Toxicity of Polycyclic Aromatic Hydrocarbons pre- and post-bioremediation using bacteria and fungi

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Executive Summary

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants that have adverse outcomes on human and environmental health. The adverse effects of the compounds include, but are not limited to teratogenicity, mutagenicity, and carcinogenicity. Presently, the former Republic Creosoting site in the Elizabeth River in Virginia, United States have become contaminated by PAHs present in creosote. Bioremediation is a strategy proposed to breakdown pollutants due to its low-cost and environmentally friendly approach. Six bacteria isolates and eighteen fungi isolates was selected for their ability to breakdown PAHs based on previous studies or enzymatic assays. The first objective of the project was to test all isolates against 3 pure PAH compounds (phenanthrene, fluoranthene, and pyrene) and check for disappearance of the parent compound using high performance liquid chromatography (HPLC). Two bacteria isolates (*Novosphingobium indicum*, and *Alcaligenes faecalis*) and two fungi isolates (*Trichoderma sp*., *Paraphaeosphaeria sp.*) were able to degrade the PAHs significantly. The two bacteria and two fungi isolates were used for toxicology endpoints. The second objective of the project was to test both bacteria and fungi isolate remediated compounds against the parent compound to find significant mutagenicity. Overall, the results demonstrated higher mutagenicity in both parent and remediated compounds with the addition of cytosolic xenotoxic metabolizing chemical (S9). The third objective was to test for teratogenicity and CYP1A activity with zebrafish embryonic development and an ethoxyresorufin-O-deethylase (EROD) assay. Overall, the results for the third objective were inconclusive. Future directions will be to test the isolates against higher molecular weight PAHs such as benzo[a]pyrene and environmentally weathered PAHs found in the actual Elizabeth River estuarine water.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of chemicals characterized by two or more benzene rings. The rings can be arranged linearly, angularly, or in cluster arrangements with only carbon and hydrogen bonds. PAHs come from the incomplete combustion of organic matter including natural sources such as forest fires and volcanic eruptions (Badger 1962). However, anthropogenic sources of PAHs are more common including automotive origins (vehicular patrol and diesel engines are the main contributors) (National Research Council 1972). Other anthropogenic origins are residential heating, industrial activities (aluminum production and coke manufacturing), incineration, and power generation (Baek et al. 1991). The United States Environmental Protection Agency (EPA) currently regulates 16 PAHs as priority pollutants (Figure 1).

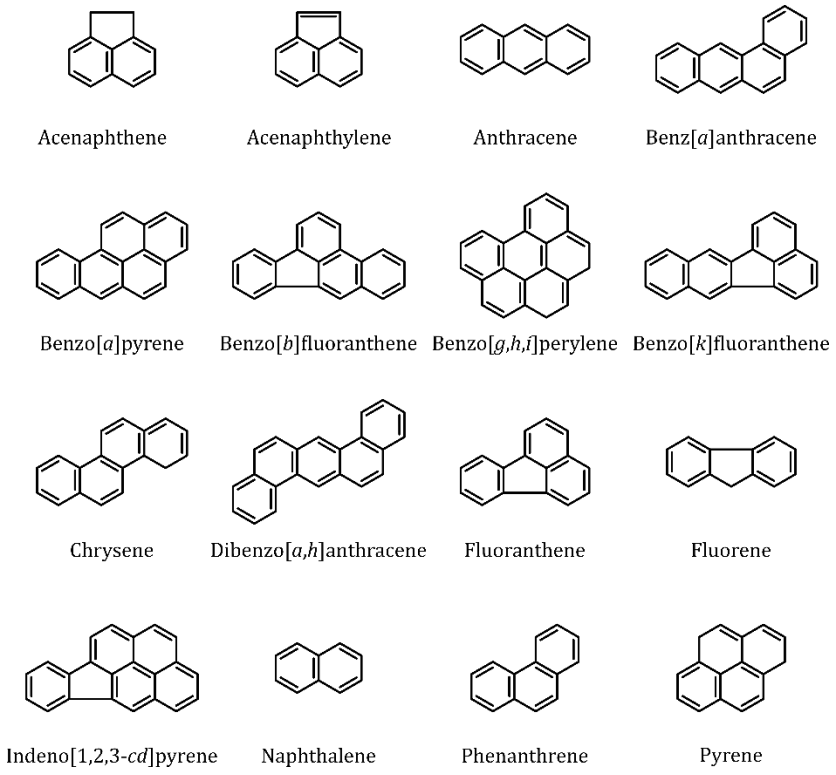


Figure 1. The sixteen EPA-regulated PAHs.

The compounds behavior in the environment depends on the size and the structure of the rings. Generally, PAHs have a high affinity for particulate and organic matter. Particularly, pyrogenic (produced from combustion) PAHs sorb readily to particulates and petrogenic (originates in rocks) PAHs bioaccumulate (Hylland 2007). PAHs are broken down via sunlight in the environment, although biological and chemical degradation can also happen (Hylland 2007). Abiotic chemical reactions of PAHs include volatilization and oxidation via sunlight and nitration (Fu et al. 2012). PAHs can reach aquatic systems from runoff (Neary and Boving 2011). The compounds can be found in marine organisms such as amoeba, diatoms, and algae (Manzetti 2013). These compounds can lead to deleterious cellular effects in living biota.

In addition to the persistence in the environment, these compounds have mutagenic, carcinogenic, and environmental toxicity potential. Some PAHs induce the aryl hydrocarbon receptor (AhR) pathway in various animals including fish and humans. The AhR is responsible for regulating Cytochrome P450 1A1(*CYP1A1*). CYP1A1 is a member of a group of xenobiotic metabolizing enzymes. AhR is present in the cytosol in a complex with Hsp90, XAP2 and p23 proteins (Mimura and Fujii-Kuriyama 2003). PAHs bind to the AhR complex which disassociates from the Hsp90 complex to form a heterodimer with partner Arnt. The heterodimer recognizes the xenobiotic responsive element on the *CYP1A1* gene and causes enhanced expression of the gene. (Mimura and Fujii-Kuriyama 2003, Figure 2).

Multiple deleterious effects of PAHs have been observed in organisms. One of the major concerns are the compound's mutagenicity. The compounds are metabolized into epoxides in mammals which bind readily to DNA, RNA, and albumin. The compounds by themselves are not carcinogenic or mutagenic but become toxic after metabolization (Pashin and Bakhitova 1979).

In humans some PAHs can cause mutagenic and carcinogenic problems. Multiple studies have found adverse carcinogenic effects stemming from exposure to PAHs. For example, haze days in China produce significant amounts of the 16 priority PAHs and can lead to health problems from inhalation of volatile compounds, including an increase in the probability of a lifetime development of cancer risk (Kang et al. 2017). Modeling studies have also indicated that inhalation of volatile PAHs can be associated with an increase in lung cancer (Hong et al. 2020).

PAHs also cause developmental defects and are known to cause embryonic defects in killifish (*Fundulus heteroclitus*) and zebrafish (*Danio rerio*) due to inhibition of CYP1A. Major deformities in these model organisms include pericardial edema, delayed development time, and yolk sac edema (Wassenberg and Di Giulio 2004; Perrichon et al. 2015). Other problems from PAH exposure includes pericardial deformities in killifish which is mediated by the AHR2 pathway (Clark et al. 2010; Chernick et al. 2021). Exposure to PAHs from vehicular and residential use can also lead to reproductive defects in humans. Relationships between pollution and congenital abnormalities were established in Eastern Europe in the 1990s. The Teplice Program in the Czech Republic, Germany, and Poland found that exposure to PAHs resulted in intrauterine growth retardation and fetal growth retardation (Lewtas 2007).

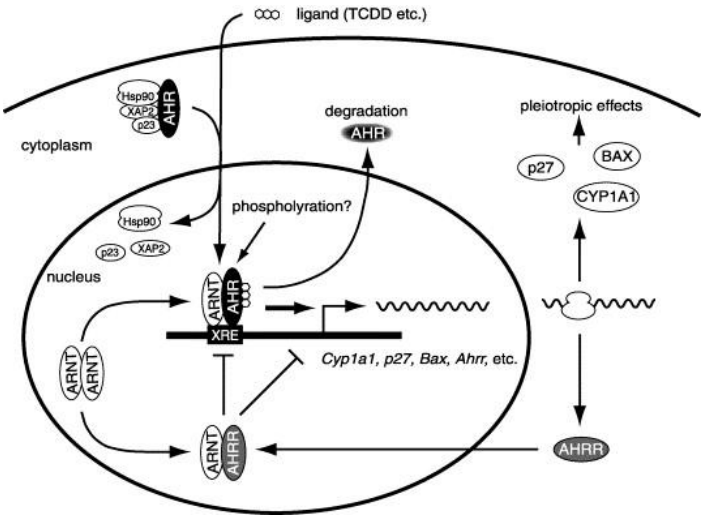


Figure 2. Schematic of the AhR pathway in drug metabolism. Figure is from Mimura and Fujii-Kuriyama 2003

Study Area

The Elizabeth River (Figure 3) is in a tidal estuary in southeastern Virginia comprising the Western, Eastern, and Southern Branches and the Lafayette River which flows through the towns of Chesapeake, Norfolk, Portsmouth, and Virginia Beach (Di Giulio and Clark 2015). The river is an important transport way for commercial and military activities. A consequence of the increased anthropogenic activity in the river has resulted in the river becoming heavily polluted with polychlorinated biphenyls (PCBs), heavy metals, pesticides from storm sewer runoff, and bacterial blooms. However, the most dominant pollutants come from historical creosote wood treatment plants in response to the increasing demand of wood for products such as railroad ties and docks (Di Giulio and Clark 2015). Creosote was used to prevent the decaying of the wood from marine wood borers (*Teredo* spp. and *Limnoria* spp, for example) and other organisms. Creosote is produced from a distillation of coal which is heavily compromised of PAHs (Di

Giulio and Clark 2015). Other sources of PAHs in the Elizabeth River are coal and petroleum storage; transport facilities, shipbuilding, and repair activities (Di Giulio and Clark 2015).

