growth strategies; 3) Many have been documented to have received plasmids previously (Ikuma, 2011; Redfern et al., 2019).

All strains were grown at 30°C on Caulobacter medium (CBM) consisting of 2 g/L peptone, 1 g/L yeast extract, and 0.2 g/L $MgSO_4 7 \cdot H_2O$ unless otherwise noted. For gene insertions and selections, the following concentrations of antibiotics and selective media were used: 50 μg/L kanamycin, 25 μg/L chloramphenicol, 100 μg/L ampicillin, 100 μg/L streptomycin, and 25% w/v sucrose. For gene insertions into F199 and pNL1, conjugation and selection were achieved on R2A medium. For gene insertions into G7 and NAH7, conjugation and selection were achieved on Luria-Bertani (LB) medium. *E.*

coli WM6026, the conjugation donor for gene insertions, was grown at 30°C on LB supplemented with 300 μM diaminopimelate decarboxylase. A list of all strains used in this study and their characteristics are presented in Table 1.

Table 1: Bacterial strains and plasmids used in this study. Relevant properties such as the 16S rRNA copy number and fluorescent protein genes are reported.

Strain or Plasmid	Relevant Properties	Reference
Pseudomonas putida G7	donor harboring NAH7; 16S copy number: 7	ATCC 17485
Novosphingobium aromaticivorans F199	donor harboring pNL1; 16S copy number: 3	DSM 12444
Sphingopyxis lindanitolerans	recipient; 16S copy number: 1	ATCC TSD-188
Caulobacter vibrioides	recipient; 16S copy number: 2	ATCC 19089
Cupriavidus metallidurans CH34	recipient; 16S copy number: 4	ATCC 43123
Acinetobacter venetianus	recipient; 16S copy number: 6	ATCC 31012
Serratia marcescens	recipient; 16S copy number: 7	ATCC 13880
Enterobacter cloacae	recipient; 16S copy number: 8	ATCC 13047
NAH7	degrades naphthalene, phenanthrene, anthracene	Sota et al. 2006
pNL1	degrades naphthalene, fluorene	Romine et al. 1999
JMN120	*N. aromaticivorans* F199; mScarlet-I, GFPmut2, kan^R	This work
PVP102	*P. putida* G7; mScarlet-I, mVenus, kan^R, cam^R	This work

3.2.2 Growth Curves of Donors and Recipients

To verify growth properties of the donors and recipients, growth curves were produced at both high levels of CBM and low levels of CBM (a 1/3 dilution of high CBM). Each strain was grown in CBM at 30°C shaking at 150 rpm overnight before 10^5 cells were added to 5 mL of respective growth medium. The growth of the organisms was tracked by measuring the OD600 every hour on a Hach DR/2500 Spectrophotometer (Hach, Loveland, CO, USA). The slopes of the growth of each strain in their exponential growth phase was calculated and reported for each growth media (Figures A.1 and A.2). The results verified that the 16S rRNA copy number of donors and recipients was directly related to their growth in both high and low nutrient media and therefore can be used as a proxy for growth strategy.

3.2.3 Sequencing of Pseudomonas putida G7

In order to develop methods for tracking conjugation of the NAH7 plasmid harbored in *Pseudomonas putida* G7 (G7), the donor strain was first sequenced. G7 was obtained from ATCC 17485 and was cultured to mid-log phase at room temperature and shaking at 150 rpm using Luria Broth (LB) medium. Total genomic DNA was extracted using Qiagen's Genomic DNA Extraction protocol with the Genomic-tip 500/G kit (Qiagen, Venlo, Netherlands). Protocol modification included growing G7 in 25 μg/mL chloramphenicol one hour before extraction. A quality score of 2.7 for 280/260 absorbance was obtained prior to sequencing which indicates high quality DNA that is

suitable for downstream genomic sequencing. Library preparation was performed using the Oxford Nanopore Technologies (ONT) Rapid Sequencing Kit (SQK-RAD004) following the provided protocol with no modifications. Sequencing was performed on the ONT MinION using a R9.4.1 flow cell and sequencing was monitored with the MinKNOW software (ONT, Oxford, UK). Reads with a length of 10 kb or more were retained for assembly resulting in 200X coverage. Reads were assembled with Flye v2.8.1 with default parameters which includes one round of polishing (Kolmogorov et al. 2019). The assembly was further polished and circular contigs were re-oriented to start at the origin of replication with Medaka model r941_min_fast_g303, v1.2.0f (ONT, Oxford, UK).

Assembly quality and completeness was assessed through several metrics. QUAST (v5.0.2) was used to report basic assembly statistics, such as N50. BUSCO (v4.1.4) was used to assess assembly complete-ness by searching for single-copy conserved orthologs using the 'auto-lineage selection' parameter to select the appropriate odb10 database (Gurevich et al., 2013; Simão et al., 2015). QUAST and BUSCO results were summarized with multiQC (v1.9) (Ewels et al., 2016). To confirm the species sequenced and check for contamination, the contig from the assembly was queried against the blast nt database using the megablast algorithm (v2.7.1). Minimap2 (v2.17) was used to assess depth of coverage across the contigs by mapping reads to the polished assemblies (H. Li, 2016). To check for mis-assemblies, each assembly was

aligned to itself using minimap2 with the "asm5" preset settings for aligning genomes to one another. No mis-assemblies were found. The chromosome sequence and annotation was deposited in the National Center for Biotechnology Information (NCBI) Genbank Sequence Database under accession number CP096581. The BioProject accession number is PRJNA831801.

3.2.4 Insertion of Fluorescent Protein Genes

To quantify conjugation via fluorescence-activated cell sorting (FACS), genes encoding constitutive fluorescence of mScarlet-I were inserted onto the NAH7 and pNL1 plasmids. A vector with the mScarlet-I protein construct under the *nptII* promoter from Schlecter et al. (2018) was synthesized by Genscript (Piscataway, NJ, USA) and cloned into the multiple cloning site (MCS) of the pAK405 vector for insertion into pNL1 replacing the Saro_RS17445 gene (Kaczmarczyk et al., 2012). Delivery of the vector was achieved through conjugation between *E. coli* WM6026 harboring the vector and *N. aromaticivorans* F199. Selection of *N. aromaticivorans* F199 colonies exhibiting successful gene insertion and constitutive fluorescence was achieved by following previously established methods and PCR/sequencing with primers targeting the insert region listed in Table 2 (Kaczmarczyk et al., 2012). A vector with the same mScarlet-I protein construct under the *nptII* promoter inserted into the MCS of the pKmobsacB vector was synthesized by Genscript for insertion into NAH7 replacing the HXC10_RS00045 gene (Schäfer et al., 1994b). Delivery of the vector was again achieved through conjugation

with *E. coli* WM6026 as the donor. Selection of *P. putida* G7 colonies exhibiting successful gene insertion and constitutive fluorescence was achieved following previous methods and PCR/sequencing with primers targeting the insert region listed in Table 2 (Schäfer et al., 1994a).

To further assist with conjugation quantification, the chromosomes of the donors, *P. putida* G7 and *N. aromaticivorans* F199, were inserted with genes encoding constitutive fluorescence of GFP. For insertion of the construct into *N. aromaticivorans* F199, a vector based on the pAK405 vector with a GFPmut2 construct under the *psyn2* promoter was synthesized for insertion into the chromosome replacing the Saro_1879 gene (Kaczmarczyk et al., 2013; Taton et al., 2014). Delivery of the vector was also achieved through conjugation with *E. coli* WM6026 as the donor. Selection of *N. aromaticivorans* F199 colonies exhibiting successful gene insertion and constitutive fluorescence was performed by following previously established methods and PCR/sequencing with primers targeting the insert region listed in Table 2 (Kaczmarczyk et al., 2012). The F199 strain fluorescing both mScarlet-I and GFPmut2 was designated JMN120 but will be referred to as F199 henceforth for simplicity. For insertion of GFP into *P. putida* G7, the pMRE-Tn7-152 vector plasmid from Schlecter et al. (Schlechter et al., 2018) was used to insert the mVenus construct at the attTn7 site via transposon mutagenesis (DeBoy & Craig, 2000). Delivery of the vector was again achieved through conjugation with *E. coli* WM6026 as the donor. Selection of *P. putida* G7 colonies exhibiting successful gene

insertion and constitutive fluorescence was achieved by following previously established methods and PCR/sequencing with primers targeting the insert region listed in Table 2 (Schlechter et al., 2018). Fluorescence of mScarlet-I and GFP in both donors was also validated by detecting fluorescence on the Tecan Sunrise microplate reader (Männedorf, Switzerland). The G7 strain fluorescing both mScarlet-I and mVenus was designated PVP102 but will be referred to as G7 henceforth for simplicity.

Table 2: Primers used to validate successful insertion of fluorescent protein gene constructs.

Insertion Target	Forward Primer	Reverse Primer
pNL1	CCCTTCCGCGTTCCACTCG	CCATACGGCGGCGAGTTGC
NAH7	ATGCGCCGAGCTTCTTTTTCAAT	CTTTCTCAGGGCCGTGTTGGG
F199 chrom.	TGCCGATCACCTACCTCAAG	AGAAGACTGGTGTGATCGGC
G7 chrom.	GCTGGCGCCGATCCTTTACAC	CTCACTTCGGCCGTCTTTATCACA

3.2.5 Conjugation into Recipient Strains

In order to validate the conjugation detection method and investigate conjugation of the degradative plasmids into select recipients, conjugation of pNL1 and NAH7 into recipients was first examined by adding one donor and one recipient in culture. G7, F199, and recipients were grown overnight in CBM at 30°C. In the morning, donors and recipients were added in triplicate to 5 mL of CBM either with or without naphthalene to assess whether or not naphthalene was acted as a significant selective

pressure for conjugation. For NAH7 conjugations, G7 and recipients were added in a 1:1 ratio. For pNL1 conjugations, F199 and recipients were added in a 10:1 ratio due to the slow growth of F199 compared to most recipients. For each culture, a total of 10^6 cells were added. Naphthalene was added to the NAH7 conjugation cultures by adding naphthalene crystals to the CBM medium which ensured the medium was continually saturated with naphthalene. For the pNL1 conjugation cultures, 100 mg/L of naphthalene was added by first dissolving in acetone, adding to the culture tube, and letting the acetone evaporate—leaving naphthalene crystals in the tubes. Conjugation frequency, determined by the number of transconjugants divided by the geometric mean of the number of donors and recipients, was measured at 24 and 48 hours after inoculation.

3.2.6 FACS Analysis

On sample days, 500 μL of reactor media was added to 5 mL polypropylene tubes prior to sorting. Cells were sorted into 200 μL of PBS which was directly used for down-stream DNA extraction. Flow cytometric detection and sorting of cells was performed using a BD FACSAria II (San Jose, CA, USA) through the Duke Human Vaccine Institute Flow Cytometry Center at Duke University (Durham, NC, USA). The following technical settings were employed: A 70 μm nozzle and sheath fluid pressure of 70 psi with chiller; GFP was excited by a 488 nm laser and detected on the FITC-A channel with a bandpass filter of 530/30 nm; mScarlet-I was excited with a 561 nm laser

and detected on the PE-Texas Red-A channel with a bandpass filter of 610/20 nm. FlowJo 10.8.1 was used for analyzing results.

Bivariate contour plots of particle FSC vs. SSC areas were employed to build a gate around the bacterial population excluding background noise. Green and red fluorescing bacterial cells were gated on bivariate contour plots using the area of PE-Texas Red vs. the area of FITC. The detection gates and example gates used in this study are depicted in Figure A.3. Donors were gated as green and red, transconjugants were gated as red-non-green, and recipients as non-green and non-red. A total of 100,000 events were recorded and a total of 20,000 transconjugant cells were sorted when applicable under purity settings.

3.2.7 Data Analysis

All data were analyzed using RStudio, version 1.4.1103. Experimental replicates for all data were averaged and reported with standard error. The effect of growth strategy, GC content, and phylogenetic relatedness on conjugation frequency in conjugation assays were analyzed using a simple linear regression to test if any of these variables had an effect on conjugation frequency. ANCOVA measurements were used to compare between naphthalene treatments for pNL1/F199 groups but not for NAH7/G7 groups due to heterogeneity of variance. All other results were analyzed using multi-way ANOVAs with post hoc Tukey's HSD and pairwise t-tests. Statistical significance was determined with a 95% confidence interval.

3.3 Results

3.3.1 Conjugation of pNL1 and NAH7

The conjugation frequencies of pNL1 and NAH7 were assessed in 1:1 conjugation assays with each donor and recipient either in the presence or absence of naphthalene at 24 and 48 hours of growth to determine: 1) conjugation preferences of the degradative plasmids; and 2) whether or not naphthalene is a significant enough selective pressure for conjugation (Figure 8). For pNL1, conjugation frequencies ranged from below detection to 0.025 (Figure 8A). Overall, there was significantly higher conjugation of pNL1 into *S. lindanitolerans*, the recipient with the lowest 16S copy number, compared to *S. marcescens* ($p = 0.04$). Additionally, there was nearly significantly higher conjugation into *S. lindanitolerans* than *E. cloacae* ($p = 0.07$). Both *S. marcescens* and *E. cloacae* have the highest 16S copy numbers of the recipients. *C. vibrioides* harbored significantly higher amounts of pNL1 when naphthalene was present after 48 hours than any other recipients throughout the study ($p < 0.001$).

For NAH7, the conjugation frequencies ranged from below detection to 0.7 (Figure 8B). There were no overall significant differences in conjugation frequencies between recipient organisms. However, there was a significant decrease in conjugation frequency at 48 hours compared to 24 hours when naphthalene was not present ($p < 0.05$). Furthermore, the addition of naphthalene led to a significant increase in

conjugation frequency across all recipients regardless of time ($p < 0.05$), suggesting that naphthalene was a selective pressure for conjugation of NAH7 but not for pNL1.

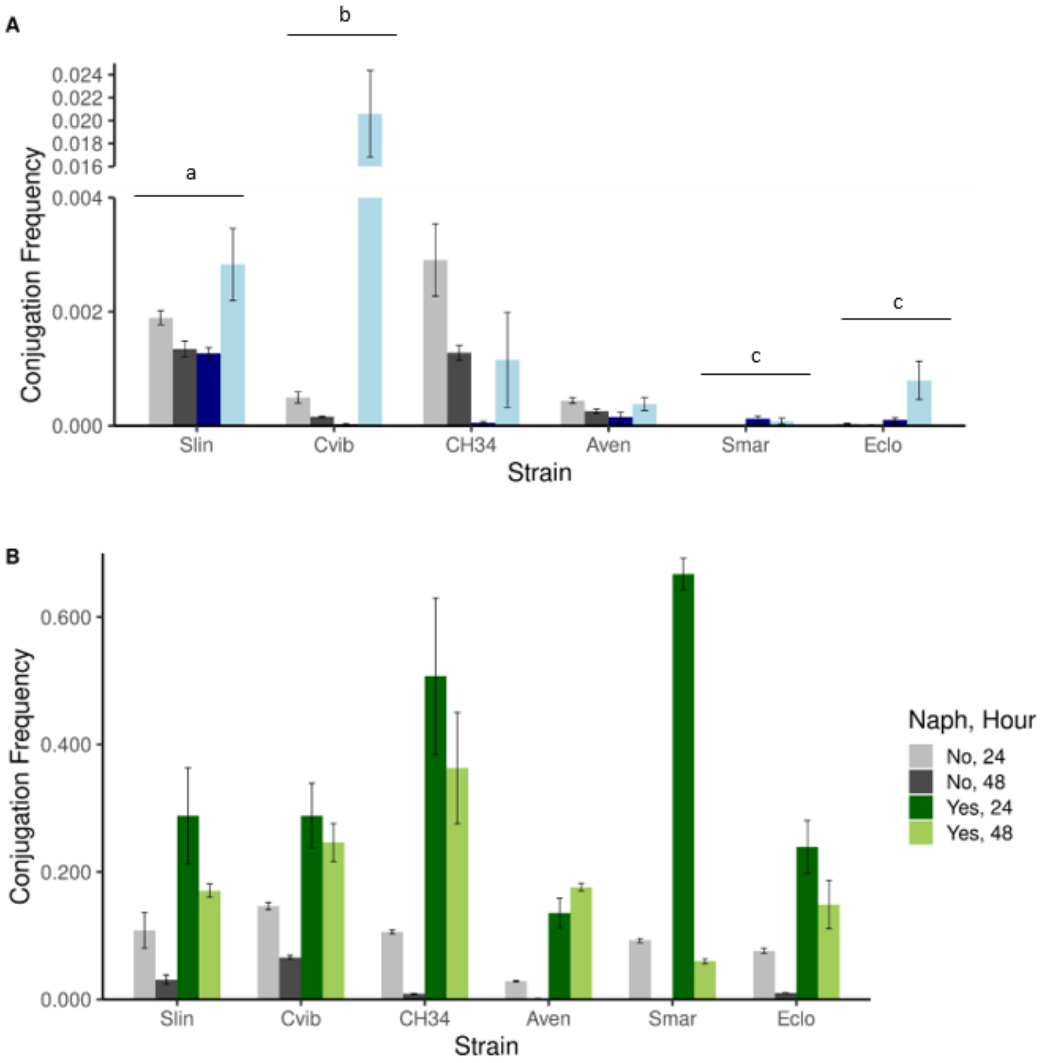

Figure 8: Conjugation frequency of A) pNL1 harbored in F199 and B) NAH7 harbored in G7 in 1:1 conjugation assays ordered from lowest 16S copy number to highest. Bars are colored by whether naphthalene was added and at which hour sampling occurred. Letters above bars indicate populations that are significantly or nearly-significantly different from one another. For B, all naphthalene-added communities had significantly higher conjugation frequency than communities with no naphthalene. "Slin" indicates *S. lindanitolerans*; "Cvib" indicates *C. vibrioides*;

"CH34" indicates *C. metallidurans* CH34; "Aven" indicates *A. venetianus*; "Smar" indicates *S. marcescens*; "Eclo" indicates *E. cloacae*. Error bars indicate standard error (n = 3).

