Toxicity of Polycyclic Aromatic Hydrocarbons Pre- vs. Post-Bioremediation

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Executive Summary

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants implicated in negative human and ecosystem health outcomes, including but not limited to carcinogenicity and teratogenicity. Bioremediation using PAH-degrading bacteria and fungi is a noninvasive, relatively low-cost technology capable of reducing environmental occurrence of PAHs. Employing analytical chemistry methods to detect the extent of degradation of PAHs, while insightful, is insufficient as the sole determinant of efficacy of bioremediation. Metabolites created during bacterial degradation of PAHs can be equally toxic or more toxic than parent compounds. Thus, toxicological assays of samples undergoing bioremediation are a crucial component for monitoring risk. The first objective of this project was to develop methods for toxicological assays that could be employed to determine the efficacy of bioaugmentation strategies currently being developed with microbial strains isolated from the heavily PAH-contaminated sediment at the former Republic Creosoting site in the Elizabeth River, VA, USA. The second objective was to use those methods to test three recently isolated PAH-degrading bacterial strains to determine their suitability for use in bioaugmentation. Experimental design included incubation of PAHs with bacteria; extraction of metabolites; analytical chemistry analysis to determine extent of degradation; then subsequent toxicological assays of extracted metabolites, including Ames assays to determine mutagenic potency and zebrafish morphological assays to determine teratogenicity. Four different PAHs were incubated with three strains of PAH-degrading bacteria. Significant degradation of only phenanthrene was observed, accompanied by a slight increase in mutagenicity and a significant decrease in teratogenicity. Visual inspection of cultures indicated potential fluoranthene degradation with a concomitant increase in mutagenic potency in monocultures, but not in co-cultures. Results for teratogenicity in fluoranthene cultures were inconclusive. Fluoranthene incubation conditions must be optimized to allow more complete degradation and to achieve more definitive results. Once optimization is attained, these assays can be employed in future studies to test additional strains of bacteria as well as fungi that may have capability of degrading a wider range of PAHs.
Introduction

Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a class of over 100 compounds comprised of two or more fused benzene rings (Figure 1). They are ubiquitous environmental contaminants present in air, water, soil, sediment, and vegetation throughout the world, with higher concentrations near urban and industrial areas (Manzetti, 2013). PAHs are lipophilic and tend to persist in the environment, binding to soils and sediments (Maletic et al, 2018).

The majority of environmental PAHs are formed during both natural and anthropogenic combustion processes, including volcanoes, forest fires, and burning of fossil fuels, trash, wood and other plant material (Maletic et al, 2018). PAHs can also be released into the environment via transport, leakage, and spills of petroleum and coal products. (Manzetti, 2013, Behera, 2018).

Toxicity of PAHs

PAH contamination in aquatic environments poses significant risk for both wildlife as well as humans, whose exposure can occur through dermal contact or consumption of contaminated seafood (Chen and Liao, 2006, Zhou et al, 2014, Nwaichi and Ntorgbo, 2016). The US EPA has classified 16 PAHs, ranging in size from 2 to 6 benzene rings, as priority pollutants under Clean Water Act guidelines (Figure 1, US EPA, 2015). Determination of priority pollutant status was decided in 1976 and was based on “toxicity, potential for human exposure, frequency of occurrence at hazardous waste sites, and the extent of information available” (ATSDR, 1995). Evidence against the adequacy of the Priority PAH list has been mounting in recent years in light of more advanced analytical chemistry methods and a wealth of toxicology research. Particularly concerning are the occurrence and toxic effects of several high molecular weight PAHs, alkylated PAHS, and PAHs containing heteroatoms (Andersson and Achten, 2015).
Of the 16 priority pollutant PAHs, the International Agency for Research on Cancer (IARC) lists one as a known human carcinogen, one as a probable human carcinogen, and six as possible human carcinogens (IARC, 2010). In general, higher molecular weight PAHs (those with 4 or more rings), including several non-priority PAHs, have higher carcinogenic potential (IARC, 2010, Andersson and Achten, 2015). Most carcinogenic PAHs are not directly mutagenic, but rather require metabolic activation by cytochrome P450 (CYP) or other metabolic enzymes for mutagenic activity (Guengerich and Shimada, 1991, Arlt et al, 2008). For example, benzo(α)pyrene, one of the most well-characterized mutagenic PAHs, is converted to

**Figure 1** – Structure of 16 Priority PAHs
benzo(a)pyrene-7,8-dihydro-diol-9,10-epoxide (BPDE). BPDE can form adducts with proteins and DNA, which leads to mutations and subsequently tumorigenesis (Melendez-Colon, 1999). A subset of PAHs, including phenanthrene, fluoranthene, and pyrene, have produced equivocal results in studies of mutagenesis and carcinogenesis, and have been deemed “not classifiable as to human carcinogenicity” (US EPA, 1990).