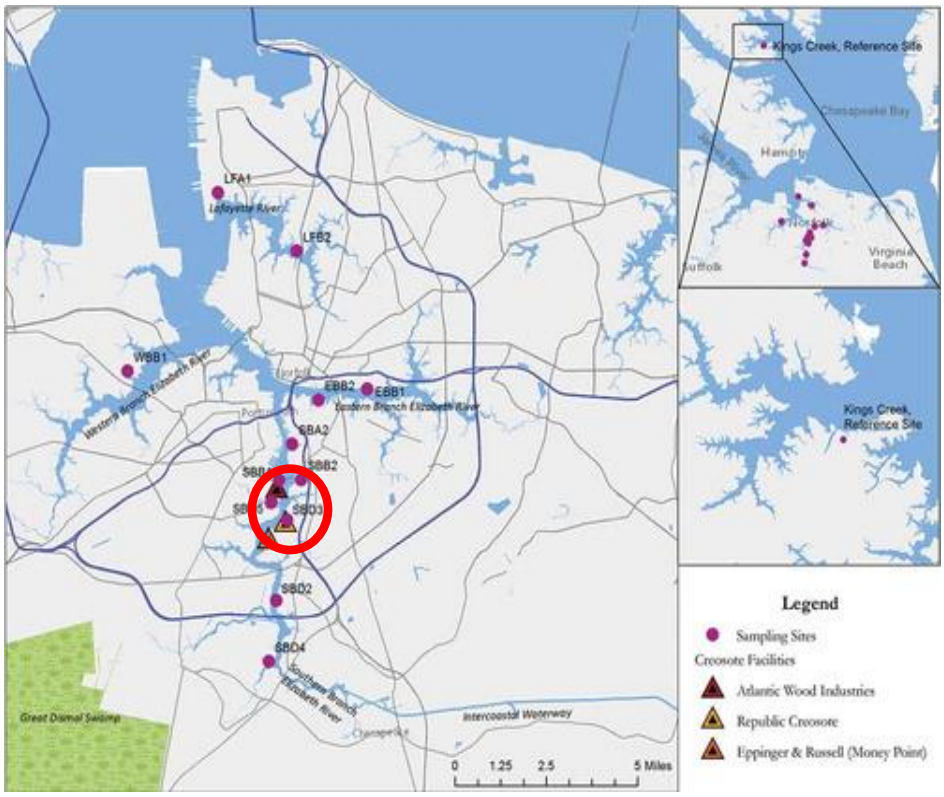


Figure 3. Map of the Elizabeth River from Di Giulio and Clark 2015. Red circle is the sampling area of the republic creosote site

Because of extensive contamination, risk assessments done on the river resulted in a "DO NOT EAT" for blue crab hepatopancreas or yellow mustard and there is a ban on eating oysters from the river due to bacterial contamination (EPA 2017). Walking on the banks of the river can result in skin burns due to the creosote present on the surface (EPA 2017). Previous efforts to remediate the Elizabeth River have included dredging and capping and as of the fall of 2017, the EPA has completed dredging 360,000 cubic yards of contaminated soil at a cost of $100 million (EPA 2017). Due to the exorbitant pricing of dredging the contaminated soil in the river, other alternative methods are being explored to remediate the contaminated sediment.

Bioremediation of PAHs

One of the more promising methods for *in situ* treatment of industrial contamination such as PAHs is bioremediation. Bioremediation is the process of degrading or rendering pollutants harmless using biological activity. The method can be low-cost with low-technological requirements and can often be performed *in situ*. As one example, as of 2017 50% of remediation projects in the United States used *in situ* treatment for groundwater cleanup, including both bioremediation and chemical treatment (United States Environmental Protection Agency 2020).

There are multiple ways to apply bioremediation to clean up contaminated sites. One commonly used and simplest method is the process of natural attenuation. Natural attenuation is letting the endogenous community degrade contaminants naturally. In addition to bioremediation, other processes that affect contaminant removal include dispersion, dilution, sorption, volatilization, radioactive decay, and chemical or biological stabilization, transformation, or destruction of contaminants (Rittman 2004). Active bioremediation schemes are split into two general methods, biostimulation and bioaugmentation. Biostimulation involves modifying the environment to stimulate indigenous microorganisms that are capable of bioremediation (Adams et al. 2015). This is achieved by adding limiting nutrients and electron acceptors (e.g., nitrogen, phosphorous, oxygen, and carbon). Bioaugmentation is adding microorganisms to the indigenous population to facilitate remediation (Adams et al. 2015). All these methods can provide environmentally friendly options to clean polluted sites. Understanding how and which microorganisms can degrade specific toxic compounds is the key to Superfund areas like the Atlantic Wood site successfully implementing a bioremediation scheme.

Microbes, particularly bacteria and fungi can degrade PAHs. Many different microbes can do this an example is bacteria are able to degrade phenanthrene and naphthalene. Typically, this is done aerobically, but degradation can happen anaerobically (Fought 2008; Seo et al. 2009; Ghosal et al. 2016). The ideal products are complete mineralization to carbon dioxide and water, but some metabolites can be created of uncertain toxicity (Ghosal et al. 2016). Select organisms can display these pathways in their metabolism.

Knowledge of organisms that can degrade PAHs will give insight to microorganisms that reside in the river that can have potential to degrade contaminants. Biostimulation has been shown to encourage PAH degradation of creosote. A study found that adding methyl-β-cyclodextrin (a type of cholesterol) stimulated bacterial degradation of smaller (2-3 ringed) PAHs in creosote (Simpanen et al. 2016). Phylogenetic analysis from the same study has determined that creosote degrading bacteria come from the Proteobacteria and Bacteriodetes phyla. Major clades include *Pseudomonas*, *Enterobacteriaceae*, *Bradyrhizobiacae*, *Cytophagacae*, and *Sphigomonadaeceae*. Another study found fungi capable of degrading creosote PAHs belong to the *Penicillium*, *Aspergillus*, *Fusarium*, *Trichoderma*, *Pleurotus*, *Cladosporium*, *Phanerochaete*, *Candida*, and *Monicillium* genera (Simarro et al. 2013; Atagana et al. 2006). The diversity of PAH degrading organisms highlights how similar genera in the rivers can be candidates in pollutant degradation.

Even though bioremediation can be an inexpensive and accessible solution to remediating pollution problems, there are concerns using the method. One of the major concerns of using microorganisms to degrade pollutants are biogenic toxic by-products. Non-ligninolytic fungi can metabolize PAHs into epoxides or dihydrodiols via cytochrome P450 which can be more toxic than the parent compounds, while quinones from the non-ligninolytic fungi metabolism have

been shown to be less toxic (Ghosal et al. 2016). Ligninolytic fungi can also produce toxic metabolites after PAH degradation such as was demonstrated in the fungus, *Pleurotus ostreatus* (Ghosal et al. 2016). These products can be genotoxic to organisms such as zebrafish and have been proposed to be produced via hydroxylated and carboxylated transformation of PAHs (Chibwe et al. 2015). Metabolic by-products of PAH bioremediation therefore warrant further study to test whether the remediated products of PAHs are more toxic than the parent compound.

Study Rationale

The purpose of the project is to evaluate bacterial and fungal isolates that can degrade three PAHs commonly found in the creosote contaminated Elizabeth River sediments (phenanthrene, pyrene, and fluoranthene) for the production of mutagenic or toxic byproducts. This work is a continuation of previous work in the Gunsch laboratory in Duke's Pratt School of Engineering. Bacterial strains used in this work are indigenous to the Elizabeth River ecosystem and have previously been shown capable of degrading PAHs commonly found in creosote (Volkoff 2019). A number of fungal isolates from the same site were recently isolated and screened for enzymatic activity related to aromatic degradation (personal communication, Joshua Crittenden) and will also be evaluated for the generation of potentially toxic PAH metabolites.

For determining deleterious effects of PAHs and metabolites, the toxicity endpoint Ames assay, which uses strains of *Salmonella* with mutations in the histidine operon to detect mutagens, was chosen due to its low cost and straightforward approach to determining mutations. When the histidine deficient bacteria are plated on a glucose minimal plate containing a trace amount of histidine, only cells that revert to the wild type (His^+) can form colonies. Thus, if a mutagen is added to the plate, then the revertant/plate should increase in a positive dose-

dependent pattern (Mortelmans and Zeiger 2000). Bioremediated extracts will be compared to the parent compound to determine if the bioremediated metabolites are mutagenic.

Two additional assays were performed to evaluate other deleterious effects of PAHs and their metabolites. Changes in zebrafish morphology give insights to teratogenicity from PAHs and PAH metabolites. Zebrafish have rapid development windows and in 3 days the embryo is differentiated enough to determine developmental problems such as yolk sac edemas, pericardial edemas, and stunted growth. The last endpoint, CYP1A induction was carried out using a 7-ethoxy-resorufin-O-deethylase (EROD) assay. As previously mentioned, some PAHs can induce CYP1A enzyme activity by binding to the AhR pathway and inducing CYP1A in teleost (fish) models (Barron et al. 2004).

Prior work was performed by Beverly deSouza to determine if the bacterial strains used in this study could degrade PAHs and analyze any toxic by-products (de Souza 2020; Volkoff 2019). Prior work determined that the bacterial strains tested did not produce any toxic by-products as tested by Ames and zebrafish morphology and all observed toxicity results were from the parent compound (deSouza 2020). This study uses higher temperature (30°C vs 25°C) and longer incubation periods (14 days vs. 7 days) to determine if those factors result in increased PAH biodegradation or toxicity than observed in prior works.

Of the large number of fungal isolates from Elizabeth River sediment, 18 isolates have been found to date to have possess phenotypes associated with PAH degrading capabilities. This project also examines PAH degradation and potential toxic by-product formation of fungi. Bacterial or fungal cultures were grown in the presence of PAHs and after 10 and 14 days of incubation for fungi and bacteria, respectively, each incubation was examined for PAH removal

via HPLC. Solvent-extracted metabolites were used in Ames assays, zebrafish morphology, and zebrafish EROD assays. The overall design of the study is shown in Figure 4.