The impact of the 16S rRNA copy number of each recipient, a proxy for growth strategy, was assessed via a regression of conjugation frequency vs. 16S copy number (Figure 9). For pNL1, there was a significant negative correlation between recipient copy number and conjugation frequency when there was naphthalene present or no naphthalene (R^2 = 0.140 and 0.286, p = 0.025 and 0.0001, respectively). ANCOVA revealed that there was no significant difference between the naphthalene and no naphthalene treatments. By contrast, for NAH7, there was only a significant negative correlation between recipient copy number and conjugation frequency when no naphthalene was present (R^2 = 0.164, p = 0.014), although this could not be tested using ANCOVA due to heterogeneity of variances. A comparison of the R^2 values between the two treatments reveals that adding naphthalene dampens any existing significant correlation (R^2 = 0.003, p = 0.77).

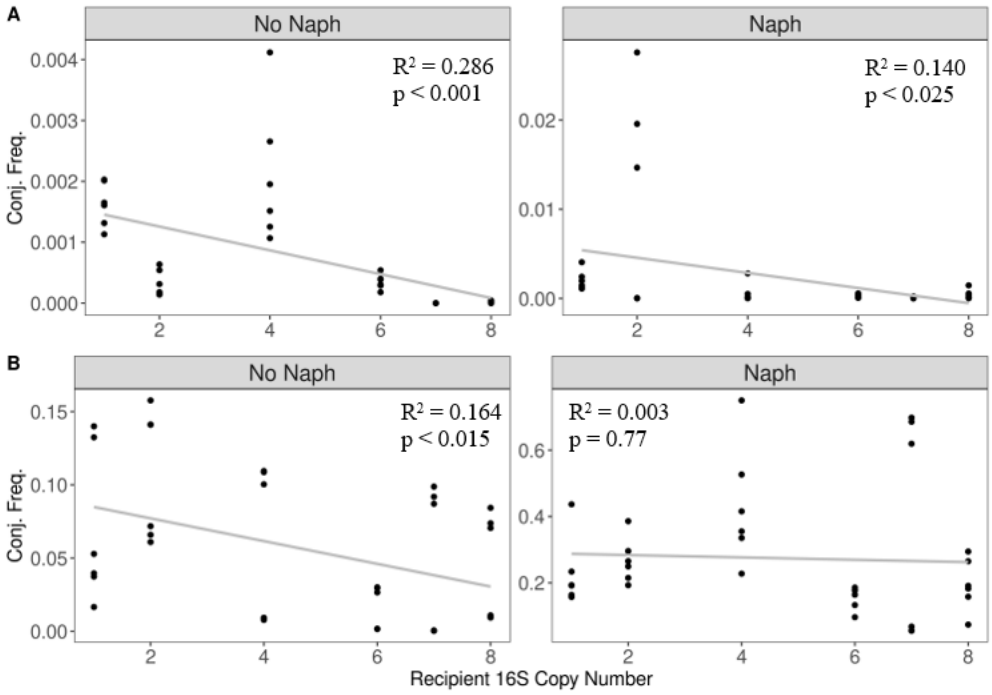

Figure 9: Regressions of conjugation frequency vs. 16S rRNA copy number of the recipient for A) pNL1 harbored in F199 and B) NAH7 harbored in G7. R^2 values and p-values are reported for each regression which are separated by whether naphthalene was present.

3.3.2 GC Content and Phylogenetic Relatedness

Both GC content and phylogenetic relatedness of donor and recipient strains have been shown to have an impact on the conjugation and survival of plasmids (Ikuma et al., 2012; Ikuma & Gunsch, 2013; C. M. Thomas & Nielsen, 2005). To assess the impact of these factors on conjugation of pNL1 and NAH7, regressions of conjugation frequency vs. recipient GC content and phylogenetic distance from the donor were assessed (Figures 10 and 11). Phylogenetic distance was calculated by quantifying the

Average Nucleotide Identity (ANI) of the genomes (Goris et al., 2007). All regressions for each plasmid and whether naphthalene was present were nearly identical between GC content and phylogenetic relatedness for respective groupings. These analyses reveal a significant correlation between both GC content of the recipient and phylogenetic relatedness, and conjugation frequency with one exception (R^2 ranging from 0.092 to 0.235; $p < 0.05$). When naphthalene was added, there was a non-significant positive correlation between GC content and conjugation frequency for both pNL1 and NAH7 (R2 = 0.092 and 0.100, p = 0.073 and 0.06, respectively). Additionally, there is a range of conjugation frequencies at similar GC contents and phylogenetic relatedness with various recipient strains which suggests conjugation preferences cannot be described entirely by these two factors for these bacteria under the given experimental conditions. Furthermore, unlike previous reports suggesting preferential carbon substrate may lead to increased conjugation, naphthalene presence did not influence conjugation frequency with respect to GC content and phylogenetic relatedness.

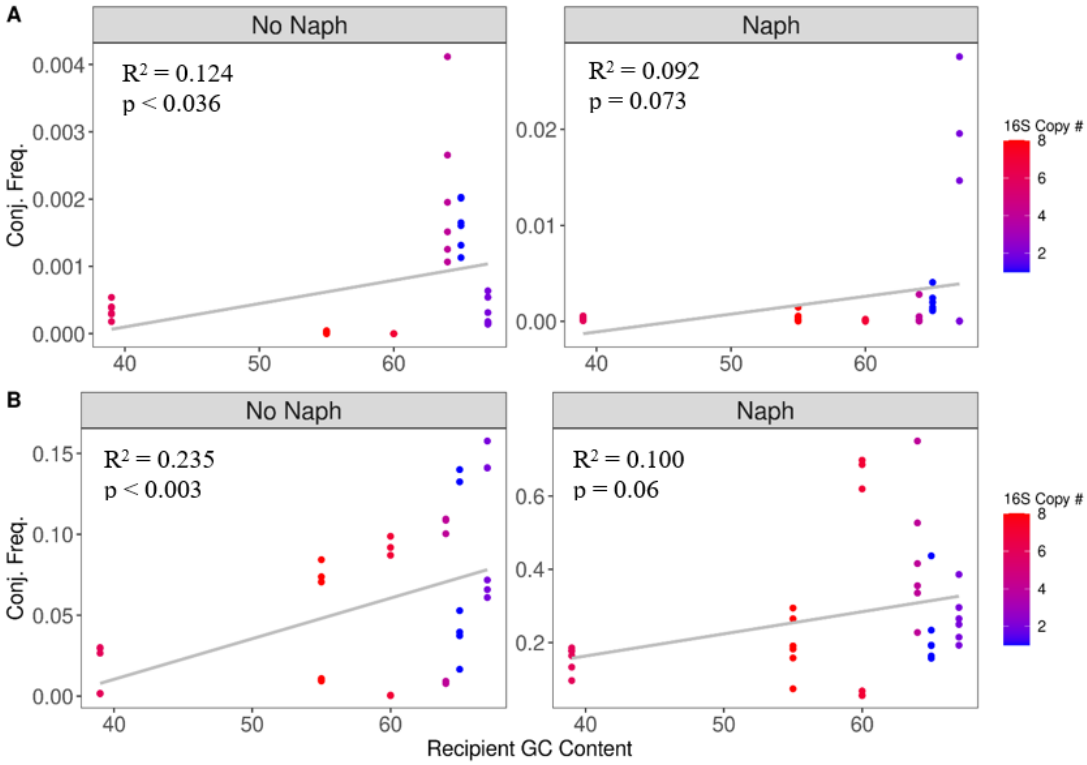

Figure 10: Regressions of conjugation frequency vs. GC content of the recipient for A) pNL1 harbored in F199 and B) NAH7 harbored in G7 colored by recipient 16S rRNA copy number. R^2 values and p-values are reported for each regression which are separated by whether naphthalene was present. GC content of F199 and G7 are 65 and 62, respectively.

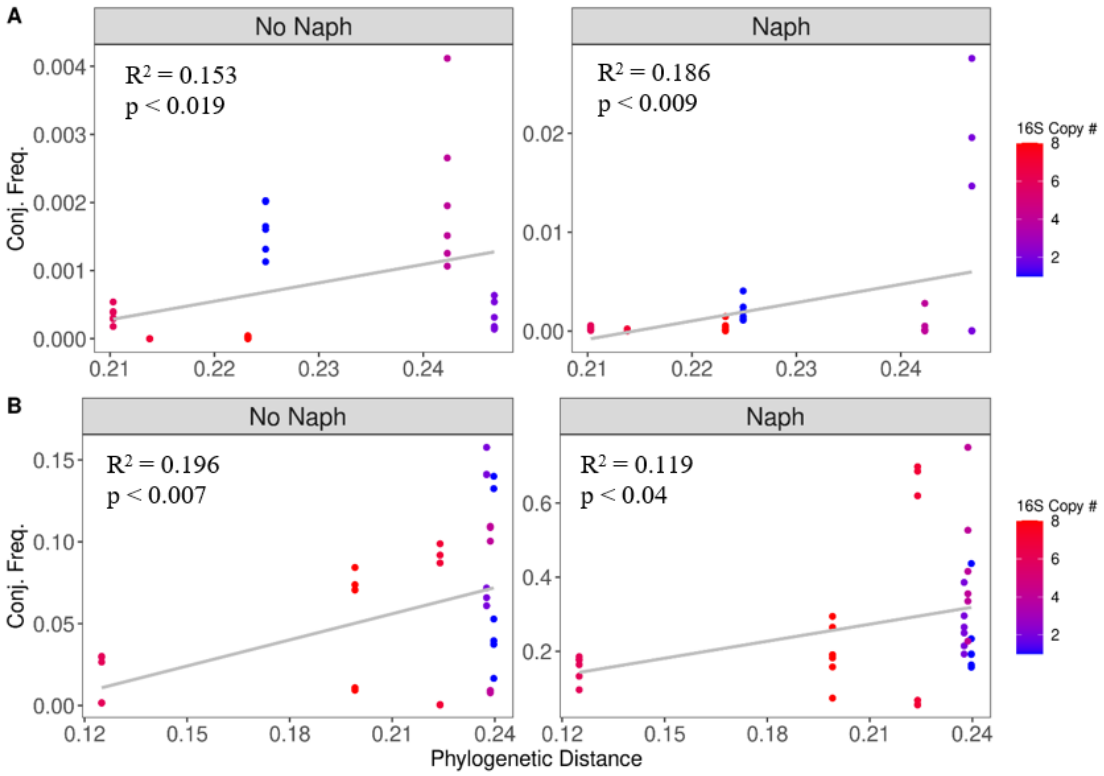

Figure 11: Regressions of conjugation frequency vs. phylogenetic distance from the donor for A) pNL1 harbored in F199 and B) NAH7 harbored in G7 colored by recipient 16S rRNA copy number. R^2 values and p-values are reported for each regression which are separated by whether naphthalene was present. A larger phylogenetic distance value indicates more species relatedness. Phylogenetic distance was calculated by quantifying the Average Nucleotide Identity (ANI) of the genomes.

3.4 Discussion

In this chapter, methods were developed to quantify conjugation using fluorescently labeled plasmids and donor chromosomes and detection/sorting via fluorescent activated cell sorting (FACS). This method sets the groundwork for

measurements in more complex combinations including mixed communities because this tool allows quantification of conjugation and identification of transconjugants *in-situ* and without the need for culturing. As only about 1% of bacteria can be cultured, this method allows for plasmid transfer tracking more accurately and with a broader range of bacteria than previously accessible (Amann et al., 1995; Hugenholtz et al., 1998; Staley & Konopka, 1985). Additionally, although using fluorescently labeled donors and plasmids paired with FACS has been used for investigating conjugation of antibiotic resistance plasmids (Klümper et al., 2015b; B. Li et al., 2018; L. Li et al., 2018, 2020), this is the first use of this method for tracking conjugation of degradative plasmids. This method presents several advantages over previously established methods necessitating qPCR because it reduces bias by not requiring DNA extraction and is able to distinguish between live and dead cells (Redfern, 2017). Although there is bias from the Nycodenz separation of cells, studies have indicated that 80-90% of bacteria are recovered from soil via Nycodenz separation (Holmsgaard et al. 2011; Khalili et al. 2019).

To validate the method and explore conjugation preferences of our selected donors and plasmids, simple 1:1 conjugation assays were performed. One donor and one recipient were added to measure conjugation frequency both with and without the presence of the model PAH naphthalene. In general, the conjugation frequencies of the pNL1 plasmid harbored in *N. aromaticivorans* F199 were 1-2 orders of magnitude lower than the conjugation frequencies of the NAH7 plasmid harbored in *P. putida* G7.

Although this phenomenon could be due to multiple different factors, the smaller size of the NAH7 plasmid may be a key contributing factor to the differences in frequency of conjugation (83 kb NAH7 plasmid as compared to the 184 kb pNL1 plasmid). Smaller plasmids are known to transfer at quicker rates and are easier to maintain in transconjugants than larger plasmids (Garbisu et al., 2017b).

In order to understand confounding factors that have an influence on conjugation preferences, conjugation frequency of pNL1 and NAH7 were explored in relation to GC content of the recipients and phylogenetic relatedness of the recipients to the donors, both of which have been shown to previously be associated with conjugation frequency (Ikuma & Gunsch, 2012; Pinedo & Smets, 2005; C. M. Thomas & Nielsen, 2005). Overall, there was a significant correlation between both GC content and phylogenetic relatedness, and conjugation frequency which supports previous studies (Ikuma et al., 2012; Ikuma & Gunsch, 2013; C. M. Thomas & Nielsen, 2005). However, the range of conjugation frequencies at similar GC contents and phylogenetic relatedness with various recipient strains suggests that conjugation preferences cannot be described entirely by these two factors. Therefore, conjugation of pNL1 and NAH7 in 1:1 communities is likely based on a combination of factors that includes, but is not limited to, GC content, phylogenetic relatedness, and growth strategy of donors and recipients.

Ecological growth strategy of recipients is another factor that is theorized to affect conjugation preferences of degradative plasmids. In the conjugation assays, the K-strategists were found to accept the pNL1 plasmid more frequently than the r-strategists. Particularly, *S. lindanitolerans* consistently had higher conjugation frequencies than the two recipients with highest 16S copy number: *S. marcescens* and *E. cloacae*. In addition, *C. vibrioides* had significantly higher levels of transconjugants than any other recipients, especially when naphthalene was present. This result suggests that K-strategists may be favored over the r-strategists for conjugation with the pNL1 plasmid. This result is further confirmed by the negative correlation observed between conjugation frequency and16S rRNA copy number. This is contrary to previous studies that suggest fast-growers, such as r-strategists, can transfer plasmids more frequently (Seoane et al., 2010; Smets et al., 1993b). However, those previous studies were strictly for smaller antibiotic resistance plasmids with different transfer and replication dynamics. Our results present the first indication that donors harboring degradative plasmids may in fact favor transfer into K-strategists when grown in 1:1 communities.

The NAH7 plasmid harbored in *P. putida* G7 was also used to explore conjugation frequencies in assays with the donor and one recipient. In this case, there were overall no significant differences between conjugation frequencies across different species of recipients. However, the addition of naphthalene was associated with a significant increase in conjugation frequency. This trend was also observed when

comparing the correlation between conjugation frequency and 16S rRNA copy number. Again, a significant negative correlation was observed suggesting that K-strategists may also accept the NAH7 plasmid more frequently. However, this correlation was not observed in the presence of naphthalene. Therefore, the presence of naphthalene exhibited a selective pressure strong enough to diminish any effect that growth strategy might have on conjugation preferences. The increase in conjugation with the selective pressure of naphthalene supports other studies on transfer and expression of degradative plasmids (DiGiovanni et al., 1996; Ikuma & Gunsch, 2012; Wang et al., 2014; Zhang et al., 2012). However, because this phenomenon was not observed for pNL1, these data suggest some dependence on the plasmid itself and highlights the difficult for identifying generalizable framework for *in situ* microbiome engineering.

4. Assessing the impact of growth strategy on conjugation frequency and naphthalene degradation in a synthetic microbial community

This chapter was published in part under the title "The effect of bacterial growth strategies on plasmid transfer and naphthalene degradation for bioremediation" in *Environmental Technology & Innovation* in 2022. It is reprinted with permission from Varner, P.M.; Allemann, M.N.; Michener, J.K.; Gunsch, C.K. (2022). The effect of bacterial growth strategies on plasmid transfer and naphthalene degradation for bioremediation. *Environmental Technology & Innovation*, *28*, 102910. https://doi.org/10.1016/J.ETI.2022.102910

4.1 Introduction

Bioremediation of organic contaminants often requires the survival of exogeneous organisms in an environment where toxic conditions exist. However, well-adapted indigenous organisms often out-compete exogenous microbes and full, complete remediation cannot be reached using traditional bioaugmentation. In contrast, genetic bioaugmentation aims to overcome this problem by transferring degradation genes from exogenous to indigenous bacteria for sustained removal of organic pollutants. Most commonly, genetic bioaugmentation is accomplished by transferring mobilizable plasmids containing degradation genes through conjugation, and

subsequent expression of degradation genes in recipient bacteria, or transconjugants (Ikuma et al., 2012; Top et al., 2002; Top & Springael, 2003).

One class of organic contaminants in which degradation genes often lie on plasmids is polycyclic aromatic hydrocarbons (PAHs). PAHs are a class of persistent organic pollutants that are produced mostly anthropogenically from either the burning of wood and fossil fuels or industrial spills (Cerniglia, 1984; Freeman & Cattell, 1990; Lim et al., 1999). PAHs are of great concern due to their toxic, mutagenic, and carcinogenic properties. Continuous exposure to PAHs can lead to biomagnification which can increase the possibility of long-term adverse heath outcomes in humans and wildlife such as kidney and liver damage and cancer (Bamforth & Singleton, 2005; Cancer, 2019; Gilbert & Blossom, 2014; Ronald, 1988; Valko et al., 2005). Due to their physico-chemical characteristics, PAHs tend to sorb to soils and sediments where they can persist for decades depending on their size (Howard, 1991a). As a result, the EPA has listed 16 PAHs as pollutants that are of high priority for remediation—the most toxic being benzo [α] pyrene (Liu et al., 2001).

Utilizing genetic bioaugmentation for remediating PAHs is promising; however, conjugation and expression of degradative plasmids, particularly PAH-degrading plasmids, has been relatively unexplored due to the lack of methods developed for tracking plasmid transfer and subsequent expression. In particular, bacterial growth strategy is one unexplored factor that likely affects plasmid transfer and expression.