PAHs can also be teratogenic. For example, zebrafish (Danio rerio) embryos exposed to phenanthrene at 4-8 hours post-fertilization (hpf) develop pericardial edema, yolk sac edema, dorsal curvature of the body axis, and reduced eye and jaw growth by 72 to 96 hpf (Incardona et al, 2004). Pyrene exposure in embryonic zebrafish is implicated in yolk sac edema as well as cardiac defects, such as elongated ventricle, string-like morphology, and pericardial edema (Incardona et al, 2006, Zhang et al, 2012). Developmental toxicity can be due to the synergistic action of PAHs, as is the case with coexposure to benzo(a)pyrene and fluoranthene. While neither produces obvious teratogenic effects by itself, coexposure causes heart defects such as elongation, improper atrial-ventricular alignment, and pericardial edema in Atlantic killifish (Fundulus heteroclitus, Clark et al, 2013).

Other health effects caused by PAHs include neurotoxicity (reviewed by Wormley et al, 2004), immune suppression (Davila et al, 1996, White and Holsapple, 1984, Wojdani and Alfred, 1984), as well as effects on reproduction, the cardiovascular system, and kidneys (Ramesh et al, 2004).

*The Elizabeth River Estuary*

PAHs comprise a large portion of creosote, a product made by the distillation of coal tar and used historically as a wood preservative. The Elizabeth River, a tidal estuary in southeastern Virginia, was home to three major creosoting facilities (shown in Figure 2) that released substantial amounts of creosote into the river during the 20th century (Di Giulio and Clark, 2015). Although the last of these companies ceased operations in the 1990s, PAH contamination in untreated sediment persists to present day.
Atlantic Woods is a designated Superfund site. Republic is the site of current bioremediation research. The site of one of these companies, Atlantic Wood Industries (AWI), was designated a Superfund site by the US EPA in 1990, due to the risk it posed to the environment and nearby communities. The AWI site has high levels of PAHs, as well as pentachlorophenol (PCP), dioxin, and metals, including arsenic, chromium, copper, lead, and zinc. The AWI site is currently undergoing a remediation process that involves building retaining walls around the most heavily contaminated areas, as well as dredging 45,000 cubic yards of soil and over 360,000 cubic yards of sediment. A portion of the dredged material is being combined with cement and used to cap other contaminated sediment, while the rest is being landfilled and capped. The remediation process has reduced short-term risks to environmental and human health but has cost around $100 million and left most of the contaminants intact with potential for future release into the environment or groundwater (US EPA, 2014).

Although not designated as Superfund sites, the locations of the other two former creosoting companies, Hess/Epping and Russell, and Republic Creosoting, were also heavily contaminated...
with PAHs. Similar to the Atlantic Wood site, Hess/Eppinger has undergone remediation involving dredging 39 million pounds of the most heavily contaminated sediment and capping the remainder with sand (Source: http://www.elizabethriver.org). Contamination at the Republic site persists, and sediment samples recently collected from the Republic site were analyzed for 31 PAHs. Over 295,000 ng/g total PAHs were detected, a concentration well above the maximum 45 ng/g goal of the EPA Superfund cleanup goal (Volkoff et al, 2019, US EPA, 2014).

**Bioremediation of PAHs**

Bioremediation takes advantage of living organisms, typically bacteria and fungi, to degrade environmental contaminants such as PAHs. Advantages of bioremediation over physical methods like dredging, capping, and retaining walls include cost-effectiveness, minimal environmental disruption, and potential to reduce PAH concentration on-site. One type of bioremediation, bioaugmentation, involves enriching a contaminated environment with microbial strains possessing degradative capability (Maletic, 2019). Bioaugmentation with microbial strains indigenous to PAH-contaminated sites confers several advantages. First, indigenous strains have adapted to exist within both the environmental conditions and the surrounding microbial community. They will likely be better equipped than exogenous strains to survive and compete for resources. Second, indigenous strains are likely to have undergone selection for PAH-degrading genes (Singh, 2006).

Of significance to this study, six bacterial strains indigenous to the Republic site in the Elizabeth River with PAH-degrading capability were recently isolated (Volkoff, 2019). They are currently being investigated for their potential to decrease environmental PAHs at the Republic site as part of a bioaugmentation strategy using bacterial/fungal biofilms and engineered bioamendments (Volkoff, 2019, unpublished data).