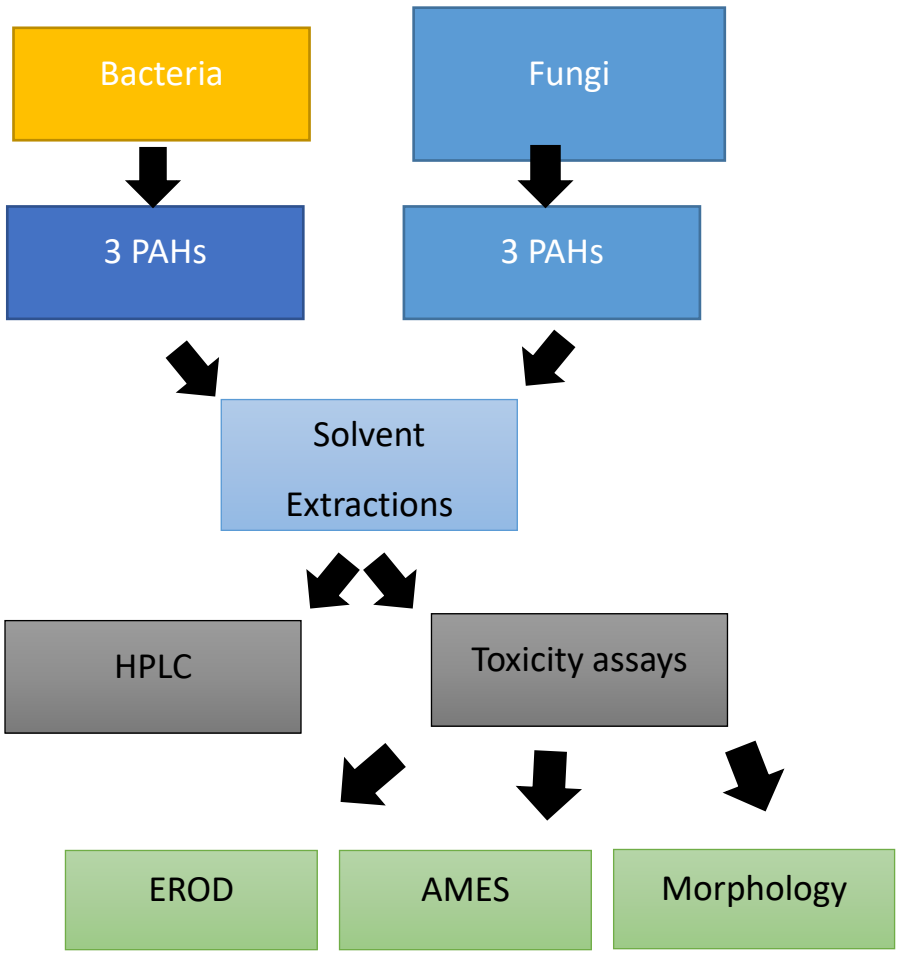


Figure 4. Schematic of the experimental design of the project

Materials and Methods

Chemicals

Phenanthrene (98%), fluoranthene (98%), pyrene (98%), sodium pyruvate (99%), nitrofluorene, sodium azide (99%), and 2-aminoanthrecene were purchased from Sigma-Aldrich (St. Louis, MO). Dimethylsulfoxide (DMSO), dichloromethane (DCM), benzene, acetonitrile (HPLC grade), water (HPLC grade), hexane, and acetone were purchased from Thermo Scientific Fisher (Bellefonte, PA). NADP and glucose-6-phosphate used in S9 extract buffer were purchased from Sigma-Aldrich.

Setup of bacterial strain incubations with multiple PAHs and extraction for HPLC quantification

Bacterial isolates were tested for the ability to degrade phenanthrene, pyrene, or fluoranthene. Stock solutions of phenanthrene (100 mg/mL), fluoranthene (50 mg/mL), and pyrene (50 mg/mL) were created in acetone. For testing of isolates for capability to degrade each all PAHs were added to a total volume of 5 mg per hungate tube. Cultures of 6 bacteria isolates (Table 1) were individually grown to turbidity using 1 mL of sRB2 minimal media described in Corteselli et al. (2017). Artificial sea salt from Instant Ocean (Instant Ocean, Foster &Smith, Rhinelander, WI, USA). was added to the sRB2 media at a concentration of 15 ppt. Fifteen mL of sRB2 media without pyruvate was added in each hungate tube of All tubes were crimped tightly and put on a shaker at 200 rpm at 30°C for 14 days. Solvent extractions with hexane were done as described in Volkoff (2019).

Table 1. Descriptions of bacteria isolates from the Elizabeth River suspected to be capable of PAH degradation

Organism name	Republic ID	Original PAH enrichment
Hydrogenophaga taeniospiralis	Rep 38	Phenanthrene
Novosphingobium indicum	Rep 36	Phenanthrene
Sphingobium RA CO3	Rep 42	Phenanthrene
Novosphingobium pentaromativorans	Rep 2	Fluoranthene
Alcaligenes faecalis	Rep 43	Fluoranthene
Stenotrophomonas sp.	Rep 4	Fluoranthene

To test selected fungal strains for PAH degradative ability, similarly to the bacterial strains each tested PAH was first added to triplicate 25 mL glass hungate tube to a final mass of 20 mg for phenanthrene, fluoranthene, and pyrene. In the multiple PAH incubations, phenanthrene, pyrene, and fluoranthene were added to each tube in triplicates and incubated in 10 mL of fungal Lignin-Modifying Enzyme (LME) basal medium (Pointing 1999) for 10 days with gentle shaking (80 rpm) at 25°C in the dark. For the single PAH incubations, each of three PAHs were added to separate tubes in triplicates with 10 mL of LME basal media. These tubes were placed in an incubator at 25°C at 80 rpm for 10 days.

Table 2. Descriptions of fungi isolates from the Elizabeth River suspected to be capable of PAH degradation

Organism name	ID number
Exophiala spp.	501
Scheffersomyces spartinae	502
Sarocladium sp.	503
Fusarium proliferatum	504
Trichoderma sp.	506

Trichoderma sp.	507
Trichoderma sp.	508
Aureobasidium sp.	509
Cladosporium sp.	510
Penicillium sp.	513
Paraphaeosphaeria sp.	514
Pleosporales sp.	515
Didymella sp.	517
Septoriella sp.	518

Solvent extractions and exchange

To extract PAHs and metabolites from fungal incubations, an equal volume of dichloromethane (DCM) was added to the bottles and Hungate tubes. Each bottle and tube were capped with a rubber stopper and shaken vigorously for 30 seconds. The organic phase with the PAHs were extracted and put in a 40 mL amber screw cap vial. This procedure was repeated twice to increase recovery. Solvents were removed under a stream of nitrogen gas and the recovered compounds were dissolved in 2-3 mL of DMSO. To extract PAHs and metabolites from bacterial incubations for HPLC analysis, at the 14 day mark 5 mL of hexane was added to each tube and vortexed for 20 seconds. The upper solvent layer with the PAHs remaining was extracted with a glass pipette and filtered through a 0.2 µm nylon membrane filter.

Quantification of PAHs

All PAHs in solvent (DMSO or hexane) was filtered in a 0.2 µm nylon membrane filter. The extracts were diluted in a 50:50 mix acetonitrile and HPLC grade water and 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 Accucore C18 HPLC column (Thermo Fischer Scientific). The method parameters are outlines in supplemental table 1. The mobile phase was

run with a 40:60 acetonitrile to water ratio for fungi and 50;50 water to acetonitrile for bacteria. A four point standard curve was generated from dilutions of analytical standards of each quantified PAH (Sigma).

AMES assay

AMES assays were conducted following the revised methods from Maron and Ames (1983). The assays used strains TA98, TA100, and YG1041 whose genotypes are outlined in Table 3. All strains were incubated as overnight cultures in 20 mL of Oxoid broth at 37°C and 130 rpm. All mutagens were prepared according to EPA standards (Claxton et al. 1987) and all extracts from the experiments were diluted to half-log and log doses in DMSO.

On the day of the experiment a S9 buffer solution was prepared with rat liver extract (Thermo-scientific, Sprague-Dawley male rats) (100µL), NADP (3.06 mg/mL), and glucose-6-phosphate salt (1.41 mg/mL) and put on ice. Extracts as well as positive and negative controls were added to prepared soft agar tubes at 100 µL and the S9 buffer was added at 500 µL. Positive control mutagens were nitrofluorene (3 µg/plate, TA 98 -S9, YG1041 -S9), 2-aminoanthracene (0.5 µg/plate, TA 100, TA 98, and YG1041+S9), and sodium azide (TA 100 -S9). DMSO was used as the negative control. The top agar was spread on minimal media VBME plates in duplicate and inverted and placed in a dark incubator for 3 days at 37°C. After 3 days the plates were taken out and the number of colonies recorded.

Table 3. Description of AMES strains and mutagen detections. Some of the information has been adapted from deSouza 2020 from her talk with David DeMarini, PhD, US EPA

Salmonella typhimurium strain	Type of his gene mutation	Additional plasmid genes	Mutagens detected
TA 98	Frameshift		**-S9-** Other environmental

			mutagens, PAHs, OPAHs **+S9-** Other environmental mutagens, PAHs, aromatic amines, and nitroarenes (Mu et al. 1985: Appendix 2)
TA 100	Base pair substitution		**-S9-** environmental mutagens, Nitroarenes, and PAHs **+S9-** Nitroarenes, other environmental mutagens, and PAHs (Dunkel et al. 1985: Appendix 2
YG1041	frameshift	Nitroreductase acetyltransferase	**+S9-** Nitroarenes, aromatic and heterocyclic amines**,** and other environmental mutagens (Mutlu et al. 2013)

Zebrafish Morphology and EROD assay

All wild-type zebrafish (Ekkwill Waterlife Resources; Ruskin, FL) were maintained in an AHAB system (Aquatic Habitats, Inc., Apopka, Fl, USA) at temperatures between 27-28°C, pH between 7-8, and nitrate levels at 100-200 ppt. The ammonium level was kept at 0 ppm and the salinity at 60 ppm. Tanks were checked daily to ensure proper water flow and that blockages in the drainage system were removed. Adult care and reproductive techniques were approved by the Duke University Institutional Animal Care and Use Committee (A109-19-05).