Growth strategy is indicated in bacteria by a high copy number of the 16S rRNA gene in fast-growing r-strategists and a lower copy number of the gene in slower-growing K-strategists (Klappenbach et al., 2000; Ortiz-Álvarez et al., 2018; Roller et al., 2016; Wu et al., 2017). To illustrate how plasmid transfer might be affected by growth strategy, conjugation of the toluene-degrading TOL plasmid has been found to increase with the specific growth rate of conjugation donor and recipients which indicates faster-growing bacteria (r-strategists) may transfer degradative plasmids more frequently (Seoane et al., 2010; Smets et al., 1993a). However, these relationships have not been explicitly explored with PAH-degrading plasmids. Furthermore, the expression of plasmids post-conjugation is required for successful remediation and therefore needs to be explored.

Because growth strategy effects on plasmid transfer and expression have not been explored at a fundamental level, we sought to elucidate these relationships in simple, synthetic communities. To this end, one of two donors harboring PAH-degradative plasmids was added in a community of two potential recipients with various growth strategies. The effect of growth strategies on plasmid transfer and expression of a model PAH (naphthalene) was explored in these synthetic communities to explore plasmid transfer preferences. Understanding these relationships is crucial to eventually precisely engineer microbiomes for PAH bioremediation.

4.2 Materials and Methods

4.2.1 Strains and Media

The donors and recipients used in this study are listed in Table 1 in Chapter 3 of this dissertation. Briefly, *Pseudomonas putida* G7 (G7) harboring the NAH7 plasmid has a higher 16S rRNA copy number compared to the other donor, *Novosphingobium aromaticivorans* F199 (F199) harboring pNL1. Donors and plasmids were inserted with fluorescent protein genes as described in Chapter 3 to track conjugation. The four recipients used in the synthetic communities from lowest 16S rRNA copy number to highest were *Sphingopyxis lindanitolerans* WS5A3p, *Caulobacter vibrioides* CB15, *Serratia marcescens*, and *Enterobacter cloacae*. All strains were grown in Caulobacter medium (CBM) consisting of 2 g/L peptone, 1 g/L yeast extract, and 0.2 g/L $MgSO_4$ x $7H_2O$ at 30°C unless otherwise noted.

4.2.2 Conjugation Assays

To assess conjugation preference and naphthalene degradation for pNL1 and NAH7, synthetic communities consisting of one donor and two recipients were created in triplicate at high or low nutrient availability. All strains were grown overnight in CBM before inoculation and sterile technique was used throughout the experiment. Naphthalene dissolved in acetone was added to 250 mL glass reactors. Subsequently, acetone was evaporated, leaving naphthalene crystals behind. The beginning naphthalene concentrations for pNL1 and NAH7 conjugations were 100 and 500 mg/L,

respectively. Reactors were then filled to 100 mL with either high nutrient medium (CBM) or low nutrient medium (CBM diluted 1:3). These media were selected for this study because the resulting nitrogen and phosphorus concentrations are relevant to those seen in the environment, with the high end being representative of a biostimulation regime (Shabir et al., 2008). For NAH7 conjugations, fluorescently labeled G7 and recipients were added in a 1:1:1 ratio while for pNL1 conjugations, fluorescently labeled F199 and recipients were added in a 10:1:1 ratio due to the relatively slower growth of F199. For each culture, a total of 10^5 cells were added. Reactors were sealed by crimping aluminum caps over sterile rubber stoppers (VWR, Radnor, PA, USA).

Four different communities were assessed at high and low nutrient conditions: 1) Controls with no bacteria present; 2) K community containing the strains most representative of K-strategists (*S. lindanitolerans* and *C. vibrioides*); 3) Mixed (M) community containing one K- and one r-strategist (*S. lindanitolerans* and *E. cloacae*); and 4) r community containing the strains most representative of r-strategists (*E. cloacae* and *S. marcescens*). For each sampling timepoint, 3 mL was taken for naphthalene analysis and 500 μL was taken for FACS analysis. Samples were taken aseptically by inserting a sterile 25G needle and syringe into reactors before placing into 1.5 mL microcentrifuge tubes for FACS analysis or glass hungate tubes for naphthalene analysis (BD, Franklin Lakes, NJ, USA). Conjugation frequency, determined by the number of transconjugants divided by the geometric mean of the number of donors and recipients, was measured

on days 1, 2, 4, and 8. Number of transconjugants, donors, and recipients was quantified via FACS (described below). Naphthalene saturation was monitored on days 1, 2, and 4 and a full extraction on day 8.

4.2.3 FACS Analysis

Flow cytometric detection and sorting of cells was performed using a BD FACSAria II (San Jose, CA, USA) through the Duke Human Vaccine Institute Flow Cytometry Center at Duke University (Durham, NC, USA). The following technical settings were employed: A 70 μm nozzle and sheath fluid pressure of 70 psi with chiller; GFP was excited by a 488 nm laser and detected on the FITC-A channel with a bandpass filter of 530/30 nm; mScarlet-I was excited with a 561 nm laser and detected on the PE-Texas Red-A channel with a bandpass filter of 610/20 nm. FlowJo 10.8.1 was used for analyzing results.

Bivariate contour plots of particle FSC vs. SSC areas were employed to build a gate around the bacterial population excluding background noise. Green and red fluorescing bacterial cells were gated on bivariate contour plots using the area of PE-Texas Red vs. the area of FITC. The detection gates and example gates used in this study are depicted in Appendix A, Figure 28. Donors were gated as green and red, transconjugants were gated as red-non-green, and recipients as non-green and non-red. A total of 100,000 events were recorded and a total of 20,000 transconjugant cells were

sorted when applicable under purity settings. Sorted cells were then used for DNA extraction and qPCR analysis.

4.2.4 DNA Extraction and qPCR

Transconjugants underwent DNA extraction and were quantified with qPCR following sorting. DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (Hilden, Germany) following manufacturer's instructions. Lysis was carried out for 30 minutes at 56°C and elution was performed twice at 25 μL. As transconjugants were sorted straight into PBS, no pelleting of cells was necessary before lysis.

The relative abundance of transconjugants in the conjugation assays was determined by qPCR. Relative abundances were calculated by comparing the copy number-corrected starting quantity of each recipient calculated via 6-point standard curves. Taqman™ probes for each strain were developed targeting the variable V3 region of their 16S rRNA genes. Primers were developed targeting the conserved area of the V3 region (Table 3). All qPCR assays were performed on a BioRad CFX96 Touch Real-Time PCR Detection System (Hercules, CA, USA) in a total reaction volume of 20 μL. Primers and probes were added to a final concentration of 250 nM with 1 μL of extracted transconjugant DNA and iTaq Universal Probes Supermix. The reaction conditions consisted of 95°C for 3 minutes, followed by 39 cycles of denaturation at 95°C for 20 seconds and annealing/extension at 60°C for 30 seconds. Reactions were

performed in triplicate and included negative controls and standards consisting of gBlocks™ of the targeted V3 region.

Table 3: Primers and probes used in qPCR assays.

Primer/Probe	Sequence
V3 Forward Primer	AKGAATTGACGGGGRCCY
V3 Reverse Primer	TCACRACACGAGCTGACG
S. lindanitolerans probe	CAGAACCTTACCAGCGTTTGACATCCTTG
C. vibrioides probe	CTTACCACCTTTTGACATGCCTGGACCGC
S. marcescens probe	CTTTACCAGAGATGGATTGGTGCCTTCGG
E. cloacae probe	CCACAGAACTTTCCAGAGATGGATTGGTG

4.2.5 Naphthalene Quantification

Liquid samples (3 mL) were collected at days 1, 2, and 4 from each reactor aseptically using a 25G1 needle attached to a glass syringe to extract naphthalene from the conjugation assays. On day 8 of the experiment, naphthalene was extracted from the whole reactor by adding 100 mL of solvent to each reactor. Naphthalene was extracted using ethyl acetate and mixed for 10 seconds followed by 20 seconds and complete separation. 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, Bellefonte, PA, USA). Naphthalene concentrations were determined using fluorescence at a 7-minute analysis window with 224 nm of excitation and 330 nm of emission and five-point standard curves.

4.2.6 Data Analysis

All data were analyzed using RStudio, version 1.4.1103. Experimental replicates for all data were averaged and reported with standard error. All results were analyzed using multi-way ANOVAs with post hoc Tukey's HSD and pairwise t-tests. Statistical significance was determined with a 95% confidence interval.

4.3 Results

4.3.1 Conjugation in Synthetic Communities

Conjugation frequency of pNL1 and NAH7 was assessed over eight days in simple synthetic communities consisting of one donor and two recipients with varying levels of ecological growth strategies to determine transconjugant preference with either high or low levels of nutrients and conjugation preferences when there were multiple recipients present in a community (Figure 12). For pNL1, the K community consisting of 2 recipients exhibiting a K-strategy had significantly higher levels of conjugation overall compared to the mixed (M) community and the r community ($p < 0.05$). Additionally, there were higher levels of conjugation frequency in the low nutrient conditions in the

mixed community consisting of one r-strategist and one K-strategist, particularly on day 8 ($p < 0.05$). There were no significant differences in conjugation frequency between high and low nutrient conditions for the K or r communities. For the NAH7 plasmid, there was significantly higher levels of conjugation in the r community compared to the K community ($p < 0.01$) and almost significant compared to the M community ($p = 0.076$). Additionally, all communities had higher levels of conjugation in the low nutrient conditions, particularly on days 4 and 8 ($p < 0.05$).

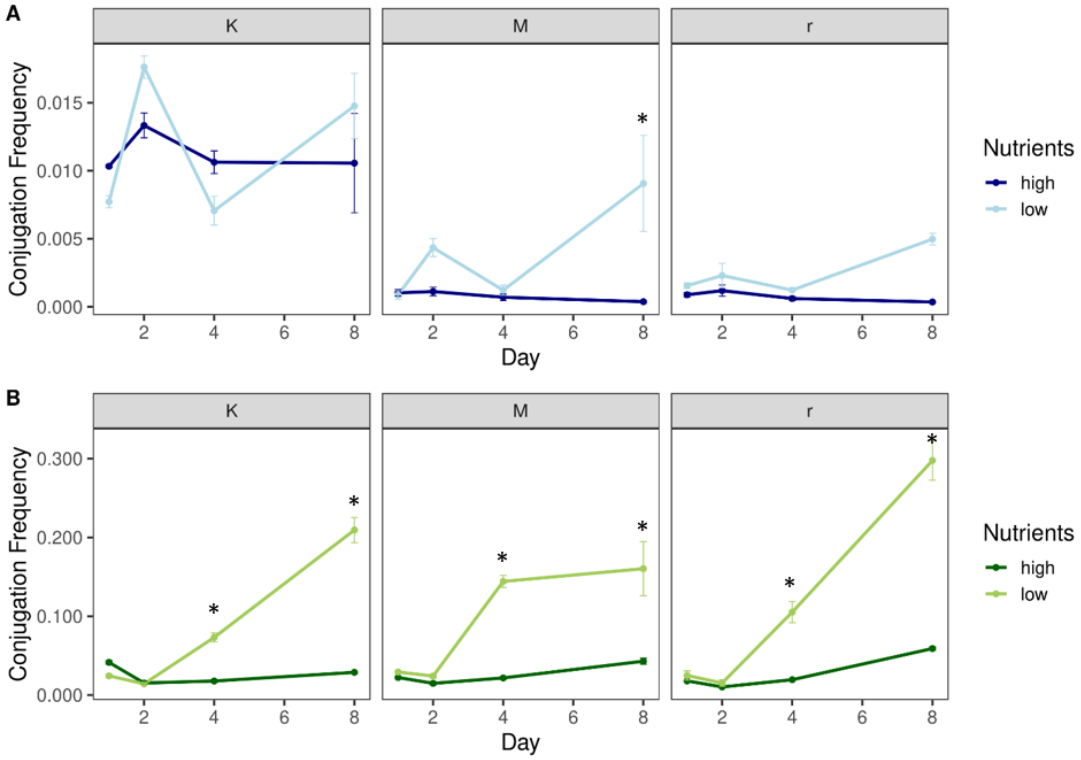

Figure 12: Conjugation frequency of A) pNL1 harbored in F199 and B) NAH7 harbored in G7 in 1:2 conjugation assays colored by high or low nutrient levels. Stars indicate significant difference in conjugation frequency between high and low nutrients per day ($p < 0.05$). "K" represents the K community consisting of *S.*

***lindanitolerans* and *C. vibrioides*; "M" represents the mixed community consisting of *S. lindanitolerans* and *E. cloacae*; "r" represents the r community consisting of *S. marcescens* and *E. cloacae*. Error bars represent standard error (n = 3).**

4.3.2 Transconjugant Relative Abundance

The relative abundance of transconjugants was assessed via qPCR after FACS for recipients that accepted the pNL1 and NAH7 plasmids (Figure 13). Transconjugant relative abundance followed similar patterns for both pNL1 and NAH7 assays in the presence of both high and low nutrients. First, in the K community which consisted of two K-strategists, nearly 100% of transconjugants were *S. lindanitolerans* which has the lower 16S rRNA copy number. In the mixed community consisting of one K- and one r-strategist, ~75-100% of transconjugants were *E. cloacae* which has the highest 16S rRNA copy number. *S. lindanitolerans* was present as a transconjugant of pNL1 in the mixed community but was not present as a transconjugant of NAH7 in the mixed community. For the r community consisting of two r-strategists, both recipients were present as transconjugants. However, *S. marcescens*, with a lower 16S copy number, was more abundant as a pNL1 transconjugant but was less abundant than *E. cloacae* as a NAH7 transconjugant.

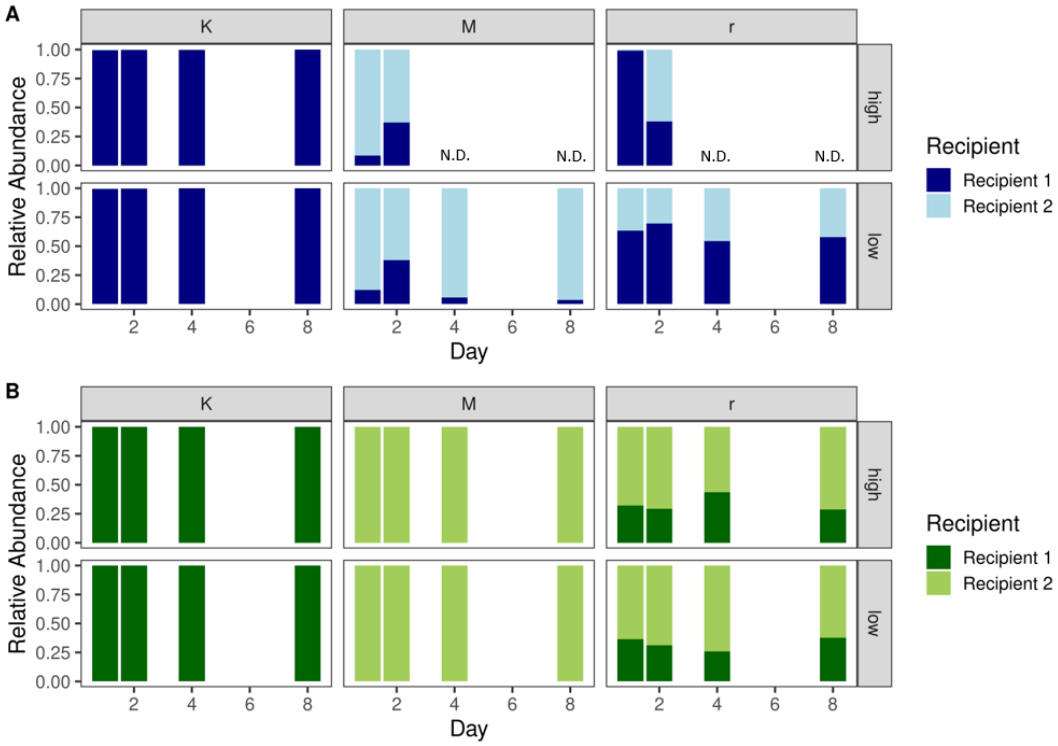

Figure 13: Relative abundances of transconjugants in 1:2 synthetic communities at high and low nutrients for A) pNL1 harbored in F199 and B) NAH7 harbored in G7. Recipient 1 has the lower 16S copy number and Recipient 2 has the higher 16S copy number. For K community: Recipient 1 is *S. lindanitolerans* and Recipient 2 is *C. vibrioides*. For M community: Recipient 1 is *S. lindanitolerans* and Recipient 2 is *E. cloacae*. For r community: Recipient 1 is *S. marcescens* and Recipient 2 is *E. cloacae*. "N.D." indicates no transconjugants detected.

4.3.3 Naphthalene Degradation

To assess the differences in expression of plasmid-mediated degradation genes in synthetic communities with various growth strategies, total naphthalene removal was measured at the end of the eight-day conjugation experiments (Figure 14). For the pNL1 experiments, all communities had significantly more naphthalene removal than the

control consisting of naphthalene with no cells added, suggesting increased biological removal of naphthalene over 8 days ($p < 0.05$). The K community had significantly higher removal of naphthalene than the mixed and r communities ($p < 0.01$). However, there were no significant differences in removal between the mixed and r communities. In addition, we observed marginally significantly higher degradation in the low medium conditions compared to the high medium conditions ($p = 0.0875$).

For the NAH7 experiments, only the K and mixed communities had marginally significantly more naphthalene removal than the controls ($p < 0.09$). The r community had no significant biological removal of naphthalene. The K and mixed communities had marginally significantly higher removal of naphthalene compared to the r community ($p < 0.08$). However, there was no significant difference in naphthalene removal between the K and mixed communities. We also observed marginally significantly higher degradation in the low medium conditions compared to the high medium conditions ($p = 0.0538$).