**Determining Efficacy of Bioremediation**

The primary determinant of the efficacy of PAH bioremediation is a decrease in concentration of parent compounds, typically determined via analytical chemistry methods, but given the overarching goal of minimizing potential risks to human and ecosystem health, chemical analysis
alone is insufficient. Studies have shown that metabolites produced during bioremediation of PAH mixtures can retain high rates of mutagenicity, genotoxicity, and developmental toxicity despite substantial PAH degradation (deSouza Pohren et al, 2018, Chibwe et al, 2015). Oxygenated PAHs (OPAHs) are among the most toxic metabolites formed during bioremediation (Chibwe et al, 2017, Tian et al, 2017). OPAHs can induce acute toxicity as well as mutagenicity, genotoxicity, developmental toxicity, metabolic modulation, and oxidative stress (Lundstedt, 2007, Tian et al, 2017, Dasgupta et al, 2014, Knecht et al, 2013, Gurbani et al, 2013, Bolton et al, 2000). Moreover, OPAHs are more mobile than parent PAHs, and thus more bioavailable in aquatic environments (Lundstedt et al, 2007). Assaying toxicity in PAH-contaminated sediment that has undergone bioremediation is thus vital to adequately monitor risk. Including toxicological assays as a supplement to analytical chemistry is critical particularly in complex environmental mixtures, as they can detect effects of unidentified compounds as well as synergistic or antagonistic effects of different components (US EPA, 2000).

**Objective and Experimental Design**

The first objective of this study was to identify and develop toxicological assays that could be used to evaluate the efficacy of bioremediation microbial strains under investigation for PAH degradation. Priorities for the assays included having relevant toxicological endpoints, being relatively easy to perform, being cost-effective, and being feasible within a short timeframe. The second objective was to validate assays by testing them with known PAH-degrading bacterial strains that have been incubated with PAHs.

The experimental design is shown in Figure 3. The first step was to incubate four selected PAHs with three different PAH-degrading bacterial strains recently isolated from the Republic site. The chosen bacterial strains, shown in Table 1, have demonstrated the capability of degrading phenanthrene and fluoranthene, and were identified as strains of: *Novosphingobium pentamativorans* (designated Strain Np), a known degrader of a wide range of PAHs with strains previously cultivated from the Elizabeth River (Sohn et al, 2004, Hilyard et al, 2008); an environmentally-relevant *Hydrogenophaga* isolate (designated Strain H) detected during stable isotope probing of the Republic sediment with $^{13}$C-labelled fluoranthene; and a
*Sphingomonas/Sphingobium* isolate (designated Strain S) which comprised the highest relative abundance of any of the bacterial isolates in microbiome analyses of Elizabeth River sediment communities (Volkoff, 2019).

![Experimental Design Diagram](image)

**Figure 3** – Experimental Design

<table>
<thead>
<tr>
<th>Strain Designation</th>
<th>Strain(s) with Closest Homology (% 16S rRNA gene similarity)</th>
<th>GenBank Accession #</th>
<th>Known to degrade</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strain Np</strong></td>
<td><em>Novosphingobium pentaromativorans</em> PY1 (100%)</td>
<td>KY511052</td>
<td>Fluoranthene</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Phenanthrene</td>
</tr>
<tr>
<td><strong>Strain H</strong></td>
<td><em>Hydrogenophaga taeniospiralis</em> Iso 10-15 (99.6%) <em>Hydrogenophaga laconesensis</em> 0-12 (99.6%)</td>
<td>AB795550 MN061010</td>
<td>Phenanthrene</td>
</tr>
<tr>
<td><strong>Strain S</strong></td>
<td><em>Sphingomonas sp. NBRC_10167</em> (99.9%) <em>Sphingobium sp. RAC03</em> (99.9%)</td>
<td>AB681367 CP016456</td>
<td>Phenanthrene</td>
</tr>
</tbody>
</table>

**Table 1** – Bacterial Strains of Closest Homology to PAH-degrading Strains Isolated from Republic site (percent similarity to 16S rRNA gene shown in parentheses) and PAHs that have been demonstrated to be degraded by them (Volkoff, 2018).

The four PAHs chosen for this study were phenanthrene, fluoranthene, pyrene, and benzo(a)pyrene (Figure 1). PAH selection was based on previously demonstrative degradative
capability of the chosen bacterial strains, prevalence in Republic sediment, and the inclusion of a range of molecular weights encompassing 3-, 4-, and 5-ring structures.

All three strains of bacteria were incubated separately for one week with each of the four PAHs. Each PAH was also incubated with co-cultures of all three strains, as consortia of bacteria can, in some cases, enhance degradation (Mikeskova et al, 2012). Upon completion, cultures were subjected to a liquid:liquid extraction with dichloromethane (DCM), which was chosen for its ability to solubilize both nonpolar PAHs and their more polar metabolites. Extracted compounds were solvent exchanged with DMSO for use in toxicological assays. A portion of each extract underwent high performance liquid chromatography (HPLC) analysis to determine the concentration of remaining parent compound.

Two different toxicological assays with relevant endpoints were chosen. The Ames assay was employed to determine mutagenic potencies of extracted cultures. It is a relatively low-cost, simple means of screening complex samples. It uses Salmonella typhimurium strains engineered with mutations in the his gene, making histidine supplementation a requirement for growth. When plated on media lacking histidine, only cells that have acquired mutations that reverse the his gene back to wild-type (called revertants) will grow into colonies. By comparing the number of revertants per plate (rev/plate) of S. typhimurium mixed with potentially mutagenic samples with that of “spontaneous revertants” mixed with vehicle only (Mortelmans and Zeiger, 2000), the mutagenic potency of samples can be determined. The extent to which bioremediation has impacted mutagenic potencies can be compared in extracted PAH + bacteria incubations vs abiotic controls.