The evening before egg collection, breeding crosses of 2-3 males and 2-3 females were separated into breeding traps. The following morning, eggs were collected within 1 hour of spawning and placed in 30% Danieau's solution at 28°C.

At 6 hours post-fertilization (hpf), batches of embryos were transferred to glass petri dishes with 10 mL of 30% Danieau's solution. The embryos were exposed to PAHs at 10 ug/mL, 1 ug/mL, 0.1 ug/mL, and 0.01 ug/mL, with DMSO (10 ug/mL) used as a control. After incubation at 28°C until 72 hpf, embryos were anesthetized with tricane (4 mg/mL stock) and 5 embryos from each group were placed into a 96 well plate imaged using a BZ-X710 microscope (Osaka, Japan) at 2X magnification with brightfield imaging. All images were analyzed using ImageJ software v2.0 to measure the area of the yolk sac and pericardial area as well as the length of the embryos in mm^2 and mm, respectively.

The remaining 5 embryos from each group were separately placed in a 96 well plate with 10 µL of ethoxyresorufin (1 mg/mL stock) diluted in 1 mL Danieau's solution. The well was covered with a plastic lid to prevent evaporation and fluorescence immediately placed in measured with a plate reader at 28°C. CYP1A activity was analyzed through measurement of fluorescence in 30 minute intervals for 24 hours using a FLUOstar Optima plate reader running OPTIMA data software (2.20R2) and analyzed using Optima data analysis software (version 3.01R2).

Statistics

All statistics were performed in Rstudio v.3.6.1. Data from the Ames assays, zebrafish morphology and EROD assays were analyzed with a 2-way ANOVA and a 0.05 alpha level.

Tukey post-hoc tests was performed on statistically different groups. All HPLC data were analyzed using a two tailed t-test with a 0.05 alpha-level.

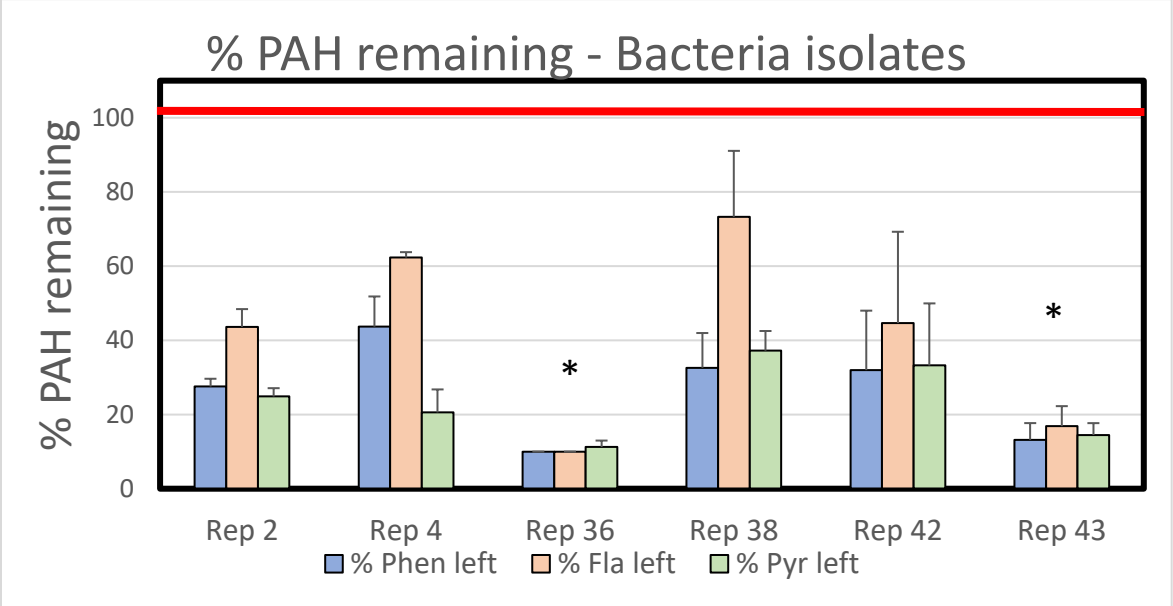
Results

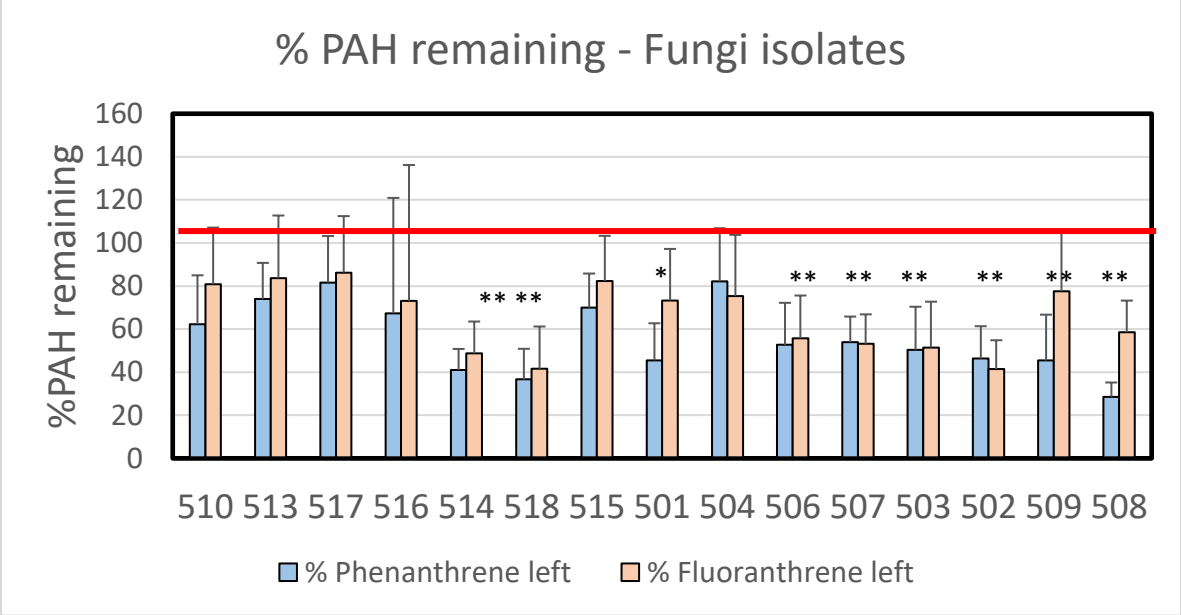
Transformation of PAHs by fungi and bacteria isolates

To test the success of degradation of PAHs by all bacteria and fungi isolates, residual PAH concentrations after incubation were measured by HPLC (Figure 5). Only bacterial strains Rep 36 and Rep 43 had a significant degradation of fluoranthene compared to the abiotic control ($p = 0.04$), although some amount of transformation (albeit not statistically significant) for at least one PAH was indicated for all strains tested

Two fungal strains, 518 and 514 displayed statistically significant degradation of all three PAHs tested ($p<0.05$, data not shown for pyrene). Strains 501, 502, 503, 506, 507, 508, and 509 degraded phenanthrene significantly ($p <0.05$). Strains 502, 503, 506, 507, and 508 degraded fluoranthene significantly ($p<0.05$). Strain 508 was the only *Trichoderma* isolate that degraded pyrene significantly ($p<0.05$, data not shown).

Figure 5. HPLC results of bacteria and fungi isolates degradation





* indicate significant degradation from control

Mutagenic effects of PAHs and fungal and bacterial metabolites

To determine if extracts of two bacterial (Rep 36 and Rep 43) and two fungal strains (506 and 514) incubated with selected PAHs contained mutagenic metabolites, Ames tests for mutagenicity was conducted. Extracts from phenanthrene incubated with bacteria isolate 43 had a high mutagenicity in Ames strain TA 98 with S9 treatment ($p<0.01$) but extracts from abiotic controls with S9 added also caused a higher mutagenicity in Ames strain TA 98 ($p =0.01$). Extracts from phenanthrene incubated with Rep 36, fungi isolate 506, and abiotic phenanthrene had higher mutagenicity in Ames strain YG1041 when S9 was added ($p =0.03$; $p<0.01$). Extracts from strain 43 and 36 did not show mutagenicity in Ames strains TA 100 and YG1041 (Table 4). No increased mutagenicity for phenanthrene or metabolites of phenanthrene was observed without S9 activation.

Table 4. Ames mutagenicity of bacteria and fungi isolates with phenanthrene (* indicates significant mutagenicity

Incubation Conditions	TA 98 +Phenanthrene (revertant/plate)		TA 100 +Phenanthrene (revertant/plate)		YG1041 +Phenanthrene (revertant/plate)	
	+S9	-S9	+S9	-S9	+S9	-S9
Rep 36	NS	NS	NS	NS	22.13±9.68*	NS
Rep 43	21.125±6.7*	NS	NS	NS	NS	NS
#514	NS	NS	NS	NS	NS	NS
#506	NS	NS	NS	NS	35.9±12*	NS
Abiotic (Bacteria)	22.25±4.0*	NS	NS	NS	NS	NS
Abiotic (Fungi)	NS	NS	NS	NS	31.5±6.3*	NS

Extracts from fluoranthene incubated with fungi isolates 514 and 506 and abiotic controls of fluoranthene had a higher mutagenicity in Ames strain TA 98 when S9 was added ($p =0.03$, 0.01). Extracts from bacteria isolate 43 and fungi isolate 514 displayed an increase in

mutagenicity in Ames strain TA 100 with S9 added (p =0.03 and 0.05). Extracts from bacteria isolates 36 and fungi isolate 506 and abiotic fluoranthene had a higher mutagenicity in Ames strain TA 100 with S9 added (p <0.01). Extracts from bacteria isolate 43 had a higher mutagenicity in Ames strain YG1041 than the control (p <0.01) (Table 5). As was observed with phenanthrene, S9 activation appeared essential to provoking any increased mutagenic response for fluoranthene or its metabolites.