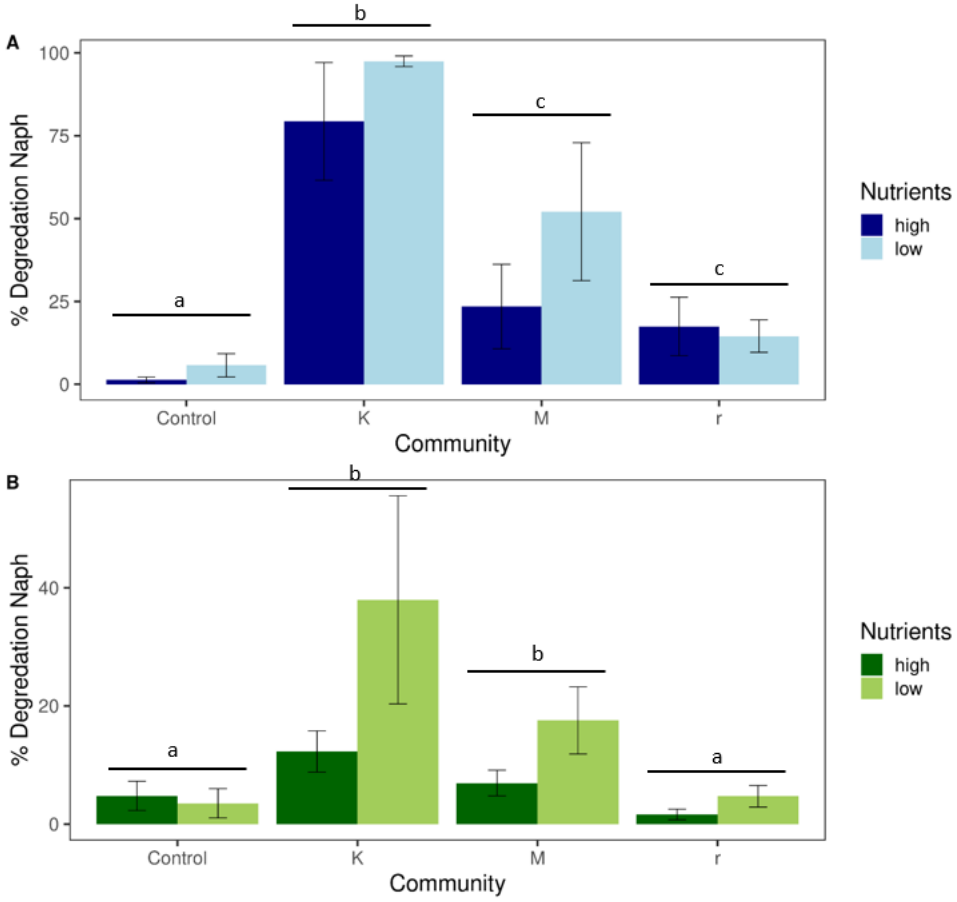

Figure 14: Percent change in naphthalene for the synthetic communities for A) pNL1 harbored in F199 and B) NAH7 harbored in G7. Letters above bars indicate populations that are significantly ($p < 0.05$) or nearly-significantly ($p < 0.09$) different from one another. "K" represents the K community consisting of *S. lindanitolerans* and *C. vibrioides*; "M" represents the mixed community consisting of *S. lindanitolerans* and *E. cloacae*; "r" represents the r community consisting of *S. marcescens* and *E. cloacae*. Error bars represent standard error (n = 3).

4.4 Discussion

To assess conjugation frequencies, preferences, and plasmid gene expression with multiple recipients present, F199 harboring pNL1 was added to three different

communities consisting of two recipients with varying growth strategies. Overall, the K community consisting of two K-strategists had higher frequencies of conjugation and higher percent removal of naphthalene. These data further support the findings from Chapter 3 conjugation experiments in which pNL1 favored conjugation into K-strategists compared to r-strategists, and findings from previous studies investigating non plasmid-mediated hydrocarbon degradation (Brzeszcz et al., 2016).

Interestingly, however, there was a higher relative abundance of the r-strategist, *E. cloacae*, as a transconjugant in the mixed community compared to the K-strategist, *S. lindanitolerans*. This finding suggests that fast growth may be a more critical factor in transconjugant prevalence as compared to conjugation preferences themselves. Furthermore, higher numbers of transconjugants were observed in the low nutrient conditions which might be explained by these conditions promoting more growth of the donor, F199, than the high nutrient conditions. These phenomena could explain why previous studies have found higher rates of transfer in r-strategists (Seoane et al., 2010; Smets et al., 1993b). Understanding relationships between conjugation and growth strategy in synthetic communities presents strategies for implementing genetic bioaugmentation and precisely engineering environmental microbiomes in the field.

NAH7 conjugation and expression of naphthalene-degrading genes was also explored in more complex synthetic communities consisting of two recipients. Contrary to both the 1:1 conjugation experiments in Chapter 3 with NAH7 and the 1:2 conjugation

experiments with pNL1, the r community, consisting of two r-strategists, had higher frequencies of conjugation than the K and mixed communities. Additionally, there were higher relative abundances of the r-strategists in the r and mixed communities. This change in preference compared to previous data provides some insight into differences in conjugation preference when there are mixed communities competing for resources and plasmid acceptance. In the present study, similarly to previously published studies, we observed that r-strategists might outcompete K-strategists as transconjugants, particularly when the plasmid donor is an r-strategist itself (Seoane et al., 2010; Smets et al., 1993b).

Interestingly, the mixed and r communities that had higher abundances of r-strategist transconjugants had less naphthalene removal than the K community consisting almost entirely of *S. lindanitolerans*, the recipient with the lowest 16S copy number. This observation supports previous findings that suggest K-strategists are able to degrade contaminants more efficiently than r-strategists (Brzeszcz et al., 2016). Thus, although conditions might favor survival of transconjugants exhibiting r-strategy, K-strategists may express plasmid genes more efficiently, a finding important to explore further for the implementation of genetic bioaugmentation for bioremediation. Finally, similar to the pNL1 experiments, the low nutrient conditions promoted significantly more conjugation in all communities compared to the high nutrient conditions. Although high nutrients would likely promote growth of the donor, G7, low nutrients

may have promoted growth of transconjugants which may explain their observed increased prevalence in the community over time.

Overall, both pNL1 and NAH7 showed conjugation preferences towards K-strategists over r-strategists, except when NAH7 was in a mixed synthetic community with two recipients. Further, removal of naphthalene via plasmid-mediated gene expression was consistently higher in communities containing K-strategists compared to either a mixed community or a community consisting of r-strategists. Although in the present experiments, some of the naphthalene degradation may be explained by favorable growth of the donor, the discrepancy between conjugation preference for r-strategists but higher degradation for K-strategists suggests that NAH7 transconjugants may be responsible for this incremental degradation. Furthermore, lower availability of nutrients consistently promoted the highest level of conjugation and naphthalene degradation.

The general preference of pNL1 and NAH7 transferring into K-strategists, or organisms with a lower 16S copy number, is a unique finding that supports previous investigations using antibiotic resistance plasmids. For example, a range of antibiotic plasmids have been found to have a preference for exponential growth phase transfer (r-strategy) or stationary phase transfer (K-strategy) depending on the plasmid donors and recipients (Sysoeva et al., 2019). Therefore, it seems that pNL1 and NAH7 show more preference for stationary phase transfer, or K-strategy. Additionally, NAH7 had higher

amounts of conjugation compared to pNL1. With G7 having a higher 16S rRNA copy number and being a faster grower than F199, it was expected that NAH7 would transfer at faster rates based on previous studies (Seoane et al., 2010; Smets et al., 1993a). More research investigating these relationships with degradative plasmids rather than antibiotic resistance plasmids is needed to further support these findings.

The data presented herein are the first to consider the impact of ecological growth strategies on conjugative plasmid transfer and expression. This information fills a critical research gap for the translation of genetic bioaugmentation and field application of microbiome engineering (Varner & Gunsch, 2021). The results from this study provide a framework for identifying the favorable conditions needed to promote the successful implementation of genetic bioaugmentation targeted to the existing recipient community present at a site. However, this work gives insight into conjugation preferences and naphthalene degradation in simple, synthetic communities. In complex communities where heterogeneous conditions exist, the functional dynamics of a microbial community may change and, therefore, the successful implementation of genetic bioaugmentation may ultimately depend on the community structure itself. Additionally, naphthalene as a sole contaminant is not entirely representative of a natural sediment community contaminated with PAHs or other complex mixtures. Therefore, more work needs to be done in natural settings to explore conjugation and plasmid-mediated degradation of contaminants for full and sustained bioremediation.

5. Investigating the impact of growth strategy on the implementation of genetic bioaugmentation of PAH-degrading plasmids in complex sediments

5.1 Introduction

Utilizing microorganisms for bioremediation of soils and sediments has been found to be less environmentally intrusive and more cost-effective than traditional, physico-chemical remediation approaches (Payne et al., 2017; Varjani, 2017). However, challenges such as survival of exogenous organisms, promotion of biodegradation, and maintenance of favorable environmental conditions need to be overcome for successful bioremediation. As discussed in the earlier chapters of this dissertation, genetic bioaugmentation may be able to help overcome these issues by increasing the abundance of biodegradative genes in well-adapted, native indigenous bacteria (Ikuma et al., 2012; Top et al., 2002; Top & Springael, 2003).

Although abiotic and biotic factors have been investigated that influence plasmid transfer and pollutant degradation for successful genetic bioaugmentation, many of the previous studies have been in simple, synthetic communities (Ikuma, 2011; Ikuma & Gunsch, 2012; Johnsen & Kroer, 2007; Seoane et al., 2010; Shintani et al., 2008). Findings from these studies help scientists and engineers understand conjugation processes at a basic level; however, challenges remain for translating these findings into natural, complex microbial communities found in polluted soils and sediments. In particular,

functionality of bacteria is known with changing abiotic and biotic environments, which are particularly different in simple communities versus in the complex communities found in natural environments.

Ecological growth strategies of bacteria and their effect on plasmid conjugation have thus far only been explored in simple, synthetic communities (Smets et al., 1993b; Sysoeva et al., 2019). However, ecological growth strategy as determined by 16S rRNA copy number is a genomic trait that has been found to be predictive of functionality in complex communities as well as synthetic simple communities (Klappenbach et al., 2000; J. Li et al., 2019; Wu et al., 2017). The effect of ecological growth strategies on conjugation of degradative plasmids has not been explored in either simple or complex communities. In previous chapters, we aimed to understand the basic relationships between growth strategy, conjugation, and plasmid-mediated degradation of polycyclic aromatic hydrocarbons (PAHs) of two PAH-degrading plasmids in simple communities. However, more work is needed to elucidate these relationships in complex communities where heterogeneous conditions and complex microbial communities exist.

To that end, herein we explored the relationship between growth strategy as determined by 16S rRNA copy number, conjugation, and plasmid-mediated degradation in natural sediment communities. Two donors harboring PAH-degrading plasmids were augmented in sediment bioreactors containing naphthalene, phenanthrene, and fluorene as model PAHs. Sediments were differentially stimulated to promote growth of bacteria

with various growth strategies to determine growth strategy impacts on conjugation and PAH degradation in complex communities. The results from this study can improve the understanding of factors that affect plasmid transfer and function to promote genetic bioaugmentation as a sustainable precision bioremediation approach in soils and sediments.

5.2 Materials and Methods

5.2.1 Chemicals, Strains, and Media

Naphthalene (98%), phenanthrene (98%), and fluorene (98%) were acquired from Sigma-Aldrich (St. Louis, MO, USA). The two plasmid donors used in this experiment were the dual-fluorescently labeled *Pseudomonas putida* G7 harboring the NAH7 plasmid and *Novosphingobium aromaticivorans* F199 harboring the pNL1 plasmid described in Chapter 3. All strains were grown in Caulobacter medium (CBM) consisting of 2 g/L peptone, 1 g/L yeast extract, and 0.2 g/L $MgSO_4 \times 7H_2O$ at 30°C unless otherwise noted.

5.2.2 Sediment Collection and Spiking

Sediment was collected from the Eno River in Durham, NC, USA. Approximately 2 kg of sediment from the top 7 cm layer was collected and sieved on-site using a 0.8 mm grain size to remove large debris. Sieved sediment was collected in a sterilized bucket and transported to the laboratory where it was immediately stored at 4°C until processing. Sediment for controls was sterilized by autoclaving three times at

121°C for 30 minutes. To improve sterilization, autoclaving was done every 24 hours (Ikuma, 2011).

For use in experiments, both sterilized and non-sterilized sediment was spiked with a mixture of naphthalene, phenanthrene, and fluorene dissolved in acetone at concentrations of 500 mg/kg dry weight for each compound. The PAH solution was manually added to 1 and 2 L glass jars until the solvent completely volatilized and solid PAHs remained on the jar interior walls. Sediment was then added to the bioreactors at greater than or equal to 50% capacity and homogenized by horizontal shaking at 215 rpm for 2 weeks. Jars were wrapped in aluminum foil to prevent photodegradation of PAHs.

5.2.3 Bioreactor Preparation

All bioreactors were prepared by partitioning 20 g of homogenized sediment in 250 mL Erlenmeyer flasks. Bioreactors undergoing a biostimulation scheme had 80 mL of CBM added. Bioreactors undergoing no stimulation throughout the experiment had 40 mL of CBM and 40 mL of sterile water added. To bioreactors receiving plasmid donors, a total of 10^6 cells were added. Bioreactors were covered with Parafilm (Bemis Company, Inc., Neenah, WI, USA) and protected from ultraviolet light by wrapping in aluminum foil. All bioreactors were maintained for 21 days in a slurry phase at room temperature at 80 rpm and flask headspace was recharged by removing the Parafilm for 10 minutes halfway through the experiment. Samples were collected for PAH analysis

on Days 0, 9, and 21 of the experiment and for conjugation on Days 1, 4, 9, 11, and 21 of the experiment. The average growth strategy of the community was assessed by the average 16S rRNA copy number of bacteria present on Days 0 and 21 of the experiment retrieved from the rrnDB (Stoddard et al., 2015).

Multiple experimental and control bioreactors were used to assess the influence of PAHs and plasmid donors on a complex sediment community, each prepared in triplicate (Figure 15). The following bioreactor conditions were prepared: 1) G7 added to nonsterile sediment spiked with PAHs, 2) F199 added to nonsterile sediment spiked with PAHs, 3) G7 added to nonsterile sediment, 4) F199 added to nonsterile sediment, 5) no donors added to nonsterile sediment spiked with PAHs and 6) no donors added to sterile sediment spiked with PAHs. For each bioreactor condition, there were two stimulation schemes to assess the impact of nutrient availability: 1) no stimulation and 2) stimulation on Day 10. Bioreactors undergoing stimulation had 1 mL of 16X concentrated CBM added on Day 10 to bring nutrient levels back to the beginning concentrations.

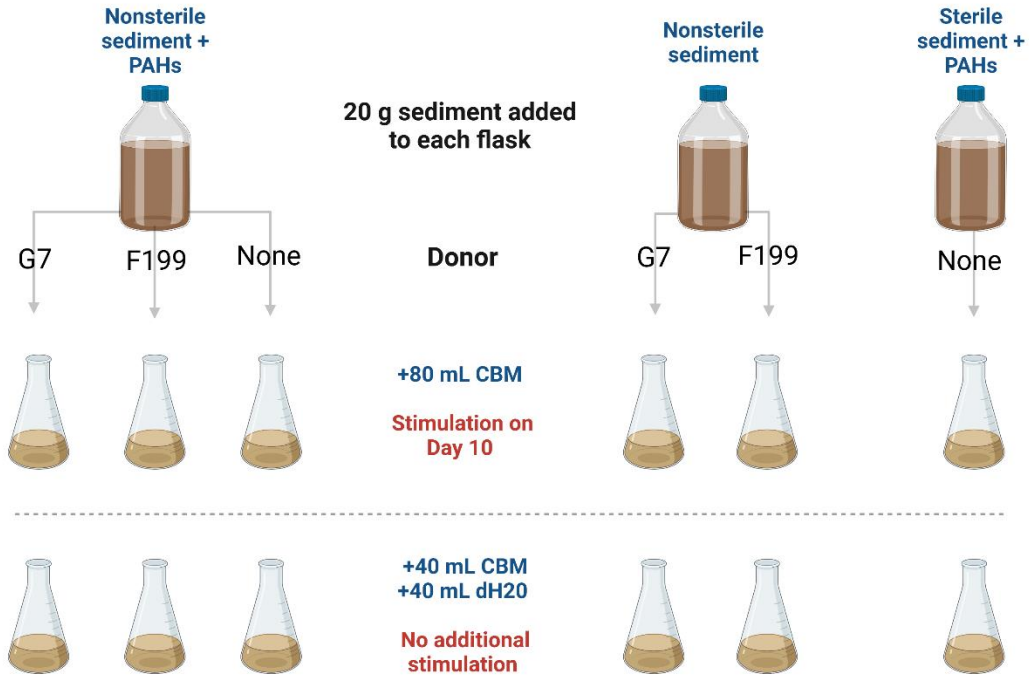

Figure 15: Experimental setup for conjugation assays in natural sediment.

5.2.4 FACS Conjugation Analysis

Flow cytometric detection and sorting of cells was performed using a BD FACSAria II (San Jose, CA, USA) through the Duke Human Vaccine Institute Flow Cytometry Center at Duke University (Durham, NC, USA). The following technical settings were employed: a 70-µm nozzle and sheath fluid pressure of 70 psi with chiller; GFP was excited by a 488 nm laser and detected on the FITC-A channel with a bandpass filter of 530/30 nm; mScarlet-I was excited with a 561 nm laser and detected on the PE-

Texas Red-A channel with a bandpass filter of 610/20 nm. FlowJo 10.8.1 was used for analyzing results.

Bivariate contour plots of particle FSC vs. SSC areas were employed to build a gate around the bacterial population excluding background noise. Green and red fluorescing bacterial cells were gated on bivariate contour plots using the area of PE-Texas Red vs. the area of FITC. The detection gates and example gates used in this study are depicted in Appendix A, Figure 28. Donors were gated as green and red, transconjugants were gated as red-non-green, and recipients as non-green and non-red. A total of 100,000 events were recorded and a total of 20,000 transconjugant cells were sorted when applicable under purity settings. Sorted cells were then used for DNA extraction and qPCR analysis.

5.2.5 DNA Extraction and Illumina MiSeq Amplicon Sequencing

Transconjugant DNA was extracted after FACS using the Qiagen DNeasy Blood and Tissue Kit (Hilden, Germany) following manufacturer's instructions. Lysis was carried out for 30 minutes at 56°C and elution was performed twice at 25 μL. As transconjugants were sorted straight into PBS, no pelleting of cells was necessary before lysis. DNA from raw sediment from the spiked Day 0 sediment and the Day 21 sediments from each bioreactor were extracted using the Qiagen DNeasy PowerSoil Pro Kit following manufacturer's instructions. A total of 500 uL of sediment slurry was used

and lysis was carried out by shaking on the Qiagen PowerLyzer 24 Homogenizer for 60 seconds.