PAHs requiring metabolic activation can be detected by including S9 extract in a subset of Ames assays. S9 extract is a microsomal liver extract isolated from rats induced with Aroclor 1254 (Mortelmans and Zeiger, 2000).

S. typhimurium strains used in this study, listed in Table 2, include TA98, TA100, and YG1041. TA98 and YG1041 have frameshift mutations in their his genes, and TA100 has a base pair substitution in its his gene. Different strains vary in their sensitivities to different classes of
mutagens (listed in Table 2), although there is considerable overlap among strains of mutagens detected (Williams et al, 2019). TA100 with S9 extract is particularly sensitive to PAHs, given that metabolically activated PAHs bind to DNA and cause base-pair mutations. Some OPAHs require metabolic activation for mutagenicity (Chesis et al, 1984), but some are directly mutagenic without metabolic activation and are detected by TA98 and TA100 without S9 (Flowers-Geary, 1995). YG1041 contains additional nitroreductase and acetyltransferase genes, enabling it to be more sensitive to nitroarenes and aromatic amines (Mutlu et al, 2015).

<table>
<thead>
<tr>
<th>S. typhimurium strain</th>
<th>Type of mutation in his gene</th>
<th>Additional Plasmid Genes</th>
<th>Mutagens detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA98</td>
<td>frameshift</td>
<td></td>
<td>-S9 – OPAHs and other environmental mutagens</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+S9 – nitroarenes, aromatic amines, PAHs, other environmental mutagens (Dunkel et al, 1985)</td>
</tr>
<tr>
<td>TA100</td>
<td>base pair substitution</td>
<td></td>
<td>-S9 – OPAHs and other mutagens</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+S9 – PAHs (Mutlu et al, 2013)</td>
</tr>
<tr>
<td>YG1041</td>
<td>frameshift</td>
<td>nitroreductase acetyltransferase</td>
<td>+S9 – nitroarenes, aromatic amines, heterocyclic amines, other environmental mutagens (Mutlu et al, 2015, David DeMarini, personal communication)</td>
</tr>
</tbody>
</table>

Table 2 – Properties of Ames S. typhimurium strains used in this study

A zebrafish morphology assay was used to determine teratogenicity of the extracted compounds. Zebrafish (Danio rerio) are model organisms for examining developmental toxicity owing to their
transparency, rapid growth, and a well-characterized developmental process. Embryonic zebrafish were exposed to extracted compounds, then screened for visible manifestations of developmental toxicity, including growth delay, pericardial edema, and yolk sac edema. The ability to scan morphology of fish larvae in 96-well plates allowed the assay to be a relatively high-throughput procedure.

**Materials and Methods**

*Chemicals*

All PAHs, sodium pyruvate, nitrofluorene, sodium azide, and 2-aminoanthracene were purchased from Sigma-Aldrich (St. Louis, MO). Analytical PAH standards for phenanthrene, fluoranthene, and pyrene were purchased from Supelco (Bellefonte, PA). Benzene, acetone, acetonitrile, water, dichloromethane, and DMSO were purchased from Thermo Fisher Scientific (Waltham, MA). Mobile phase reagents of acetonitrile and water were HPLC grade. NADP and glucose-6-phosphate used in S9 extract buffer were purchased from Sigma-Aldrich.

*Single PAH Incubations with Bacterial Strains*

Phenanthrene, fluoranthene, and pyrene were dissolved to a nominal concentration of 100 mg/ml in acetone and subsequently were added separately to 100 ml culture bottles at 200 µl per bottle (final mass 20 mg per bottle). Solvent was allowed to evaporate prior to the addition of bacterial cultures. Cultures of the three PAH-degrading bacterial strains (shown in Table 1) were grown to turbidity in sRB2, a minimal defined medium previously described in Corteselli et al. (2017), supplemented with 0.2% (w/v) pyruvate as a carbon source and 1.5% (w/v) artificial seawater (ASW, Instant Ocean, Cincinnati, OH) to mimic estuarine conditions. Cells were washed 3x in sRB2 supplemented with ASW but lacking pyruvate and resuspended in the same medium at a concentration of 10^7 CFU/ml (determined by OD_{600}). 20ml of culture was added to each culture bottle (final PAH concentrations 1 mg/ml). Each PAH, in addition to being incubated with the three monocultures, was incubated with one co-culture of all three strains (with each strain at 3.33 x 10^6 CFU/ml for a total of 10^7 CFU/ml) and abiotic controls. Triplicate samples of each treatment group were covered in aluminum foil to minimize photodegradation, then incubated
at approximately 22°C for 1 week with shaking at 200 rpm. Cultures containing benzo(a)pyrene underwent the same procedures as described for the other three PAHs, but with initial dissolution in benzene, and a total of 4ml culture in 25 ml culture bottles (final PAH concentration 1 mg/ml).