Table 5. Ames mutagenicity of bacteria and fungi isolates with fluoranthene (* indicate significant mutagenicity)

Incubation Conditions	TA 98 +Fluoranthene (revertant/plate)		TA 100 +Fluoranthene (revertant/plate)		YG1041 +Fluoranthene (revertant/plate)	
	+S9	-S9	+S9	-S9	+S9	-S9
Rep 36	NS	NS	NS	NS	70.5±23.87*	NS
Rep 43	NS	NS	34.38±7.40*	NS	94.63±35.16#	NS
#514	29.38±5.75*	NS	91.13±12.38*	NS	NS	NS
#506	28.13±7.17*	NS	NS	NS	7.88±4.99*	NS
Abiotic (Bacteria)	NS	NS	NS	NS	76.63±30.41*	NS
Abiotic (Fungi)	27.89±5.00*	NS	NS	NS	NS	NS

Extracts incubated with fungi isolate 514 with pyrene and the abiotic pyrene had a higher mutagenicity in Ames strain TA 98 and YG1041 with S9 added (p<0.01). The rest of the Ames strains did not show mutation rates significantly different than controls (Table 6).

Table 6. Ames mutagenicity of fungi isolates with pyrene (* indicates significant mutagenicity)

Incubation condition	TA 98 + Pyrene		TA 100 +Pyrene		YG1041 + Pyrene	
	+S9	-S9	+S9	-S9	+S9	-S9

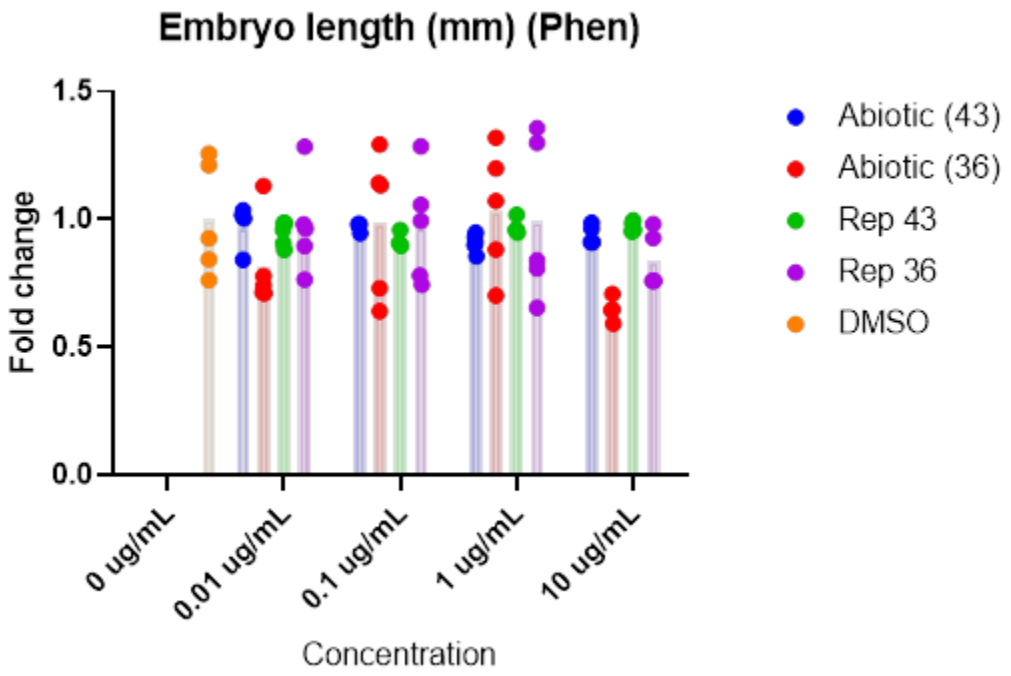
#514	29.1±4.9*	NS	NS	NS	37.5±5.9	NS
Abiotic	31.6±7.4*	NS	NS	NS	41.8±10*	NS

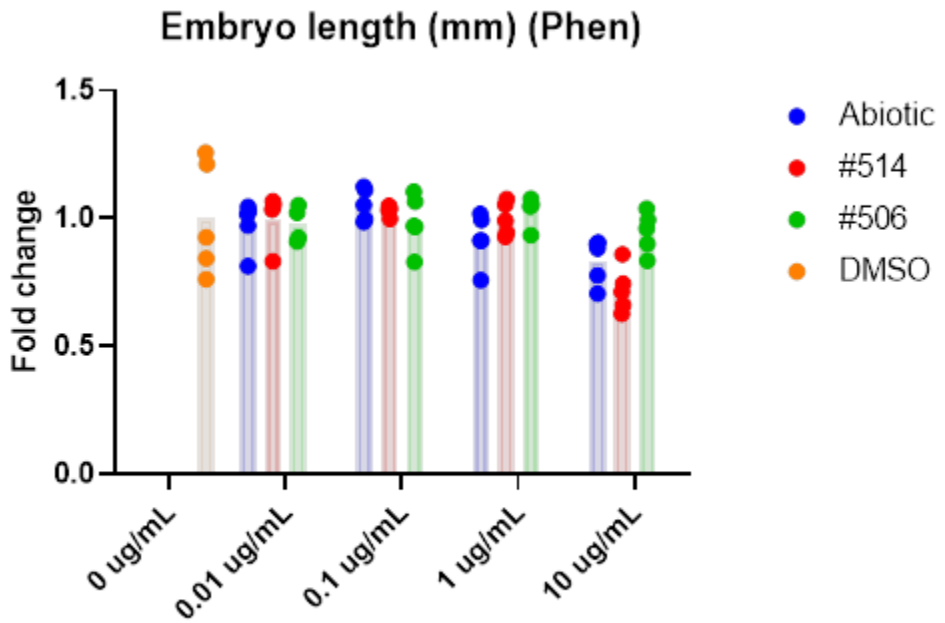
Effects of PAHs and PAH metabolites on the development of Zebrafish

Zebrafish morphology and CYP1A induction were used to test for teratogenicity of PAH metabolites. Few statistically significant impacts were observed on pericardial area (Figure 8 and 9), yolk sac area (Figure 7 and 10), or embryo length (Figure 6 and 11) (Figure 12) of zebrafish when exposed to PAHs or metabolites of PAHs incubated with the selected bacterial and fungal strains. The exceptions include extracts of phenanthrene with bacteria isolate 36 had a higher pericardial area at 0.01 ug/mL than the top dose (10 ug/mL) ($p<0.01$) than at the lower doses.

In addition, embryos exposed to extracts of phenanthrene incubated with fungi isolate 514 had a higher pericardial area than the abiotic controls ($p<0.01$). Embryos exposed to abiotic phenanthrene had a higher yolk sac area at 10 ug/mL than those exposed to extracts from phenanthrene incubated with fungi isolate 506 ($p<0.01$).

Figure 6. Length of zebrafish embryos exposed to phenanthrene and isolates extracts

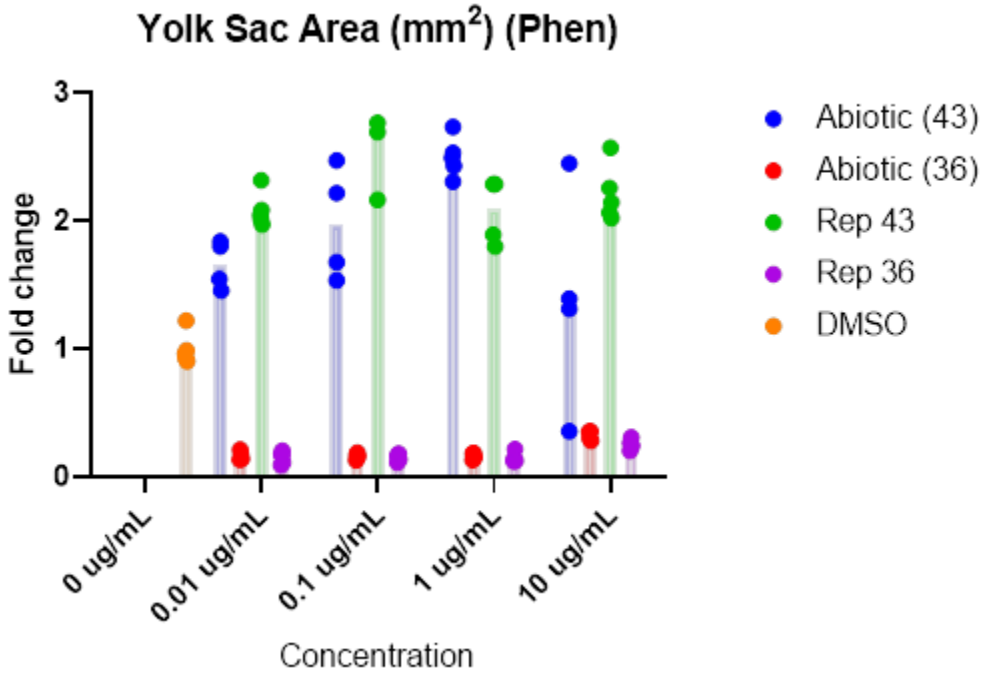


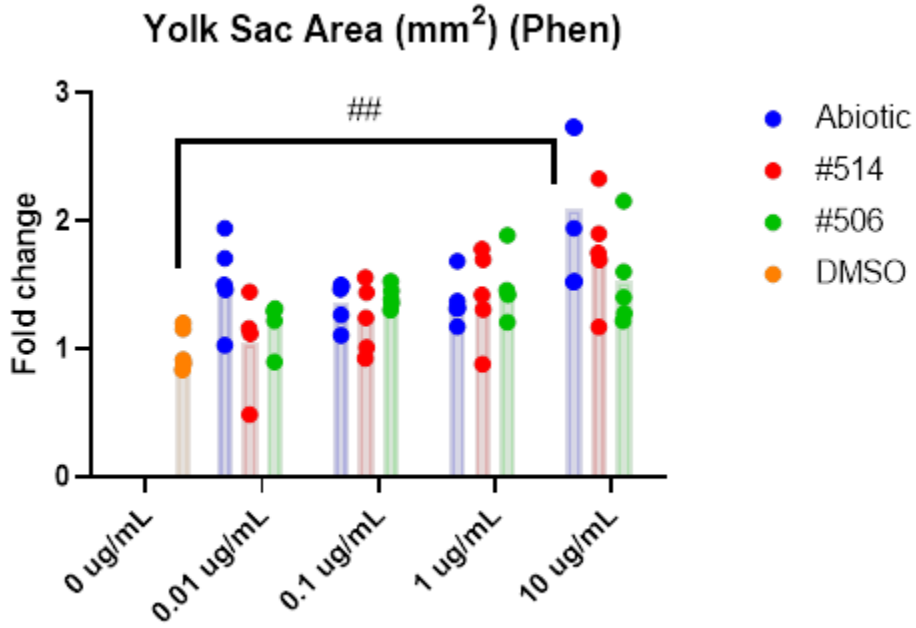


Top graph represents bacteria isolates and bottom graph represents fungi isolates