Extracted DNA was sent to Argonne National Laboratory for library preparation and amplicon sequencing of the V4 region of the 16S rRNA gene (Argonne National Laboratory, Lemont, IL). Sequencing was conducted on the Illumina MiSeq via 250 paired-end sequencing. The 16S libraries were demultiplexed, filtered, trimmed, and analyzed using QIIME's DADA2 workflow in R. Reference libraries were developed using SILVA v. 138.

5.2.6 Quantification of PAHs in Sediment

Sediment samples, taken at Days 0, 9, and 21 of the experiment, were stored at -20°C until processed. Sediment slurries were centrifuged at 500 rpm for 10 minutes and approximately 0.5 g of sediment was removed and dried with anhydrous sodium sulfate. Each sample was then spiked with surrogate standard mix (200 uL of 2,000 ng/mL spiking solution). Each sample was then extracted in a 50:50 acetone:hexane mixture (4 mL) via ultrasonication for 5 minutes. The sample was then centrifuged at 500 rpm for 10 minutes and supernatant was collected. This process was repeated three times and supernatants were pooled. 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. 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 6 mL hexane. Samples were once again concentrated to approximately 0.5 mL with SpeedVac concentrator (Thermo Scientific, Waltham, MA, USA). Samples were spiked with internal standards (100 μL, final concentration 200 ng/mL), transferred to muffled autosampler vials, and final volumes were brought to 1mL. 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.

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 flowrate of 1.3 mL/min. The GC oven was programmed 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: d8-Naphthalene, d10-Anthracene, d10-fluoranthene, d12-benz(a)anthracene, d12-benzo(a)pyrene, and d14-dibenzo(a)pyrene. Surrogate standards included: d10-2-methylnaphthalene, d10-phenanthrene, d-12-perylene, and d12-indeno(1,2,3-c,d)pyrene. All isotopically labelled standards were purchased from Cambridge Isotopes (Tewskbury, MA) and used without further purification.

5.2.6 Statistics and Analysis

All data were analyzed using RStudio, version 1.4.1103. Experimental replicates for all data were averaged and reported with standard error when applicable. Conjugation and PAH results were analyzed using multi-way ANOVAs with post hoc Tukey's HSD and pairwise t-tests. Statistical significance was determined with a 95% confidence interval.

Several R packages were used for sequence and data analysis including: phyloseq for beta diversity PCOA plots, alpha diversity, and relative abundance plots, rstatix for running various statistical analyses, and ggplot2 for visualization of plots. ANCOM-BC was used to determine differently abundant taxa from the beginning of the experiment to the end. The average community 16S rRNA copy number was calculated by averaging the 16S copy number for each operational taxonomic unit (OTU) in each sample. The 16S rRNA copy number was estimated for each OTU through the rrnDB database based on its closest relatives with known 16S rRNA copy number (Stoddard et al., 2015). Alpha diversity, conjugation frequencies, and average community 16S rRNA copy number was assessed with multivariate ANOVAs followed by post-hoc Tukey's HSD. Beta diversity was assessed via PERMANOVA. All data were normally distributed.

5.3 Results

5.3.1 Alpha Diversity of Whole Community

The alpha diversity of the whole sediment community was assessed on Days 0 and 21 for both the non-PAH and PAH-spiked sediments to determine the changes in community diversity after augmentation of donors and/or stimulation with nutrients. The alpha diversity of the Day 0 sediment communities was significantly lower for the PAH-spiked sediment community compared to the non-PAH sediment communities ($p < 0.0001$; Figure 16). On Day 21, the alpha diversity of F199 and G7 stimulated and non-stimulated communities in the non-PAH sediment significantly decreased compared to the Day 0 alpha diversity ($p < 0.05$). However, in the sediment spiked with PAHs, there were no significant differences in alpha diversity from Day 0 to Day 21 regardless of the donor augmented, no donor augmented, or stimulation scheme.

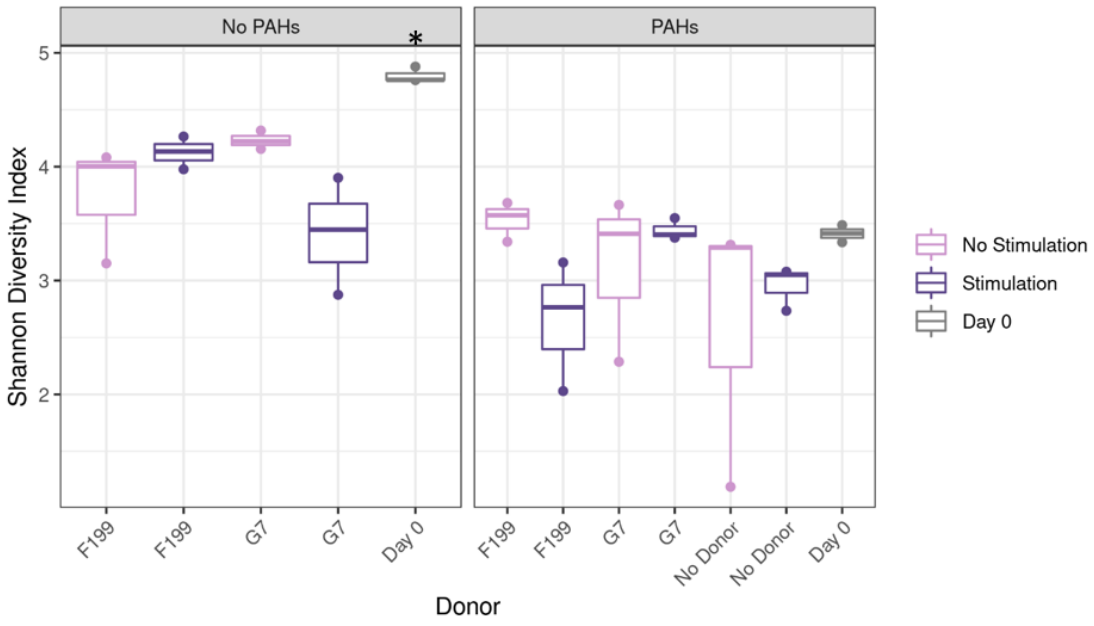

Figure 16: Alpha diversity of sediment communities reported by the Shannon Diversity Index for non-PAH sediment and PAH-spiked sediment. Gray boxes indicate alpha diversity of the sediment communities on day 0 and purple boxes indicate alpha diversity on day 21. Light purple boxes indicate communities that were not stimulated, and dark purple boxes indicate communities that were stimulated. Star indicates significant difference of this values to all other values in the graph ($p < 0.05$, $n = 3$).

5.3.2 Beta Diversity of Whole Community

The beta diversity, or the differences in the community structure between samples, was assessed for the whole sediment communities of the bioreactors on Day 21 to quantify differences between stimulated and non-stimulated communities and differences between donor-augmented or no donor communities. First, principal coordinates analysis (PCoA) using Bray-Curtis dissimilarity metric was used to assess

differences in beta diversity in PAH-spiked sediment communities. There was a significant difference between stimulated and non-stimulated sediment communities by the end of the experiment on Day 21 ($p < 0.001$; Figure 17). For the stimulated communities, there was no difference between F199-augmented, G7-augmented, or no donor communities. However, for the communities undergoing no stimulation, the communities were all significantly different from each other depending on the donor or no donor augmented ($p < 0.001$).

The relative abundances of phyla in each community was also used to assess differences in community structure between Days 0 and 21 of the whole community, PAH-spiked and non-PAH sediment, stimulation schemes, and donor augmentation schemes. Relative abundance comparisons qualitatively support the qualitative alpha and beta diversity findings (Figure B.2).

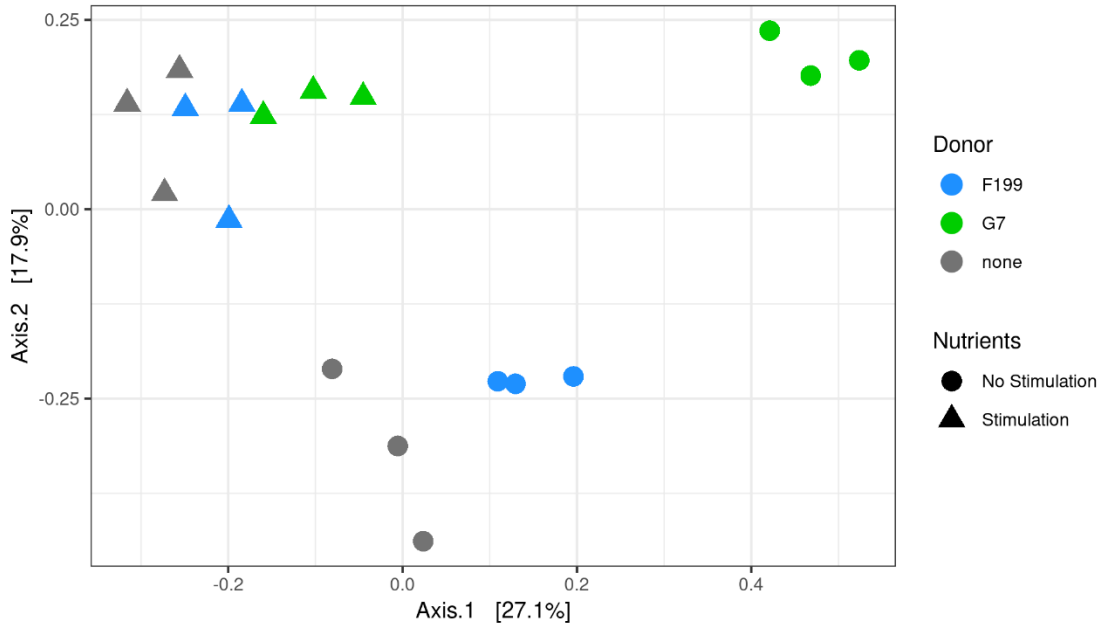

Figure 17: Principal coordinates analysis (PCoA) using Bray-Curtis dissimilarity metric of the PAH-spiked sediment. F199-augmented communities are blue, G7-augmented communities are green, and no donor communities are gray. Bioreactors undergoing stimulation are represented by triangles and bioreactors without stimulation are represented by circles.

5.3.3 Conjugation in Sediment Communities

The conjugation frequency of pNL1 and NAH7 was assessed over the 21-day experiment in natural, complex sediment communities that were either spiked or not spiked with PAHs and received either stimulation or no stimulation to determine conditions that promote conjugation of PAH-degradative plasmids. For pNL1, there was overall significantly more conjugation on Day 21 compared to Days 1-11 ($p < 0.05$; Figure 18A). Additionally, there was no significant difference in conjugation frequency between

stimulated and non-stimulated communities. Particularly in communities spiked with PAHs, conjugation frequency was nearly identical between stimulated and non-stimulated replicates.

For NAH7, there was a significant difference in conjugation frequency between stimulated and non-stimulated communities when PAHs were present ($p = 0.048$; Figure 18B). Particularly, there was one replicate in the stimulation/PAH group that had a much higher frequency of conjugation on Days 9, 11, and 21 that created high variability in this group. However, even when this replicate was removed in the statistical analysis, there was significantly higher conjugation frequency in the stimulated communities than the non-stimulated communities when PAHs were present ($p < 0.05$; Figure B.1 This replicate was kept for analysis, however, due to its biological relevance.

Overall, marginally higher conjugation frequency of NAH7 was observed compared to pNL1 when PAHs were present ($p = 0.070$). This difference was mostly driven by the higher amount of conjugation of NAH7 in stimulated communities compared to pNL1 stimulated communities ($p = 0.066$). There were similar conjugation frequencies of both plasmids when there was no stimulation.

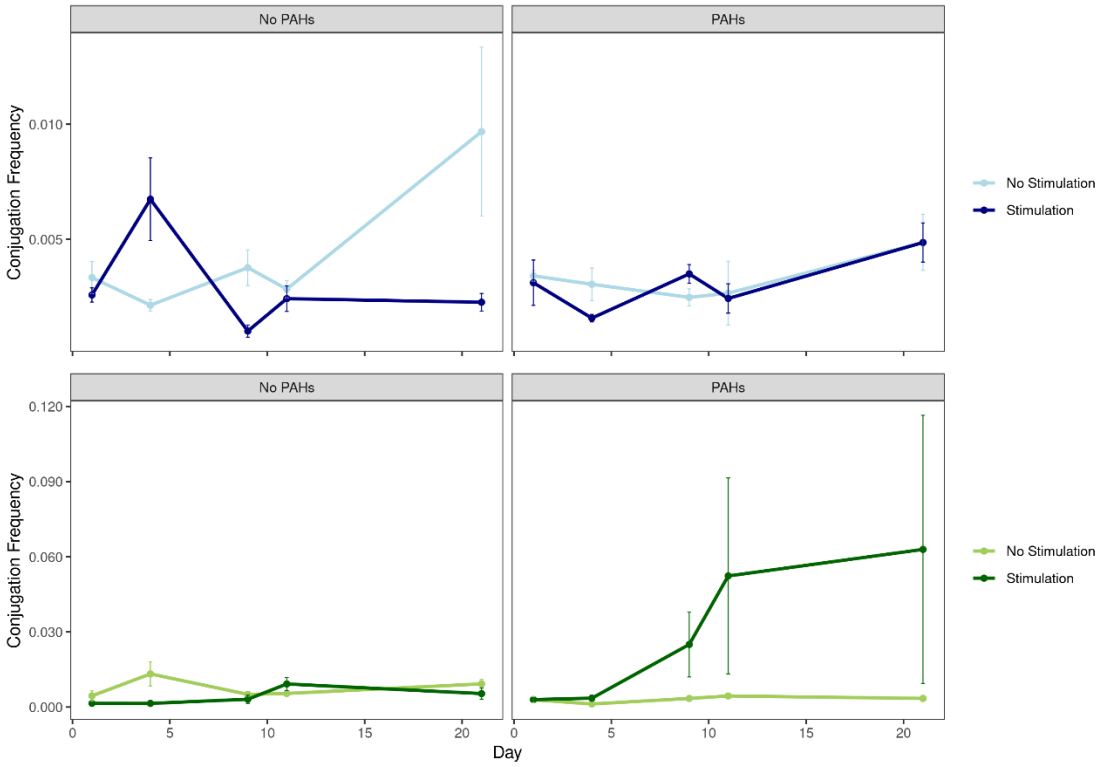

Figure 18: Conjugation frequency over time of A) pNL1 harbored in F199 and B) NAH7 harbored in G7 in sediment communities separated by PAH presence and colored by stimulation/no stimulation. Error bars represent standard error (n = 3).

5.3.4 pNL1 Transconjugants

The taxonomy of the transconjugants of pNL1, harbored in F199, and their 16S rRNA copy numbers were assessed to investigate the structure of the pNL1 transconjugant community and the effect of the average transconjugant 16S rRNA copy number on conjugation and PAH degradation. Changes in the relative abundances of genera of pNL1 transconjugants was assessed qualitatively in both the stimulated and

non-stimulated communities in sediment spiked with PAHs (Figure 19). Species from 17 distinct genera made up the pNL1 transconjugant community. On Day 1 in the non-stimulated communities, the most common transconjugant genera were high-16S rRNA copy number genera including *Bacillus, Raoultella*, and *Citrobacter*. However, by Day 21, the transconjugant community shifted and the most common genera were lower 16S rRNA copy numbers genera including *Acinetobacter* and *Novosphingobium*. On Day 1 of the stimulated communities, most pNL1 transconjugants were once again the high-16S rRNA copy number genera such as *Bacillus, Raoultella, Citrobacter,* and *Solibacillus*. On Day 21 of the stimulated communities, transconjugants were overwhelmingly of the genera *Acinetobacter* and *Escherichia-Shigella*.

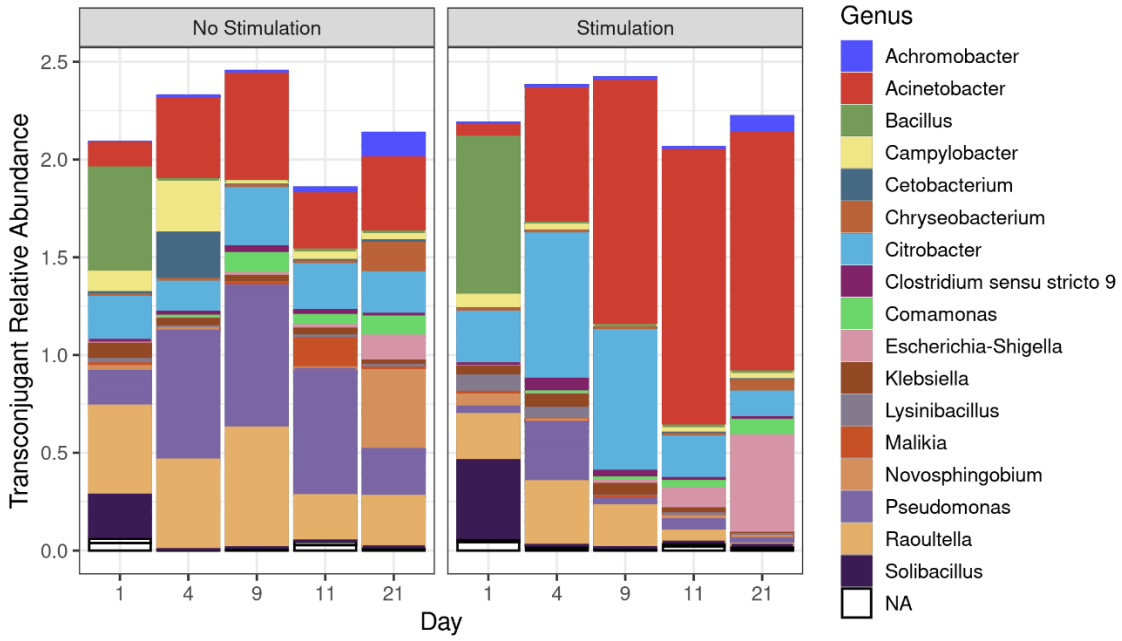

Figure 19: The relative abundances of genera of pNL1 transconjugants from day 1-21 separated by stimulation schemes. Relative abundances of each biological replicate are added so that the total abundance is equal to 3 (n = 3).

The 16S rRNA copy number of pNL1 transconjugants throughout the experiment were assessed to determine how the transconjugant community growth strategies changed over the 21 days and whether this variable had an effect on conjugation and PAH degradation. The average transconjugant copy number decreased from Day 1 to 21 for both stimulated and non-stimulated communities (Figure 20). There was also a consistently higher average 16S copy number in stimulated transconjugants compared to non-stimulated transconjugants ($p < 0.05$).