**Extraction and Solvent Exchange**

Two successive liquid:liquid extractions of the PAH incubations were performed with dichloromethane (DCM) at a volume equal to the culture volume. Mixtures were shaken vigorously for 30 seconds. The organic phase was transferred to an amber glass bottle, gently blown down under nitrogen gas, and solvent exchanged with dimethyl sulfoxide (DMSO) to final nominal concentrations of 20 mg/ml (phenanthrene, pyrene, and benzo(a)pyrene) and 2 mg/ml (fluoranthe). Nominal concentration values are based on concentration of starting material and were used in methods of subsequent assays. Fluoranthe was diluted 10x compared to the other PAHs due to insolubility in DMSO at 20 mg/ml.

**HPLC Analysis**

All extracted samples were diluted in acetonitrile (AcN), filtered with 0.2µm PTFE filters (VWR International, Radnor, PA) and quantified with 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 C-18 column. The mobile phase consisted of 50:50 AcN:water for the initial 5 minutes, followed by a gradient up to 100% AcN at 25 minutes with a return to initial conditions after 30 minutes. PAHs were detected with fluorescence at various analysis windows corresponding to absorption for individual PAHs. Four-point calibration curves were constructed using analytical standards for phenanthrene, fluoranthene, and pyrene, and a laboratory stock of benzo(a)pyrene. Statistical analysis was conducted using a two-tailed paired t test with a significance level (α) of 0.05 (Microsoft Excel) to evaluate percentage of PAH concentrations remaining in bacterial incubations compared to corresponding abiotic controls.
**Ames Assay**

Ames assays were conducted as described in Maron and Ames (1983) using strains TA98, TA100, and YG1041 (generously supplied by David DeMarini, US EPA, Durham, NC). Briefly, 100 µl of Ames cultures were mixed with 100 µl of extracts diluted to different doses in DMSO before being mixed with top agar and plated. For each extract, 4 log doses, with 2 replicates per dose, were included. S9 extract (Moltox, Boone, NC) was added to selected samples to a final concentration of 8%. Negative controls contained 100 µl DMSO and positive controls included nitrofluorene (3 µg/plate, TA98 -S9), sodium azide (3 µg/plate, TA100 -S9), and 2-aminoanthracene (0.5 µg/plate, TA98 +S9, TA100 +S9, and YG1041 +S9).

The linear portion of the rev/plate to dose/plate curve was used to generate mutagenic potencies. Mutagenic potencies are expressed as rev/dose and are derived from the slope of the regression line, in cases in which the slope is significantly different from zero (p<0.05). Flat or downward sloping portions of curves were indicative of toxic doses and were disregarded. Samples deemed mutagenic were those that produced a dose-related increase in rev/plate with a maximum rev/plate at least twice that of DMSO controls. Mutagenic potencies of PAH + bacteria extracts were compared to those of abiotic control extracts using a two-tailed paired t test with 0.05 significance (α) in Microsoft Excel.

**Zebrafish Morphology Assay**

Wild-type adult zebrafish (*D. rerio*, Ekkwill Waterlife Resources; Ruskin, FL) were maintained in a recirculating AHAB system (Aquatic Habitats, Inc., Apopka, FL) at 28°C on a 14:10-hour light-dark cycle. Fish were fed twice daily, first with brine shrimp (INVE Aquaculture, Inc., Salt Lake City, UT, USA), then with Zeigler’s Adult Zebrafish Complete Diet (Aquatic Habitats, Inc). Adults spawned naturally, and embryos were collected and maintained in 30% Danieau solution. Adult care and reproductive techniques were noninvasive and approved by the Duke University Institutional Animal Care and Use Committee (A109-19-05).

At 6 hours post-fertilization, embryos were screened for viability and transferred to glass petri dishes filled with 10ml of 30% Danieau at a density of 10 embryos per dish. PAH + bacteria
extracts were diluted in 4 log doses DMSO, and a volume of 10 µl was added each dish (0.1% maximum DMSO per dish). Both 0.1% DMSO and media only were used as negative controls. No significant differences were observed between the two for all measured endpoints. At 72 hpf, larval zebrafish were washed in 30% Danieau and anesthetized with MS-222 (Sigma-Aldrich), then placed in 96-well plates with 100 µl 30% Danieau per well. Plates were imaged at 2x magnification with a Keyence BZ-X710 microscope (Osaka, Japan). Starting from the left side of the 96-well plate, the first five larvae in a position amenable to measuring were used for analysis. Fiji ImageJ v2.0 was used to measure larval body length, yolk sac area, and pericardial area. Statistical differences between fish exposed to extracts were compared to unexposed DMSO controls in Microsoft Excel using one-way ANOVA and Tukey’s HSD at a 0.05 level of significance (α).