Bars are means for each group

Figure 7. Yolk Sac area of zebrafish embryos exposed to phenanthrene and isolate extracts



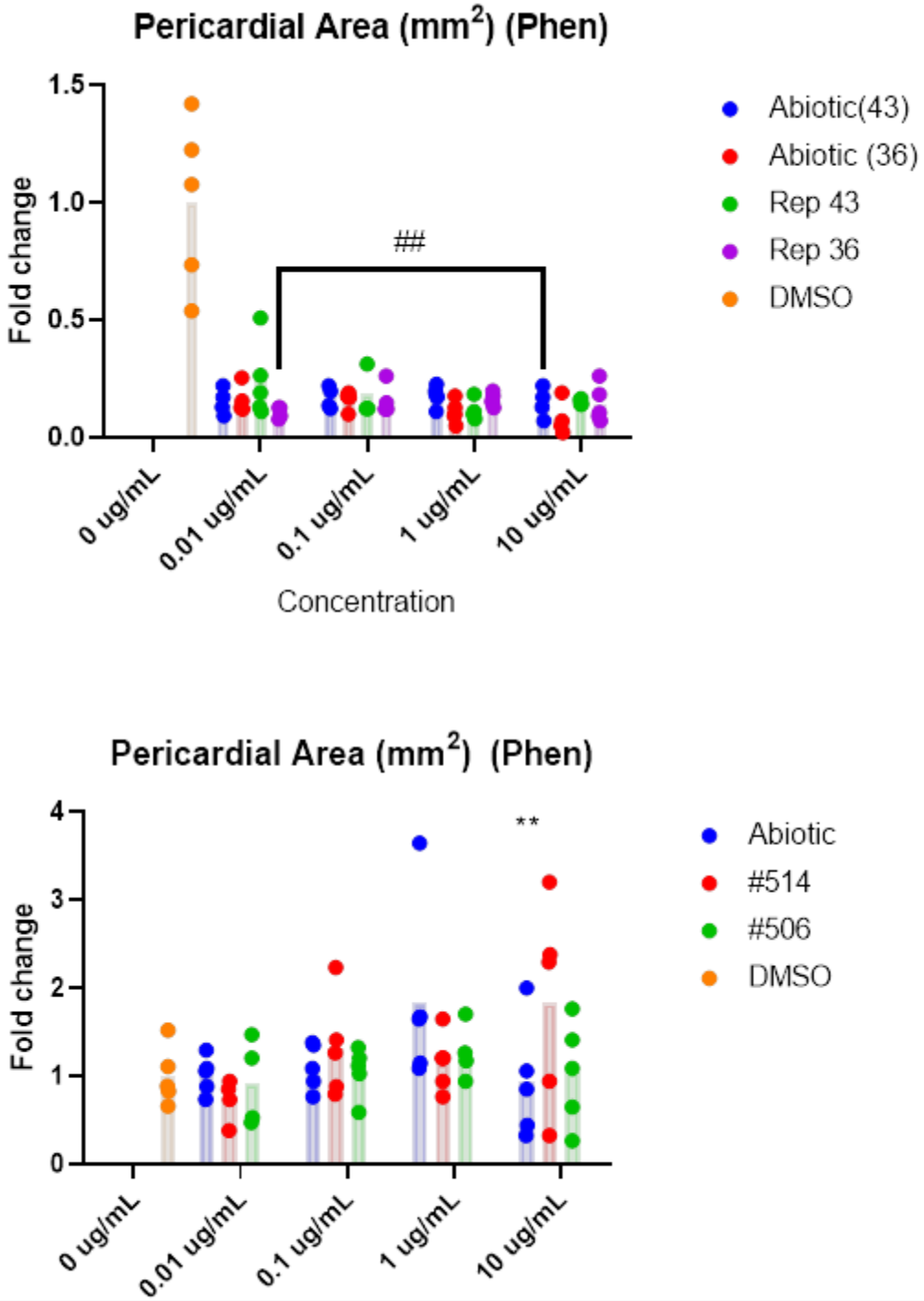


Top graph represents bacteria isolates and bottom graph represents fungi isolates

Bars are means for each group

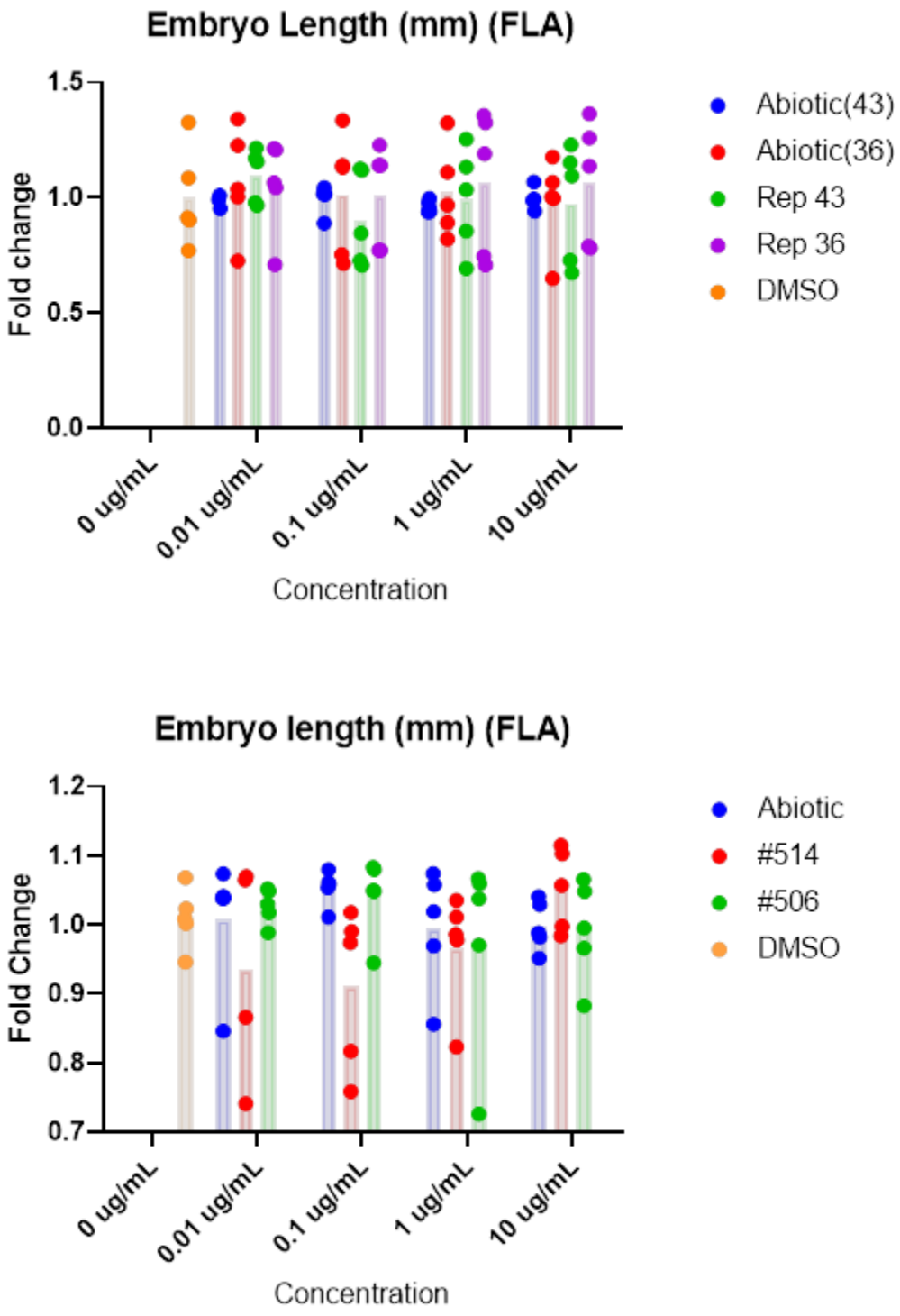
are statically different groups ($p<0.01$)

Figure 8. Pericardial area of zebrafish embryos exposed to phenanthrene and isolate extracts



Top graph represents bacteria isolates and bottom graph represents fungi isolates. Bars are means for each group ## are statistically different groups ($p<0.01$)** indicates significantly higher area ($p<0.01$)