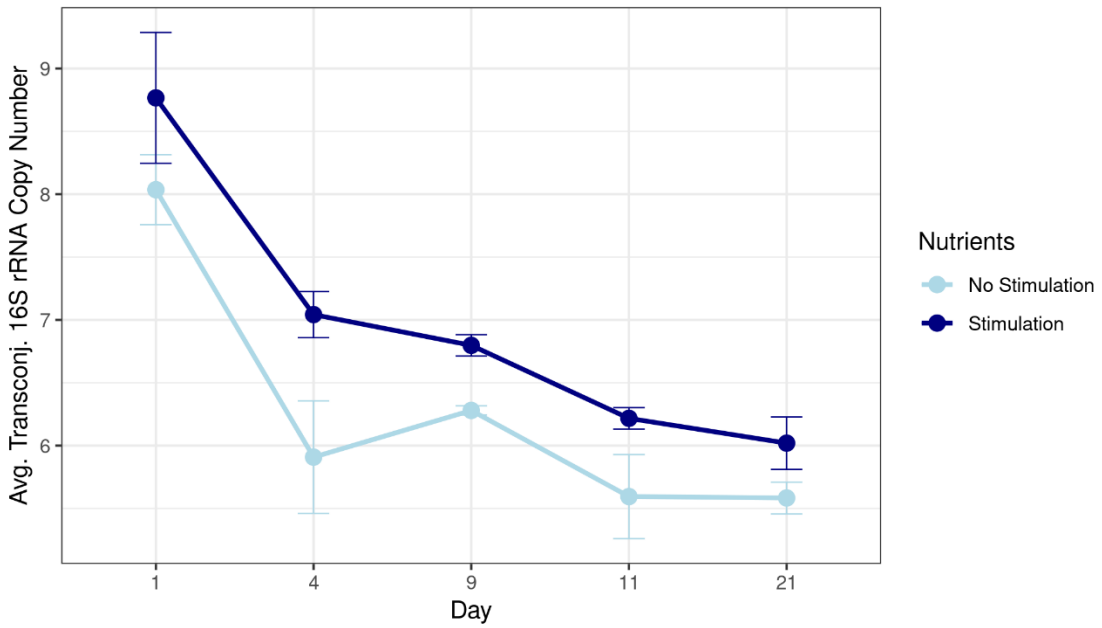

Figure 20: The average 16S rRNA copy number of pNL1 transconjugants from Day 21 colored by no stimulation (light blue) or stimulation (dark blue). Error bars represent standard error (n = 3).

5.3.5 NAH7 Transconjugants

The taxonomy of the transconjugants of NAH7, harbored in G7, and their 16S rRNA copy numbers was assessed to investigate the structure of the NAH7 transconjugant community and the effect of the average transconjugant 16S rRNA copy number on conjugation and PAH degradation. Similarly to the analysis performed for the pNL1 transconjugants, the relative abundances of genera of NAH7 transconjugants was assessed qualitatively in both the stimulated and non-stimulated communities in sediment spiked with PAHs (Figure 21).

Species from 12 distinct genera made up the NAH7 transconjugant community, which is slightly less than the 17 genera that make up the pNL1 transconjugant communities. The majority of NAH7 transconjugants in the non-stimulated communities consisted of *Pseudomonas* species. However, there was a large abundance of *Chryseobacterium, Escherichia-Shigella*, and *Novosphingobium* species by Day 21 in the non-stimulated communities in addition to *Pseudomonas* compared to the first 11 days of the experiment. In the stimulated communities, the NAH7 transconjugants consisted primarily of *Bacillus* and *Pseudomonas* on Day 1. However, by Day 21, there was a much smaller relative abundance of *Pseudomonas* and a higher abundance of *Acinetobacter, Escherichia-Shigella, Citrobacter*, and *Chryseobacterium* as NAH7 transconjugants compared to Days 1-9.

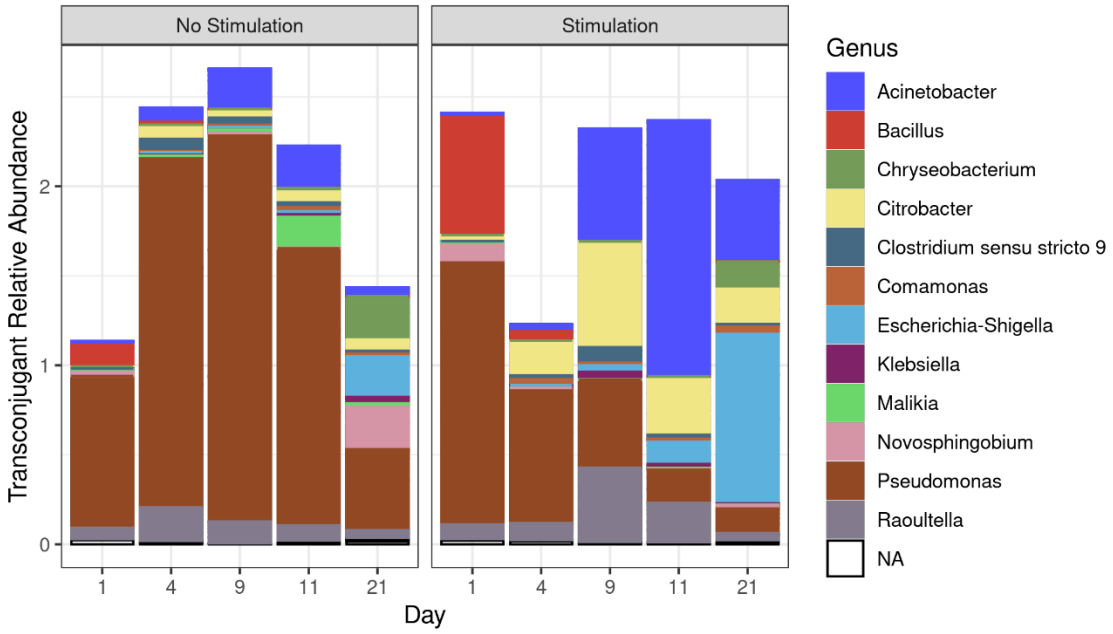

Figure 21: The relative abundances of genera of NAH7 transconjugants from day 1-21 separated by stimulation schemes. Relative abundances of each biological replicate are added so that the total abundance is equal to 3 (n = 3).

The 16S rRNA copy number of NAH7 transconjugants throughout the experiment were assessed to determine how the transconjugant community growth strategies changed over the 21 days and whether this variable had an effect on conjugation and PAH degradation. The average transconjugant copy number decreased from Day 1 to 21 for non-stimulated communities, similarly to pNL1 transconjugants (Figure 22). However, in the stimulated communities, there was an increase in average transconjugant 16S rRNA copy number from Day 1 to 9, and then a decrease in copy number was observed from Day 9 to 21. There was also a higher average 16S rRNA copy

number in stimulated transconjugants compared to non-stimulated transconjugants from Day 9 to 21 ($p < 0.05$).

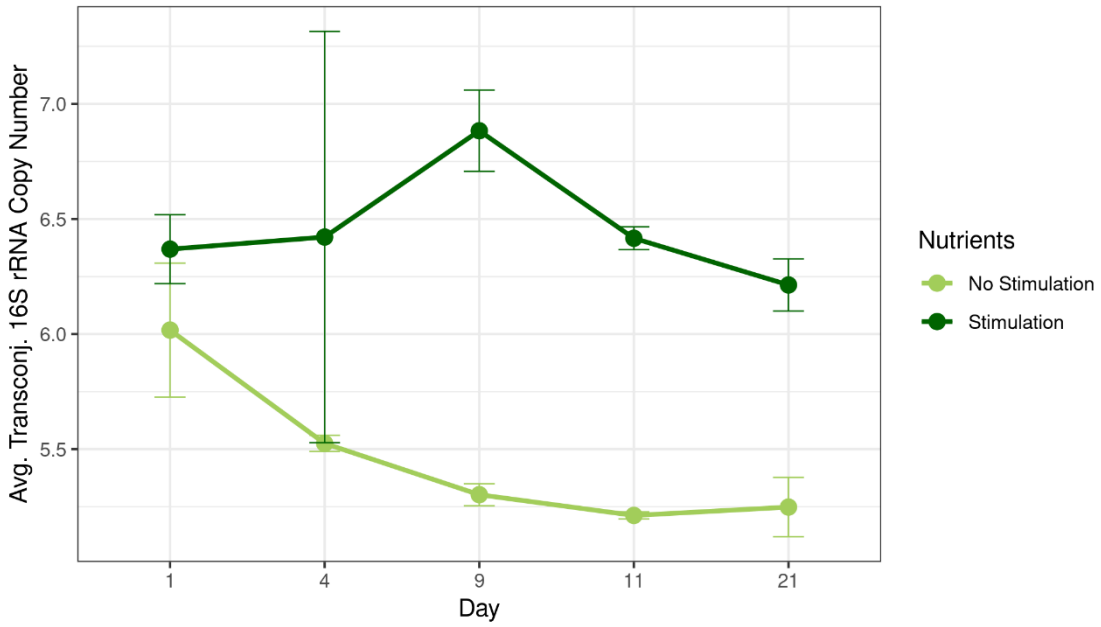

Figure 22: The average 16S rRNA copy number of NAH7 transconjugants from day-21 colored by no stimulation (light green) or stimulation (dark green). Error bars represent standard error (n = 3).

5.3.6 Differentially Abundant Taxa

To determine taxa that might be good targets for genetic bioaugmentation, ANCOM-BC was used to determine which taxa, present as transconjugants, significantly increased in abundance from the beginning of the experiment to the end. Taxa whose abundance increased also in the no donor controls were eliminated because these taxa may have increased simply from stimulation and not due to either conjugation or

presence as a transconjugant. The F199-augmented, non-stimulated communities had 7 species that increased in abundance which was the highest number of taxa compared to F199-augmented, stimulated communities (1 taxa), and G7-augmented communities (2 taxa in each stimulated and non-stimulated communities).

Species that significantly increased in abundance across all donor-augmented communities were of the genera *Novosphingobium, Achromobacter, Raoultella, Comamonas, Pseudomonas, Clostridium,* and *Chryseobacterium* (Figure 23; $p < 0.05$). All of these species were transconjugants that increased in abundance in the F199-augmented, non-stimulated communities, with *Novosphingobium, Achromobacter,* and *Comamonas* having the highest change in abundance from Day 0 to 21. Only *Achromobacter* increased from Day 0 to 21 as a transconjugant in the F199-augmented, stimulated communities. For the G7-augmented communities, *Chryseobacterium* and *Clostridium* increased in the stimulated communities, and *Clostridium* and *Novosphingobium* increased in the non-stimulated communities.

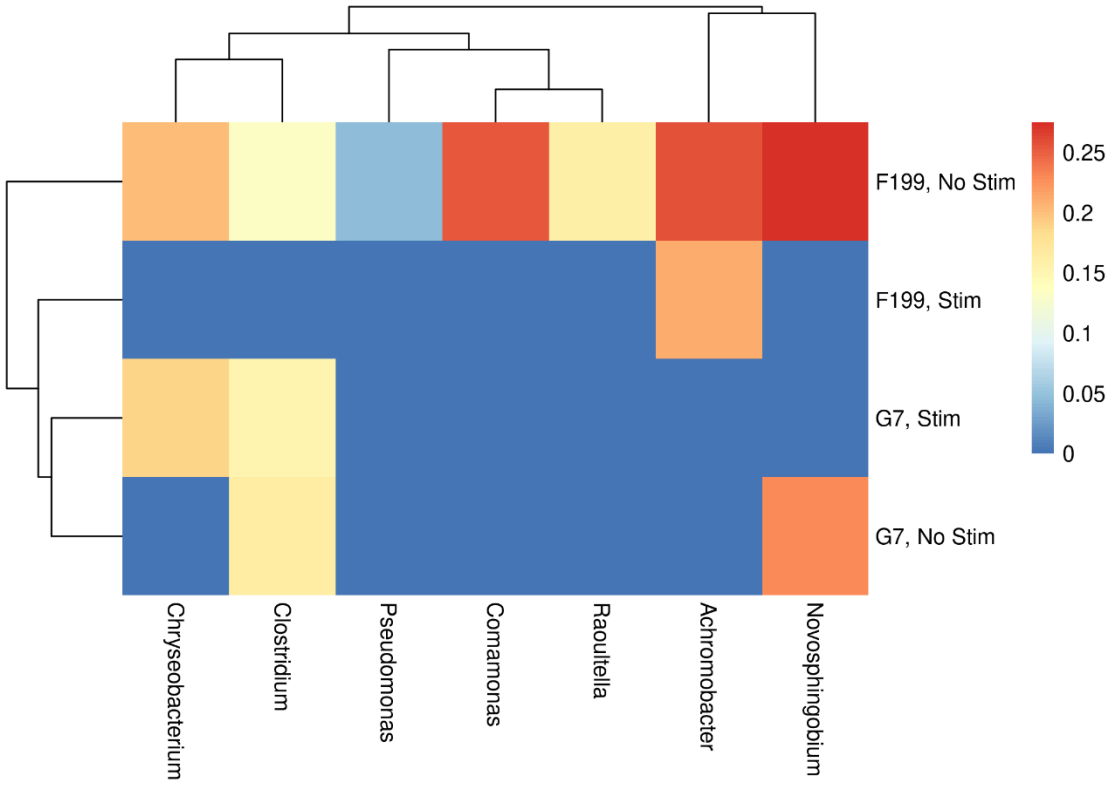

Figure 23: Heatmap of differentially abundant taxa in donor-augmented communities, separated by donor and stimulation scheme, as determined by ANCOM-BC. Taxa included significantly increased from Day 0 to Day 21, were present as transconjugants, and were not present in the no donor controls. The log fold change from Day 0 to Day 21 is represented by color with no change in blue and the highest log fold change in red (n = 3).

5.3.7 Whole Community vs. Transconjugants

The average 16S rRNA copy number of the PAH-spiked whole sediment communities and transconjugant communities was assessed at the end of the experiment on Day 21 to determine if transconjugants and their 16S rRNA copy numbers were representative of the whole sediment community. The average 16S rRNA copy number

of the transconjugant community was 5.47 compared to 5.77 for the whole community (Figure 24). The average community copy numbers were not significantly different and therefore, the transconjugant community was not significantly different that the whole sediment community.

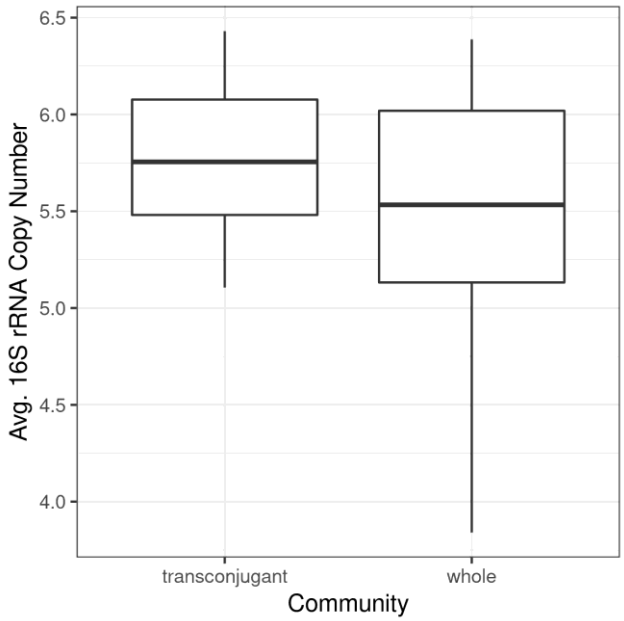

Figure 24: Boxplot of the average 16S rRNA copy number of the transconjugant community and whole sediment community in PAH-spiked sediment on Day 21 (n = 12).

5.3.8 PAH Degradation

The concentrations of naphthalene, phenanthrene, and fluorene were measured at the end of the experiment to assess the differences in plasmid-mediated removal of PAHs in communities augmented with NAH7 or pNL1 as well as control communities

with no augmented donor or no bacteria present (Figure 25). Overall, there was no significant measurable removal of the combined PAHs in donor-augmented communities compared to control communities. When comparing removal of PAHs between G7 and F199 communities, there were no significant differences in removal with one exception: there was significantly more removal of naphthalene in G7-augmented communities and F199-augmented communities undergoing stimulation ($p = 0.05$).

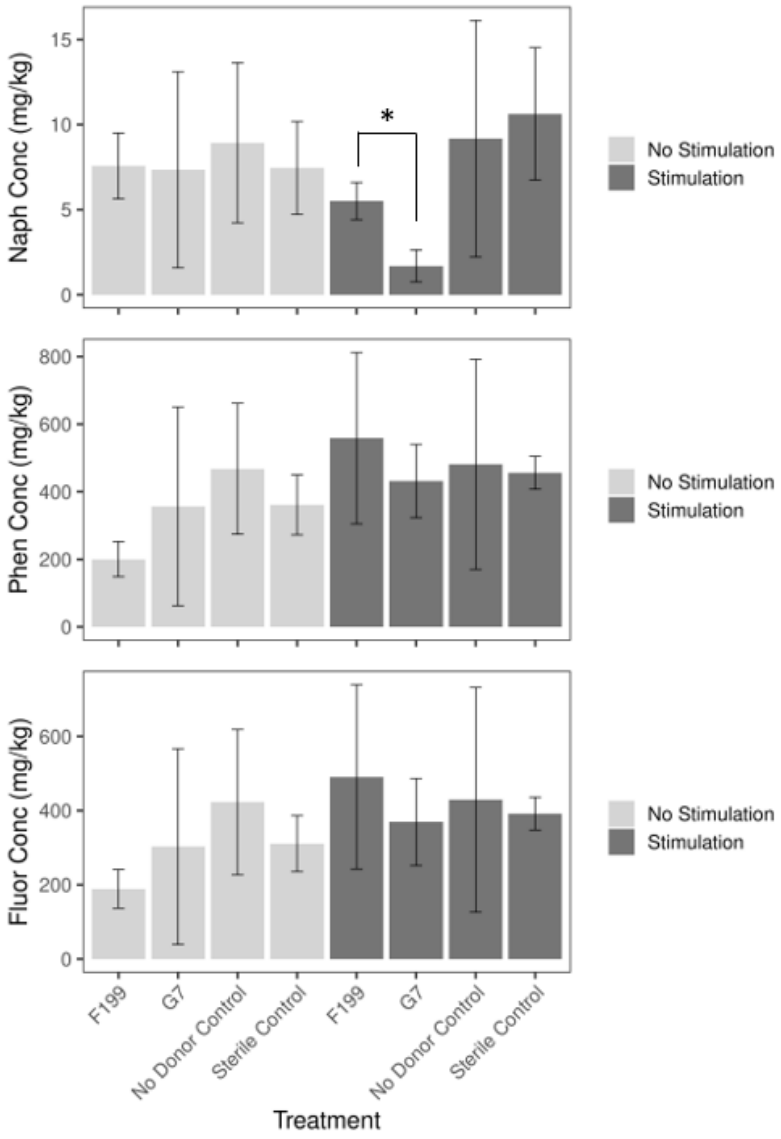

Figure 25: Concentrations in mg/kg wet weight on day 21 for A) Naphthalene, B) Phenanthrene, and C) Fluorene in various communities colored by stimulation or no stimulation. "F199" community had F199 augmented, "G7" community had G7 augmented, "No Donor Control" had no donor augmented but had non-sterile sediment, and "Sterile Control" had no donor augmented into sterile sediment. Stars indicate significant differences between communities indicated ($p < 0.05$). Error bars represent standard error ($n = 3$).