Results

Degradation of PAHs

Concentrations of remaining PAHs following a 1-week incubation with bacteria were determined via HPLC. Figure 4 shows concentrations of PAHs remaining as a percentage of the abiotic control. Significant degradation of phenanthrene was observed in the presence of both Strain H and co-culture, with 29.9 ± 6.5% and 31.0 ± 2.2% of abiotic control remaining, respectively. Neither Strain Np nor Strain S degraded phenanthrene significantly, nor did any strains significantly degrade fluoranthene, pyrene, or benzo(a)pyrene compared to abiotic controls.
Upon visual inspection of culture bottles of phenanthrene incubations with both Strain H and co-culture, turbidity confirmed bacterial growth and color change provided evidence of chemical transformation. Although Strain Np and Strain S incubations with phenanthrene did not yield significant degradation as determined by HPLC, turbidity and color change in these cultures suggested that limited bacterial growth and degradation were occurring. Similarly, although no
significant degradation of fluoranthene by any strains was indicated by HPLC, some turbidity and color change were evident in incubations with Strain Np, which has been previously identified as a fluoranthene degrader, as well as Strain S. Visual inspection of all pyrene and benzo(a)pyrene cultures confirm HPLC results that little or no degradation occurred, as evidenced by clear cultures with visible undissolved PAH similar to the abiotic controls. Due to their degradation and potential degradation, phenanthrene and fluoranthene cultures were selected for analysis in subsequent toxicological assays.

**Figure 5** – PAH + bacteria incubations.

**Ames Mutagenicity Assays**

Results of Ames mutagenicity assays for extracts of phenanthrene and fluoranthene cultures are shown in Table 3. Without any bacterial augmentation, extracts of phenanthrene were not mutagenic in any Ames strains, either with or without metabolic activation. Among phenanthrene + bacteria incubations, only the Strain H monoculture exhibited mutagenicity, and only in Ames strain TA98 with metabolic activation. Strain H was the only monoculture that had significant degradation, and a mutagenic potential above that of the abiotic control could be
indicative of one or more mutagenic metabolites being produced during degradation. The relatively low mutagenic potency of 1.5 rev/µg suggests that metabolite(s) are either weakly mutagenic or are occurring at low concentrations. Interestingly, the phenanthrene + co-culture extract was not mutagenic, despite having Strain H as one of its components.

Extracts of fluoranthene without bacterial augmentation were mutagenic with metabolic activation in all Ames strains, but not mutagenic without activation. In Ames strain TA98 +S9, all extracts of fluoranthene treated with bacteria retained their mutagenic potency, with the exception of the co-culture. The same phenomenon was observed in strain TA100 +S9, in which all bacterial incubations of fluoranthene retained their mutagenicity except for the co-culture. In strain YG1041 +S9, mutagenic potencies for extracts of fluoranthene treated with Strain Np and Strain S were significantly greater than that of the abiotic control, while the mutagenic potency of the co-culture did not show an increase compared to the control.
Table 3 – Mutagenic potencies (rev/µg) of extracted bacterial cultures incubated with a. phenanthrene (Phe) and b. fluoranthene (FL). Abiotic controls are indicated as “Phe + No bacteria” and “FL + No bacteria”.

NM: not mutagenic.

\(^{a}\)Mutagenic potency significantly greater than that of abiotic control (p < 0.05).

\(^{b}\)Mutagenic potency significantly less than that of abiotic control (p < 0.05).

**Zebrafish Morphology Assays**

Body length, pericardial area, and yolk sac area of zebrafish larvae were measured after exposure to extracts of PAHs incubated with bacteria (Figure 6). An example of a larva with decreased body length, pericardial edema, and yolk sac edema as a result of exposure to the phenanthrene abiotic control extract (10 µg/ml) is shown in Figure 7.
Regardless of bacterial treatment, all phenanthrene extracts caused a significant decrease in body length at the highest dose (10 µg/ml), as did all at the second highest dose (1 µg/ml) except the extract of incubation with Strain H. At 10 µg/ml, extracts of phenanthrene incubated with Strain H and co-culture, both of which underwent significant degradation, caused less deviation in length from the DMSO control than did extracts of phenanthrene incubated with Strain Np or Strain S, neither of which significantly degraded phenanthrene. Similar trends were observed for both pericardial area and yolk sac area. Exposure to extracted phenanthrene incubated with Strain H or co-culture did not result in any significant pericardial edema, though it was observed in fish exposed to the phenanthrene abiotic control or phenanthrene incubated with Strain Np or Strain S. Yolk sac edema was less pronounced in fish exposed to the phenanthrene + Strain H or co-culture extracts compared with those exposed to the phenanthrene abiotic control or phenanthrene + Strain Np or Strain S extracts. Thus, degradation of phenanthrene by Strain H in monoculture and a co-culture of all three strains tested was accompanied by a decrease in developmental toxicity. There were no significant differences in any of the endpoints between exposures to extracts of phenanthrene + Strain H and phenanthrene + co-culture.