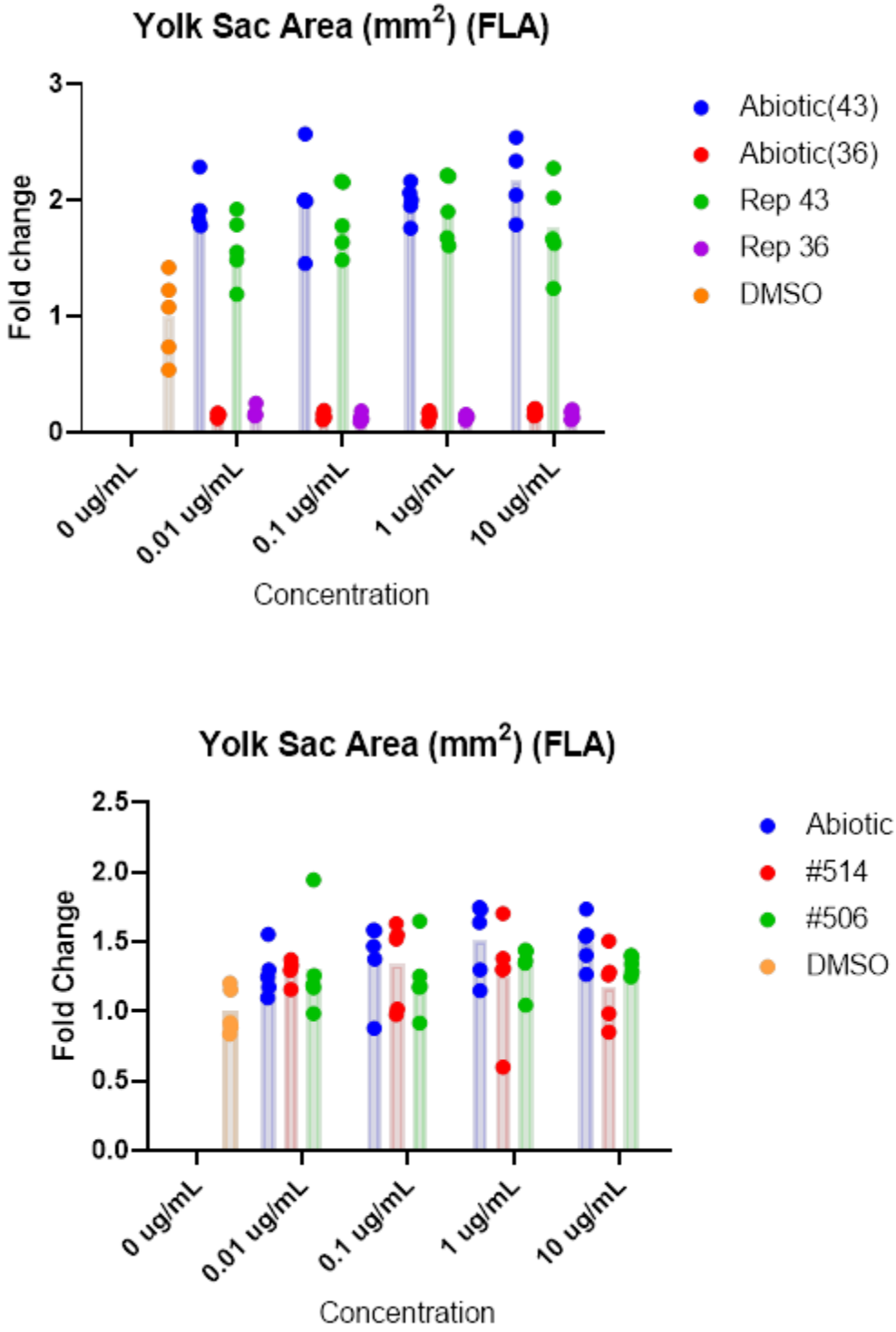
Figure 9. Lengths of zebrafish embryos exposed to fluoranthene and isolate extracts



Top graph represents bacteria isolates and bottom graph represents fungi isolates

Bars are means for each group

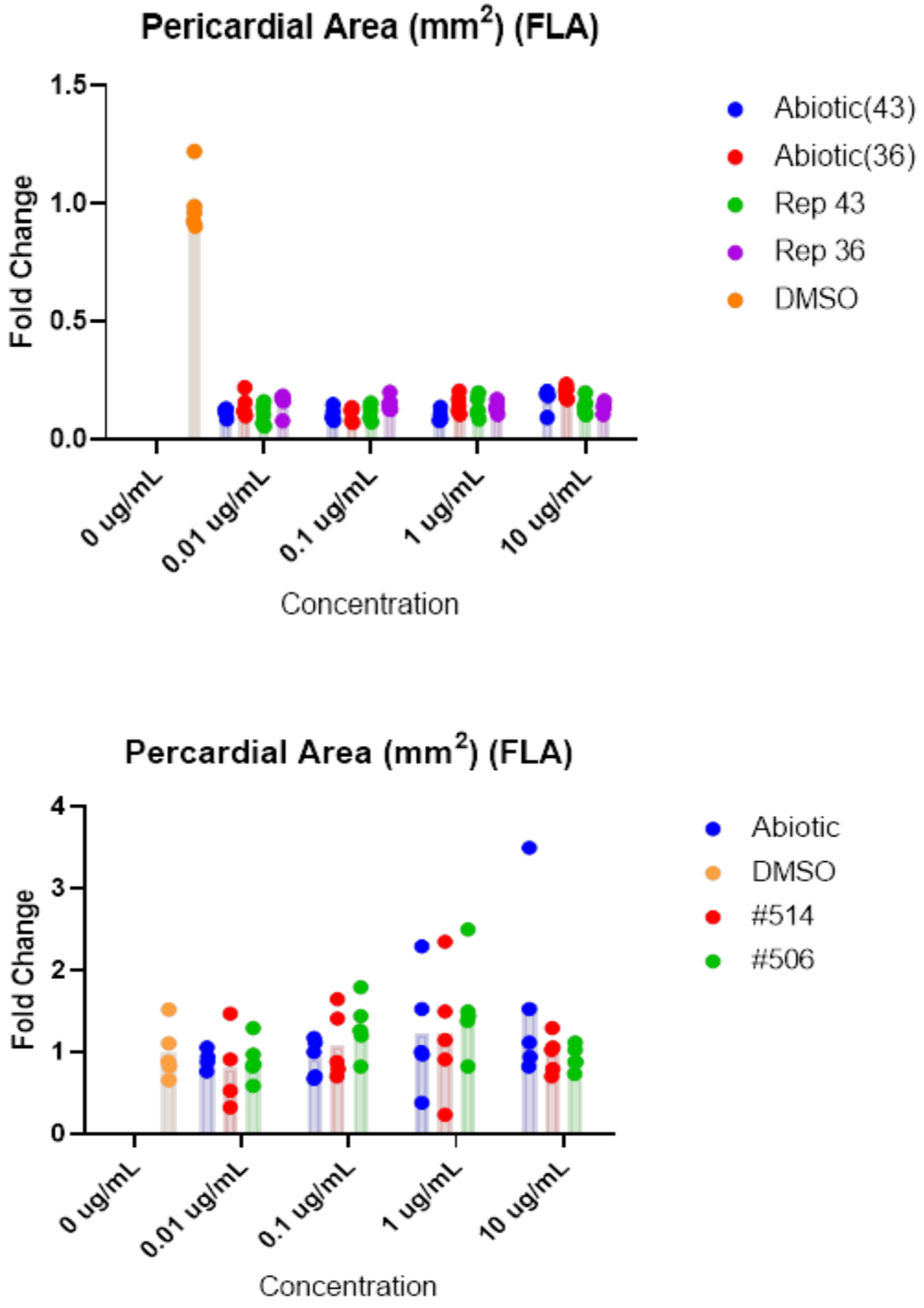
Figure 10. Yolk Sac area of embryos exposed to fluoranthene and isolate extracts



Top graph represents bacteria isolates and bottom graph represents fungi isolates

Bars are means for each group

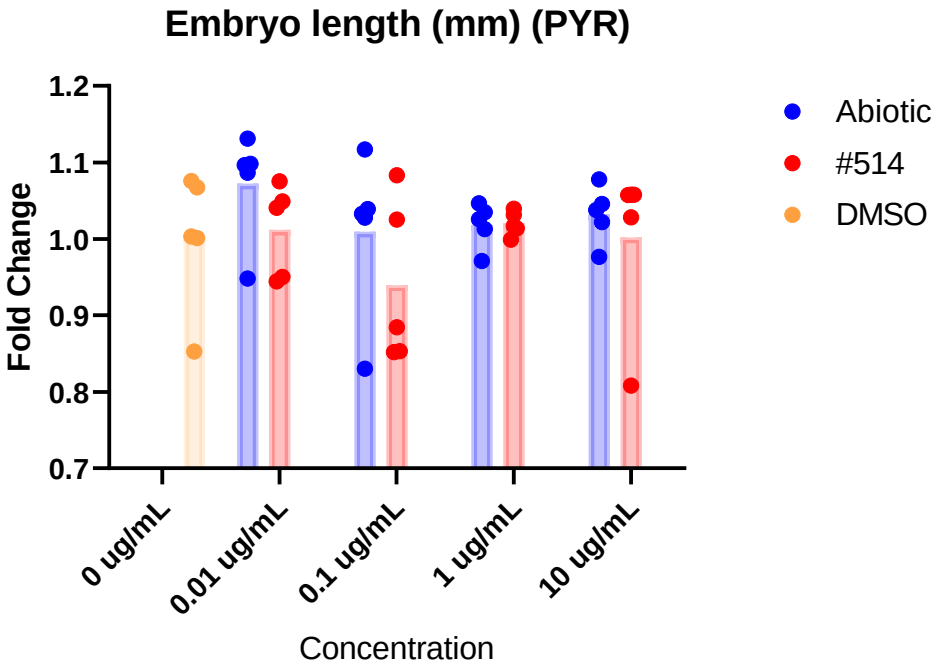
Figure 11. Pericardial area of zebrafish embryos exposed to fluoranthene and isolate extracts