5.4 Discussion

5.4.1 Whole Community Changes

The changes in the microbial structure of the sediment community as a result of stimulation and donor augmentation was explored to assess microbial community changes under various genetic bioaugmentation and biostimulation scenarios and to determine if community structure changed as a function of the augmented bacterium. Previous studies have shown that bioaugmentation of bacteria for the purpose of bioremediation results in a shift of the native bacterial community structure (S. Chen et al., 2021; Haleyur et al., 2019; Liang et al., 2020; Yang et al., 2016). Here, we observed a decrease in alpha diversity of the microbial community from Day 0 to 21 in sediments lacking PAHs—supporting the findings of previous studies. However, in sediments homogenized with PAHs, there was no change in alpha diversity in augmented and stimulated communities from Day 0 to 21. No change in alpha diversity in PAH-spiked sediment is likely due to the presence of PAHs themselves inherently decreasing bacterial diversity prior to augmentation and stimulation compared to the non-PAH sediment. The presence of recalcitrant contaminants, such as PAHs, have previously been found to decrease the microbial diversity in soils and sediments (Deng et al., 2011; el Fantroussi et al., 1999; Zhou et al., 2009). This is likely due to the toxic effects of PAHs on many microorganisms and the limited number of microbes capable of utilizing PAHs as a carbon and energy source.

The beta diversity of the bacterial communities at the end of the genetic bioaugmentation experiment was assessed via Bray-Curtis dissimilarity PCoA and PERMANOVA to determine if the whole bacterial community could be differentiated based on stimulation of donor augmentation. The stimulated communities were indistinguishable from each other regardless of the plasmid donor or no donor added. However, in the non-stimulated communities, the bacterial community structure was distinguishable depending on whether F199, G7, or no donor was augmented. This finding suggests that it may be possible to promote changes in community structure particularly in low-nutrient environments but not with highly stimulated remediation schemes. This is likely due to a predictable pattern of microbial community structure when a high amount of nutrients is added to an environment. Particularly, in high nutrient scenarios, microbes are taxonomically and functionally less diverse than in low nutrient environments (Ortiz-Álvarez et al., 2018; Song et al., 2016). Therefore, small changes in bacterial community makeup in low nutrient environments where bacteria are more diverse may result in greater changes in beta diversity which are observed here.

5.4.2 Conjugation and Transconjugants

The conjugation of two PAH-degradative plasmids, the identity of transconjugants, and the effect of transconjugant growth strategy on conjugation was explored in complex sediments differentially stimulated to determine whether

conjugation could be promoted. For augmentation into PAH-spiked sediment of the pNL1 plasmid harbored in *N. aromaticivorans* F199, conjugation was highest at the end of the experiment on Day 21. Conjugation frequency was not different between stimulation schemes. This is contrary to results found in earlier chapters of this dissertation where low nutrient conditions promoted more conjugation in synthetic communities. In synthetic communities (Chapter 4), the low nutrient conditions contained 20% less nutrients than the low nutrient conditions in the complex community experiments herein. The higher amount of nutrients may explain the lack of differences in conjugation in the complex community experiments and suggests that the community conjugation response can be regulated by nutrient availability.

The average pNL1 transconjugant 16S rRNA copy number decreased in both nutrient schemes from Day 1 to 21. This result suggests either more conjugation into slower growers towards the end of the experiment or persistence of slow-growing K-strategist transconjugants as the experiment progressed. Conjugation preference of pNL1 into K-strategists, as observed in synthetic communities in previous chapters, could account for this transition. Furthermore, because K-strategists are known to thrive in low nutrient, bacterially-dense communities, these transconjugants could have been simply better at survival than fast-growing r-strategists (Fierer et al., 2007). Finally, stimulated transconjugant communities always had higher 16S rRNA copy numbers than the non-stimulated communities—supporting previous studies that determined

growth strategy can be promoted with various nutrient concentrations (Ortiz-Álvarez et al., 2018; Wu et al., 2017).

For augmentation into PAH-spiked sediment of the NAH7 plasmid harbored in *P. putida* G7, conjugation frequency stayed consistent throughout the 21-day experiment in communities undergoing low nutrient conditions (no stimulation). However, conjugation in the stimulated communities increased throughout the experiment. High variability in the stimulated communities was due to one replicate with extremely high amounts of conjugation compared to the other replicates.

The average NAH7 transconjugant 16S rRNA copy number for the non-stimulated communities followed similar patterns to pNL1 transconjugants: there was a steady decrease in transconjugant 16S rRNA copy number and, therefore, conjugation or persistence of slower growers towards the end of the experiment. In the stimulated communities, however, there was an increase in transconjugant 16S rRNA copy number until Day 9, after which the transconjugant 16S rRNA copy number decreased to eventually reach levels on Day 21 that were indistinguishable from Day 1 levels. NAH7 conjugation into higher copy number r-strategists, especially in stimulated communities, supports previously reported findings that fast-growing r-strategists donors and recipients might conjugate more frequently (Merkey et al., 2011; Smets et al., 1993a; Wu et al., 2017). However, persistence of transconjugants from resilient K-strategists may

have led to the decrease in the average transconjugant 16S rRNA copy number observed towards the end of the experiment (Brzeszcz et al., 2016; Sysoeva et al., 2019).

Identifying transconjugants of pNL1 and NAH7 may provide some indication of potential targets for genetic bioaugmentation and, consequently, a nutrient scheme to promote conjugation and growth of these organisms. Overall, 7 genera of bacteria, identified as transconjugants, were found to significantly increase in abundance in the whole community from Day 0 to 21, likely due to conjugation. *Clostridium* species were found to increase in both stimulation scenarios for G7-augmented communities and also for F199-augmented, non-stimulated communities. However, this increase was small in comparison to: 1) *Novosphingobium* found in both G7- and F199-augmented, non-stimulated communities; 2) *Achromobacter* found in both stimulation schemes for F199-augmented communities; and 3) *Comamonas* found only in non-stimulated F199-augmented communities. Overall, the non-stimulated pNL1 transconjugants had the highest number of differentially abundant genera suggesting that pNL1 affects the bacterial community more significantly in the sediment, especially under lower nutrient stimulation. Although both plasmids are similar in that they contain PAH degradation genes, other factors differentiating the plasmids have created differences in the microbial community structure. Therefore, unique plasmids augmented to the environment may alter the microbial community.

Although some bacteria increased in abundance, these bacteria often did not make up a large portion of the overall transconjugant population—typically making up less than 1% of the transconjugant community, but no more than 15% (Figures 19 and 21). Genera such as *Acinetobacter*, *Pseudomonas*, *Escherichia-Shigella*, and *Citrobacter* were highly abundant as transconjugants in both pNL1 and NAH7 transconjugants but did not change in total whole community abundance from Day 0 to 21. Therefore, conjugation preferences of these degradative plasmids seem to be reliant on the community structure inherently in the native soil and sediment and, therefore, do not significantly change abundances of many native bacteria (de Gelder et al., 2005; Musovic et al., 2010b; Sheth et al., 2016). The average community 16S rRNA copy number of transconjugants was nearly identical to the whole community by the end of the experiment, further supporting this fact. Although there might be conjugation preferences into, or persistence of, K-strategists, the transconjugant community was consistently representative of the whole sediment community which means that augmentation of plasmid donors did not drastically change the community due to conjugation. As genetic bioaugmentation aims to introduce degradative genes into the native community, these findings are promising as the transconjugant community is generally representative of the native bacterial communities (Ikuma et al., 2012; Top et al., 2002; Top & Springael, 2003).

5.4.3 PAH Degradation

The degradation of 3 PAHs—naphthalene, fluorene, and phenanthrene—was investigated in various genetic bioaugmentation scenarios to determine whether bacterial growth strategies had an effect on the removal of PAHs from sediment and if those changes were associated with conjugation. The concentrations of all 3 PAHs were extremely variable on Days 0, 9, and 21 within replicates and between replicates. Within-replicate treatments, variability may point to instrument variability and issues with homogenization (Banjoo & Nelson, 2005; Marfil & Albaigés, 1984). Although there were not many significant differences due to this variability, there were a few trends of interest that warrant follow up. First, there were significantly lower concentrations compared to controls of naphthalene in G7 and F199 augmented communities that had undergone stimulation. However, there was no removal of naphthalene in the non-stimulated, plasmid-augmented communities. This finding suggests that removal of naphthalene could be increased with continual stimulation indicative of a biostimulation scenario (Shabir et al., 2008). However, due to the variability observed, likely due to homogenization/equilibration issues or instrument variability, we are unable to determine whether growth strategies of transconjugants had an effect on the removal.

Further, there was no clear removal of fluorene and phenanthrene regardless of plasmid augmentation or stimulation with one exception: non-stimulated communities augmented with F199 showed a qualitative decrease in fluorene and phenanthrene

concentrations compared to controls. Removal of these higher-molecular weight PAHs compared to naphthalene might be due to promotion of slow-growing, lower 16S rRNA copy number K-strategists which were promoted by the lower nutrient availability (Brzeszcz et al., 2016). However, due to the variability of the data, no significant correlation between transconjugant growth strategies and PAH degradation was measured. These findings suggest that a longer-term experiment might be necessary to show differences in degradation similar to that observed by others (Juhasz & Naidu, 2000b; Kanaly & Harayama, 2000).

5.5 Conclusion

Translating findings from simple, synthetic communities has often been difficult due to differences in bacterial behaviors and community structure when translated to complex environments where heterogeneous conditions exist. Here, we aimed to investigate genetic bioaugmentation of PAHs in complex, natural communities to determine if growth strategies and differential nutrient promotion had an effect on plasmid transfer and PAH degradation. Changes in community structure based on the plasmid donor added could be promoted with low nutrient conditions. Additionally, slow-growing K-strategists, promoted with lower amounts nutrients, persisted and increased in abundance towards the end of the experiment and might have contributed to more conjugation. These findings fill a critical research gap for translation of genetic bioaugmentation and field application of precision microbiome engineering and

provides the beginning of a framework for identifying favorable conditions needed to promote successful implementation of genetic bioaugmentation for bioremediation of PAHs.

6. Conclusions

6.1 Key Findings and Future Work

6.1.1 Conclusion 1: Conjugation of PAH-degrading plasmids can be monitored *in situ* using fluorescent protein insertions and fluorescent activated cell sorting

Fluorescent protein genes inserted into plasmid donor chromosomes and PAH-degrading plasmids allowed for tracking conjugation of these plasmids both in simple, synthetic communities and *in situ* using fluorescent activated cell sorting (FACS). This method allows for tracking plasmid transfer in more complex combinations of bacteria in mixed communities without the need for culturing. Advantages of this method over previously established methods, such as using qPCR, include reducing bias by not requiring DNA extraction, the ability to distinguish between live and dead cells, and distinction between transconjugants and recipients without the plasmid.

This method was validated for monitoring the conjugation of two PAH-degradative plasmids in simple, synthetic communities and was further used *in situ* in complex communities. Both the pNL1 plasmid harbored in *Novosphingobium aromaticivorans* F199 and the NAH7 plasmid harbored in *Pseudomonas putida* G7 were transferred to recipients with naphthalene as a selective pressure for conjugation. These methods were also successfully used to determine that similar GC content between donor and recipient, closer phylogenetic relatedness of the strains, and the slower growth strategy of the recipients determined by a lower 16S rRNA copy number were

all biological factors that impacted transfer of these plasmids. Overall, this work suggests that this approach could be broadly used to study plasmid transfer in the context of bioremediation.

The main limitation of this monitoring approach is the need for genetic insertion of fluorescent protein genes into plasmids and donor chromosomes and thus, necessitates significant *a priori* knowledge of the plasmid of interest as well as knowledge of genetic manipulation. Nonetheless, new genetic modification methods are continuously being developed that are reducing the barriers for carrying out these complex modifications in a wide range of bacteria. The development of more streamlined approaches will ultimately make these monitoring approaches more accessible to a wide range of scientists and engineers to explore plasmid transfer processes for a wide range of technologies.

6.1.2 Conclusion 2: Slow-growing K-strategists are better naphthalene degraders and preferred as pNL1 transconjugants in simple, synthetic communities

The conjugation of PAH-degradative plasmids pNL1 and NAH7 was investigated in simple communities consisting of one donor and two recipients to track conjugation and assess the influence of bacterial growth strategies on conjugation and naphthalene degradation. The goal of this part of the dissertation was to assess the effects of bacterial growth strategies on genetic bioaugmentation for remediation of PAHs. A clear conjugation preference into slow-growing K-strategists for the pNL1

plasmid was observed, however there was no clear preference for a specific growth strategy for the NAH7 plasmid. Furthermore, more conjugation was consistently observed when a lower amount of nutrients was added to the reactors for both plasmids, an environmental growth condition that would preferentially advantage the slower growing K-strategists.

Growth strategies of recipients significantly affected the overall community's ability to degrade of naphthalene over 8 days. A higher amount of degradation was observed in the community consisting of 2 slow-growing K-strategists compared to either 2 fast-growing r-strategists or a mixed community for both pNL1 and NAH7 plasmids. These findings suggest that slow-growing K-strategists, which typically have a lower 16S rRNA copy number, may be better targets for genetic bioaugmentation. Although this work was performed in simple, synthetic communities, this information provides some initial insights for the design and implementation of strategies to promote remediation of contaminants in soils in sediments via precision bioremediation. In addition, follow up work should be focused on isolating the transconjugants to determine their individual contributions to naphthalene degradation compared to donor-mediated naphthalene degradation.

6.1.3 Conclusion 3: Increased conjugation of K-strategists can be promoted by differential *in situ* biostimulation in complex natural sediment communities

The implementation of genetic bioaugmentation was further assessed in complex, natural sediment communities to determine if findings in simple, synthetic communities could be translated to more complex environments. Conjugation of pNL1 and NAH7, and PAH degradation, was investigated in natural sediment communities treated with naphthalene, fluorene, and phenanthrene to determine if differential *in situ* nutrient biostimulation could be used to stimulate targeted bacterial growth strategies and impact overall genetic bioaugmentation efficacy. The growth strategies of transconjugants were most closely associated with effects on conjugation or persistence of transconjugants. Specifically, the highest conjugation frequencies of pNL1 and NAH7 were most commonly detected in the latter experimental stages, when transconjugants had lower overall 16S rRNA copy numbers. These findings suggest that conjugation in complex communities may either be favored by transconjugants exhibiting a slower-growing K-strategy, or that these transconjugants are able to persist longer than their faster-growing counterparts.

Differential nutrient biostimulation was a successful approach for promoting K-strategist transconjugants. Transconjugants with higher 16S rRNA copy numbers were consistently observed under high nutrient biostimulation conditions compared to low/no additional nutrient biostimulation conditions. These findings provide some

insights as to approaches for promoting the growth of bacteria exhibiting particular growth strategies *in situ* in complex environment that may be useful for the implementation of precision bioremediation. Furthermore, the observed bacterial community structure was different in low nutrient concentrations depending on the donor and plasmid augmented. This finding suggests that it may be possible to engineer targeted community structure changes depending on the selection of donor and plasmid used for genetic bioaugmentation.

Interestingly, although growth strategy was found to impact conjugation and persistence of transconjugants, its impact on overall PAH degradation was not as clear. We hypothesize that this may be due to the length of our experiments and that equilibrium/homogenization of PAHs may require longer than 21 days to see degradation effects. For this reason, we recommend that future studies on genetic bioaugmentation in complex communities, especially with PAHs, should be carried out for a longer period than 21 days to be able to see decreases in contaminants via plasmid-mediated degradation. Finally, these data amplify the difficulty of detangling the biological, chemical, and physical criteria in complex media. Future work in evaluating the relative contributions of transconjugants via proteomics or RNA sequencing would be beneficial to identify key components that lead to the promotion of more efficient bioremediation of PAHs.

6.2 Engineering Significance

This dissertation aimed to identify precision bioremediation targets by investigating how bacterial growth strategies affect plasmid transfer and PAH degradation for the implementation of genetic bioaugmentation. The work presented in this dissertation suggests that bacterial growth strategy can be stimulated by differential nutrient stimulation. Furthermore, this work demonstrates that slow-growing K-strategist bacteria with lower 16S rRNA copy numbers appear to be optimal targets for genetic bioaugmentation. This dissertation work also demonstrates that utilizing *in situ* conjugation tracking techniques via fluorescent protein gene insertions and fluorescent activated cell sorting are beneficial for understanding properties that affect genetic bioaugmentation for successful and sustainable removal of contaminants from the soils and sediments.

The information presented in this dissertation fills a critical research gap for the translation of genetic bioaugmentation and field application of precision microbiome engineering. This work provides the beginning of a framework for identifying the favorable conditions needed to promote the successful implementation of genetic bioaugmentation targeted to the existing recipient community present at a PAH contaminated site. Beyond their relevance to the field of bioremediation, these findings are broadly applicable to other fields including the development of strategies for

preventing the spread of antibiotic resistance or microbiome engineering technologies for the agriculture, biomedical and environmental fields.