Results for treatment with fluoranthene were inconclusive, possibly due to poor fluoranthene degradation across all treatment groups. Statistically significant differences between treatment groups and DMSO controls did not consistently occur in a dose-response pattern, and were not of similar magnitude observed in the phenanthrene group. Further study in which incubation conditions allow significant degradation would likely produce more conclusive results.
a. Phenanthrene

### Body Length (mm)

<table>
<thead>
<tr>
<th>Condition</th>
<th>0.01 µg/ml</th>
<th>0.1 µg/ml</th>
<th>1 µg/ml</th>
<th>10 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe + No bacteria</td>
<td>*</td>
<td>**</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>Phe + Np</td>
<td></td>
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<td>Phe + H</td>
<td></td>
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<td></td>
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<tr>
<td>Phe + S</td>
<td></td>
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<tr>
<td>Phe + Co-culture</td>
<td></td>
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</tr>
<tr>
<td>DMSO</td>
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### Pericardial Area (mm²)

<table>
<thead>
<tr>
<th>Condition</th>
<th>0.01 µg/ml</th>
<th>0.1 µg/ml</th>
<th>1 µg/ml</th>
<th>10 µg/ml</th>
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</thead>
<tbody>
<tr>
<td>Phe + No bacteria</td>
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<tr>
<td>Phe + Np</td>
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<tr>
<td>Phe + H</td>
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<tr>
<td>Phe + S</td>
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<tr>
<td>Phe + Co-culture</td>
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<tr>
<td>DMSO</td>
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### Yolk Sac Area (mm²)

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<th>Condition</th>
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<th>0.1 µg/ml</th>
<th>1 µg/ml</th>
<th>10 µg/ml</th>
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</thead>
<tbody>
<tr>
<td>Phe + No bacteria</td>
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<td>Phe + Np</td>
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<td>Phe + S</td>
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<td>Phe + Co-culture</td>
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<tr>
<td>DMSO</td>
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b. Fluoranthene

Figure 6 – Measurements, including body length, pericardial area, and yolk sac area, of 72 hpf zebrafish larvae (n=5) exposed to increasing doses of extracts of a. phenanthrene treated with PAH-degrading bacteria and b. fluoranthene treated with PAH-degrading bacteria. Error bars represent standard error. *0.01<p<0.05, **p<0.01 compared to DMSO control.
Figure 7 – Zebrafish larvae at 72 hpf
a. DMSO control
b. exposed to phenanthrene + no bacteria extract (10 µg/ml)

Discussion

In this study, procedures were devised for the incubation of PAH-degrading bacteria with PAHs, with subsequent metabolite extraction and toxicological assays in order to be able to determine the extent to which bioremediation alters mutagenic and teratogenic potencies of PAH-containing samples. The procedures were used to determine the impacts of treating four different PAHs with three PAH-degrading bacterial strains recently isolated from the Republic site in the Elizabeth River, VA.

*Hydrogenophaga* sp. strain H was capable of degrading phenanthrene, and in doing so, decreased its teratogenicity but slightly increased its mutagenic potency. *Hydrogenophaga* spp. have been previously implicated in phenanthrene degradation and were among indigenous strains enriched in soil consortia upon addition of phenanthrene (Martin et al, 2012, Jiao et al, 2016). Of the possible OPAHs generated by degradation of phenanthrene, most are nonmutagenic or weakly mutagenic, resembling the weak mutagenicity of the phenanthrene metabolites in this study (Flowers-Geary et al, 1996, Chesis et al, 1984, Wood et al, 1979). Diol epoxides of phenanthrene are directly mutagenic, and the fact that the extract in this study required metabolic activation suggests an alternative mutagenic metabolite (Flowers-Geary et al, 1996, Wood et al, 1979).
The co-culture containing *Hydrogenophaga* sp. degraded phenanthrene to the same extent as the *Hydrogenophaga* sp. monoculture, but did not contain mutagenic metabolites, pointing to the possibility of a co-metabolic process, in which one or both of the other bacterial strains present were able to further degrade the metabolite that the *Hydrogenophaga* sp. in monoculture could not. Co-metabolic phenomena have been previously observed in the degradation of other PAHs. For example, a *Pseudomonas* sp. strain, unable to degrade fluorene, degrades the metabolite 9-fluorenone, a dead-end product of metabolism of fluorene by an *Arthrobacter* sp. strain (Casellas et al, 1998). Additionally, Rodgers-Vieira et al (2015) identified bacterial strains capable of degrading anthraquinone, an oxygenated form of anthracene, that were unique from anthracene-degrading strains, in PAH-contaminated soil. This strengthens the assertion that consortia can be more effective than monocultures for bioremediation.

Enhanced mutagenicity of fluoranthene cultured with *Novoshphingobium pentaromativorans* and *Sphingobium/Sphingomonas* sp. strains in Ames Strain YG1041 +S9 suggest that these strains produce mutagenic metabolite(s) requiring metabolic activation. Although fluoranthene degradation in these strains is not well-characterized, oxygenated metabolites created during the degradation of fluoranthene by *Pseudomonas* and *Pasteurella* spp. have been identified (Gordon and Dobson, 2001, Sepic et al, 2003). Of those identified, to this author’s knowledge, only 9-fluorenone-1-carboxylic acid has been identified as a mutagen, suggesting a possible component of degradation here (Hauser et al, 1997). Mutagenic potency of the co-culture of fluoranthene was below that of the *N. pentaromativorans* and *Sphingobium/Sphingomonas* sp., again raising the possibility of a co-metabolic process in which the other *Hydrogenophaga* strain can degrade the mutagenic metabolite(s).

In Ames strains TA98 and TA100, mutagenic potencies for all fluoranthene monocultures were not statistically different from the abiotic control, suggesting that metabolites are equally as mutagenic as fluoranthene in these strains. The co-culture, however, had a lower mutagenic potency than the monocultures and abiotic control, again providing evidence that a co-metabolic degradation may be happening.
In zebrafish, phenanthrene degradation by *Hydrogenophaga* sp. was accompanied by a decrease in markers for developmental toxicity, with no further decrease in the co-culture incubation. Metabolites created thus appear to be less teratogenic than the parent compound. Knecht et al (2013) identified two oxygenated phenanthrene compounds, phenanthrene-1,4-dione, and phenanthrene-quinone, that are acutely toxic to zebrafish embryos at the lowest concentrations tested. These were not likely present to a large extent in the *Hydrogenophaga* sp. culture.

The results from effects of fluoranthene bioremediation in zebrafish were inconclusive. One weakness of the fluoranthene experiments is that no statistically significant degradation was observed via HPLC. Given the high concentration of fluoranthene added to incubations, significant growth may not have been reflected in the percentage of PAH remaining after only one-week incubation. Fluoranthene, a 4-ring PAH, has been previously shown to biodegrade more slowly than 3-ring phenanthrene (Pagnout et al, 2006), and it is plausible that extending incubation time would result in more complete degradation. During a prior stable isotope-probing experiment, a smaller concentration of fluoranthene amended to sediment from the Republic site required more than 10 days for complete removal (Dr. David Singleton, personal communication).

Another weakness in the fluoranthene data is the dosing for Ames assays. Slopes of several of the dose-response curves began to decrease as the doses increased, sometimes beginning as early as the second-lowest dose, an indication that fluoranthene and/or its metabolites were toxic at the chosen doses. Repeating the experiment with lower maximum doses and decreased intervals between doses (such as half-log instead of log) would likely produce more definitive slopes, and consequently, mutagenic potencies.

Pyrene and benzo(a)pyrene were not degraded by the three bacterial strains in the indicated incubation conditions. Bacterial strains with the capability of utilizing benzo(a)pyrene as a sole source of carbon and energy have not been identified; however, when supplemented with additional carbon sources, some strains, including *Novosphingobium pentaromativorans* and *Sphingomonas* sp., can transform benzo(a)pyrene co-metabolically (Sohn et al, 2004, Juhasz et
al, 2000). Future research could include testing additional Republic isolates for pyrene-degrading ability, and all isolates for benzo(α)pyrene-degrading ability with carbon source supplementation.

An important next step to this research is determining how bioaugmentation might impact toxicity in actual complex environmental matrices. This would entail incubating PAH-degrading bacteria with Republic sediment samples, extracting compounds that are remaining, and performing the toxicological assays laid out in this study. It is important to note that different results would be expected depending on extraction technique. Performing a complete extraction procedure would help to identify effects of all possible compounds in a worst-case scenario, while using porewater or partitioning techniques for extracts may provide a more environmentally relevant exposure (Mehler et al, 2010, Fang et al, 2014, Bandow et al, 2009).

Davie-Martin et al (2017) conducted a meta-analysis of 26 studies in which cancer risk was evaluated following PAH-contaminated soil bioremediation. They concluded that overall, soil bioremediation was insufficient to reduce risk, due largely to polar transformation products and undegraded high molecular weight PAHs. They suggest that future studies need to focus on strategies that can specifically tackle these compounds. Determining the optimal consortia of microorganisms capable of reducing both parent compounds and toxic metabolites will likely play a role in future research.

This research serves to expand the tools used to assess the efficacy of Republic sediment bioremediation strategies currently being developed beyond PAH concentration endpoints to include toxicological factors. A follow-up project is underway to identify fungal strains from the Republic site that may be co-cultured with PAH-degrading bacteria, with the potential to more completely degrade high molecular weight PAHs. The methods developed in this project could help determine the impact of fungi on toxicity. The results can help inform whether bioaugmentation strategies are reducing human and ecosystem health risk and can be applied to future research of the Republic site as well as other similarly contaminated environments.
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Resources