Appendix A. Supporting Material for Chapter 3

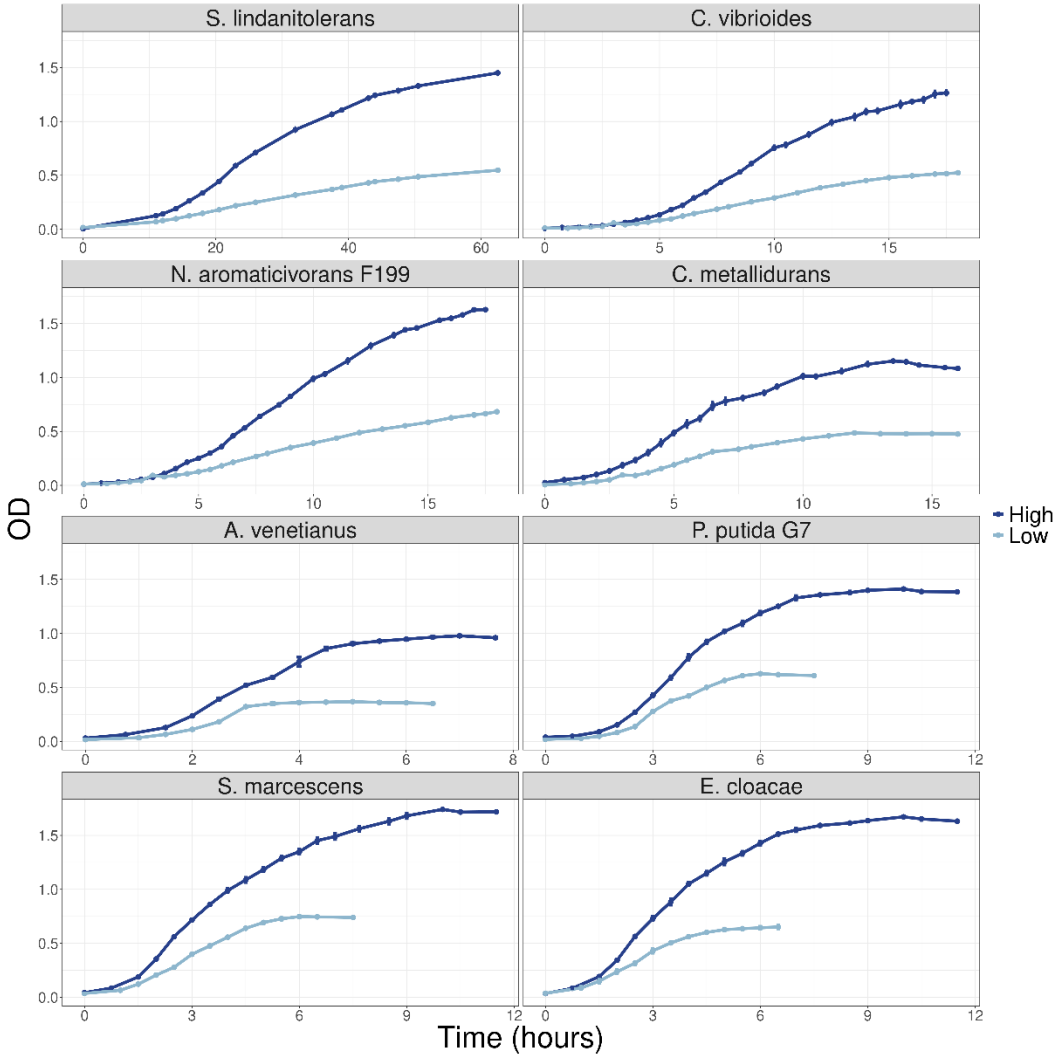

Figure A.1: The growth curves of each donor and recipient strain at either high nutrient levels (dark blue) or low nutrient levels (light blue) in monoculture. Note the difference in x-axis scale for *S. lindanitolerans*.

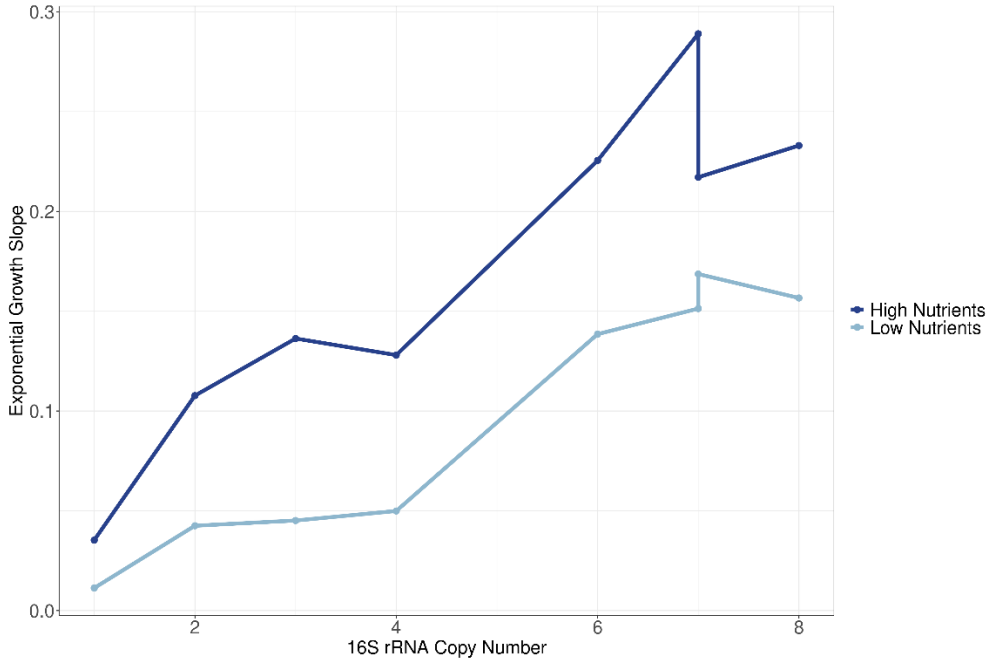

Figure A.2: The slope at the exponential growth stage of each donor and recipient vs. the 16S rRNA copy number of the organism at either high nutrient levels (dark blue) or low nutrient levels (light blue) in monoculture.

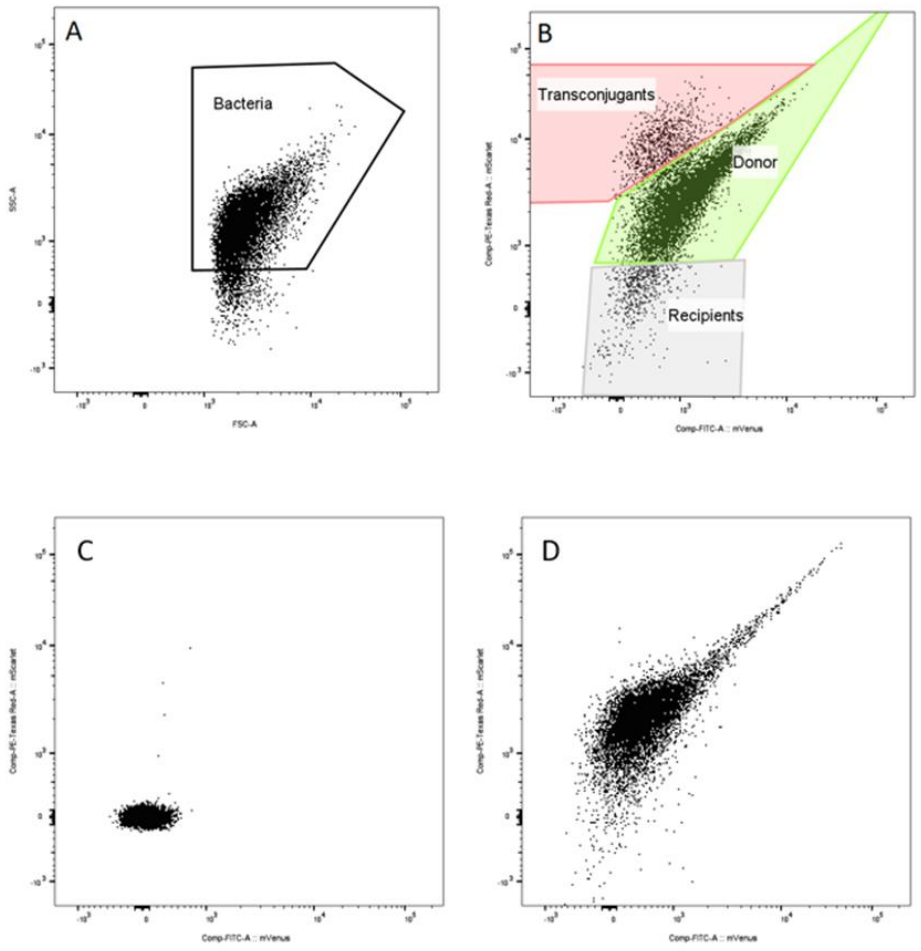

Figure A.3: Overview of the gating scheme for flow cytometry and FACS employed for counting and sorting donor, recipient, and transconjugant cells. A) Bacterial gate used to sort out debris and dead cells. B) Gating employed for a mating example between G7 and *S. marcescens* consisting of red-non-green fluorescing transconjugant cells, green and red fluorescing donor cells, and non-fluorescing recipient cells. C) Example of a recipient-only monoculture on FACS. Recipient is *A. venetianus*. D) Example of a donor-only monoculture on FACS. Donor is F199.

Appendix B. Supporting Material for Chapter 5

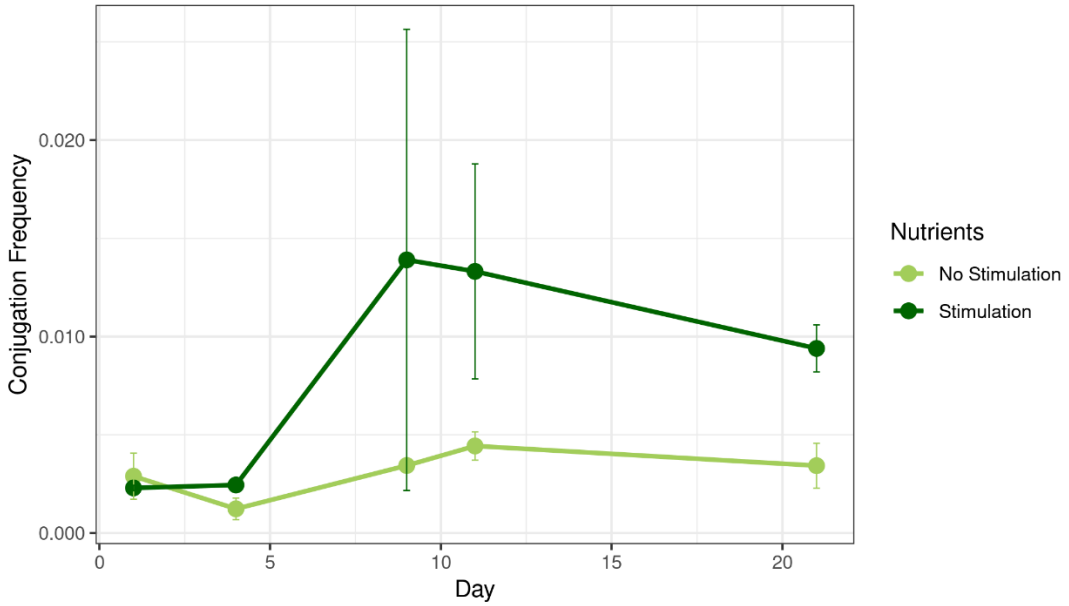

Figure B.1: Conjugation frequency over time of NAH7 harbored in G7 in PAH-spiked sediment communities without the stimulated replicate with extremely high amounts of conjugation. Error bars represent standard error (n = 2 or 3).

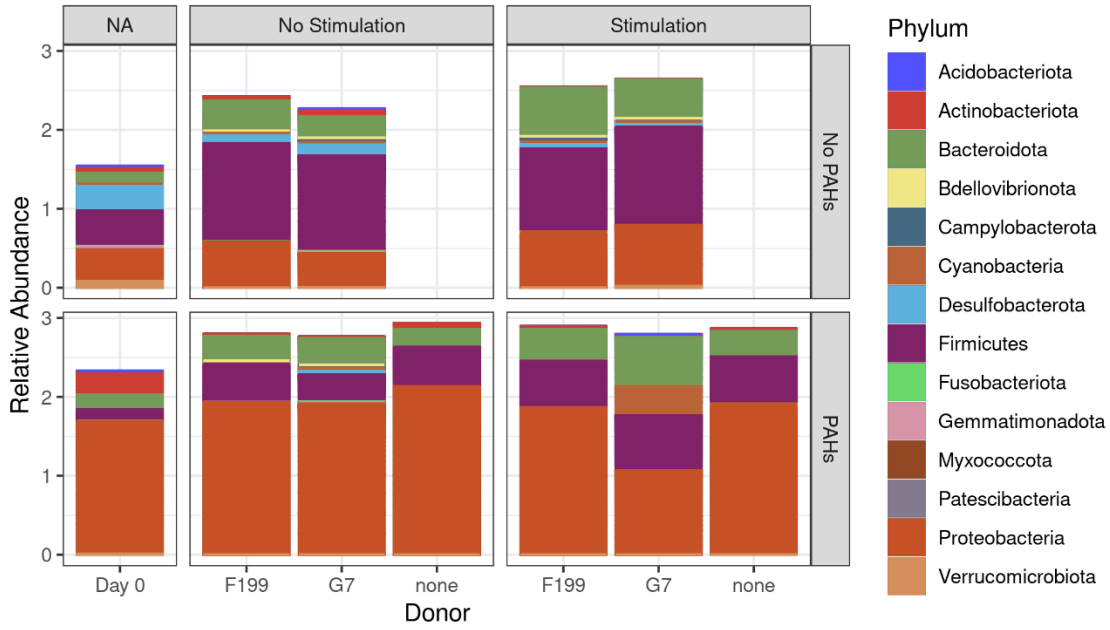

Figure B.2: The relative abundances of phyla on Day 21 in each community separated by PAH-spiked and non-PAH sediment, and stimulation schemes. The relative abundance of each sediment community on Day 0 is also plotted on the left. Relative abundances of each biological replicate are added so that the total abundance is equal to 3 (n = 3).

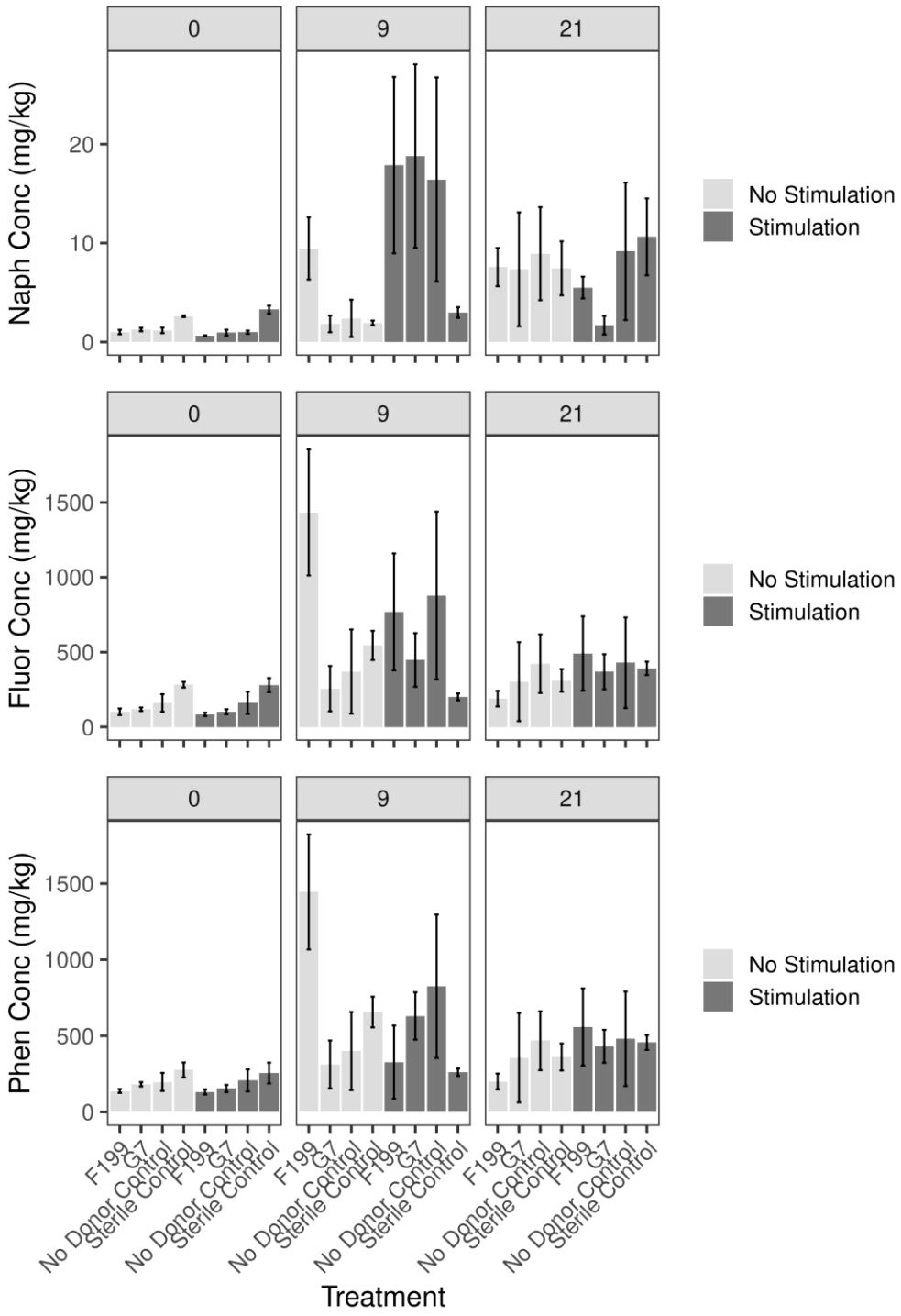

Figure B.3: Concentrations in mg/kg wet weight on Days 0, 11, and 21 for Naphthalene, Phenanthrene, and Fluorene in various communities colored by

stimulation or no stimulation. "F199" community had F199 augmented, "G7" community had G7 augmented, "No Donor Control" had no donor augmented but had non-sterile sediment, and "Sterile Control" had no donor augmented into sterile sediment. Error bars represent standard error (n = 3).

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