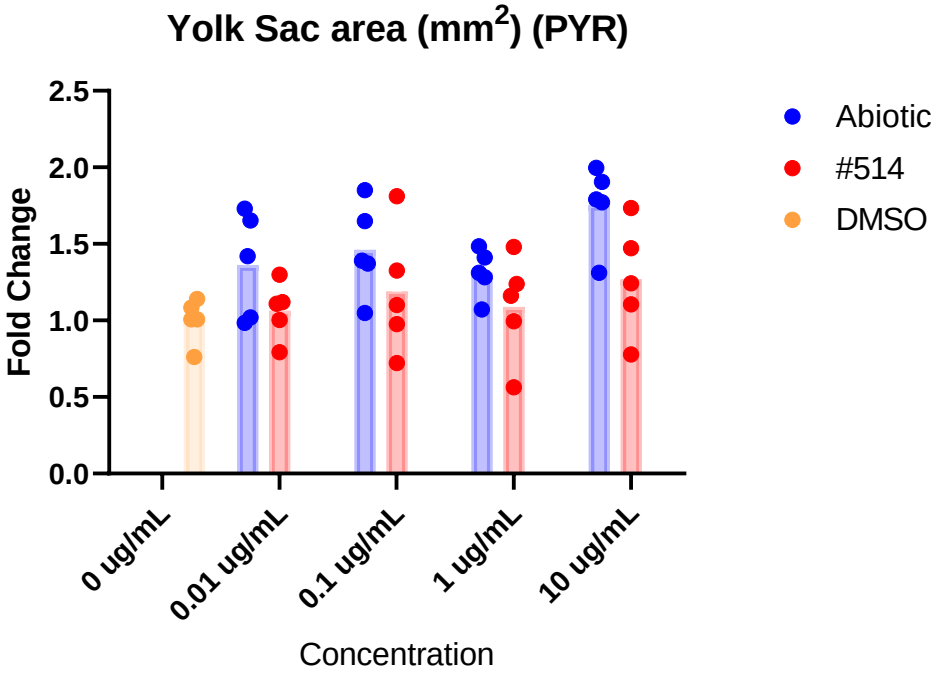


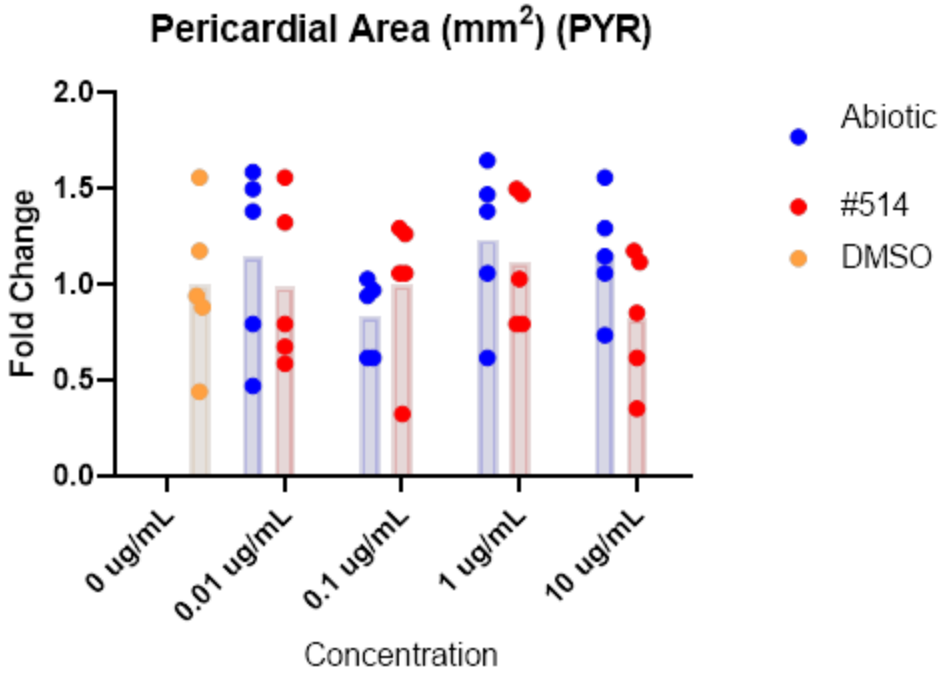
Top graph represents bacteria isolates and bottom graph represents fungi isolates

Bars are means for each group

Figure 12. Length, pericardial area, and yolk sac area of zebrafish exposed to pyrene and isolate extracts



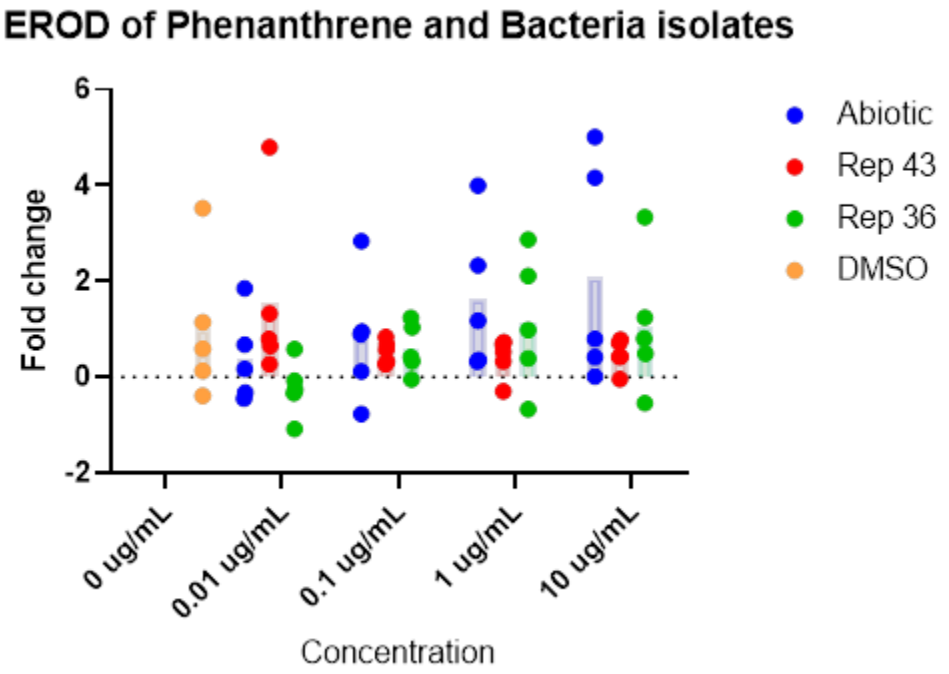


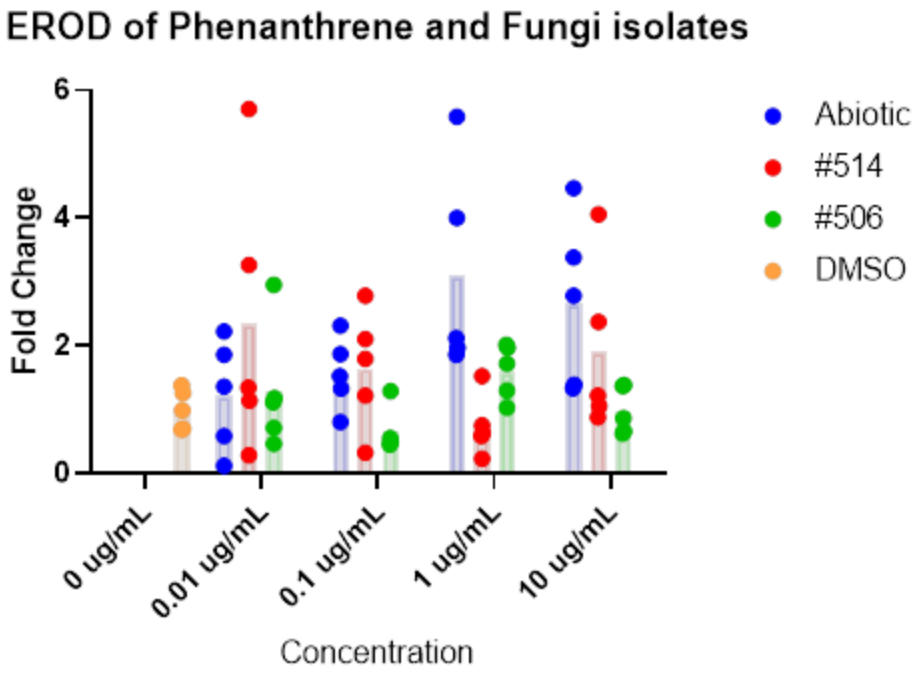


Bars are means for each group

The EROD assay in zebrafish embryos (Figures 13, 14, and 15) did not produce significant CYP1A induction in the embryos for the abiotic controls nor the three PAHs incubated with bacterial and fungal isolates. However, for the bacteria isolates, Rep 36 and Rep 43, higher fluorescence values in the top doses (10 ug/mL and 1 ug/mL) and a decrease in the lower doses (0.1 ug/mL and 0.01 ug/mL) for both phenanthrene and fluoranthene were observed. The same trend applied to fungal isolates 514 and 506 for phenanthrene and fluoranthene. Pyrene had lower induction at the top dose but followed a dose-dependent trend for the lower doses.

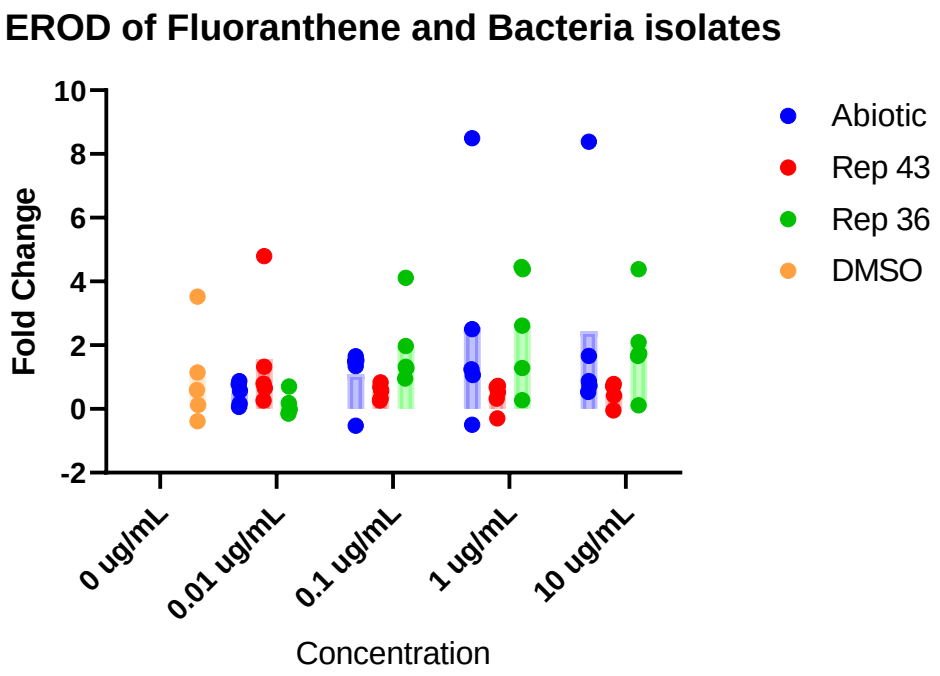
Figure 13. EROD assay results for phenanthrene

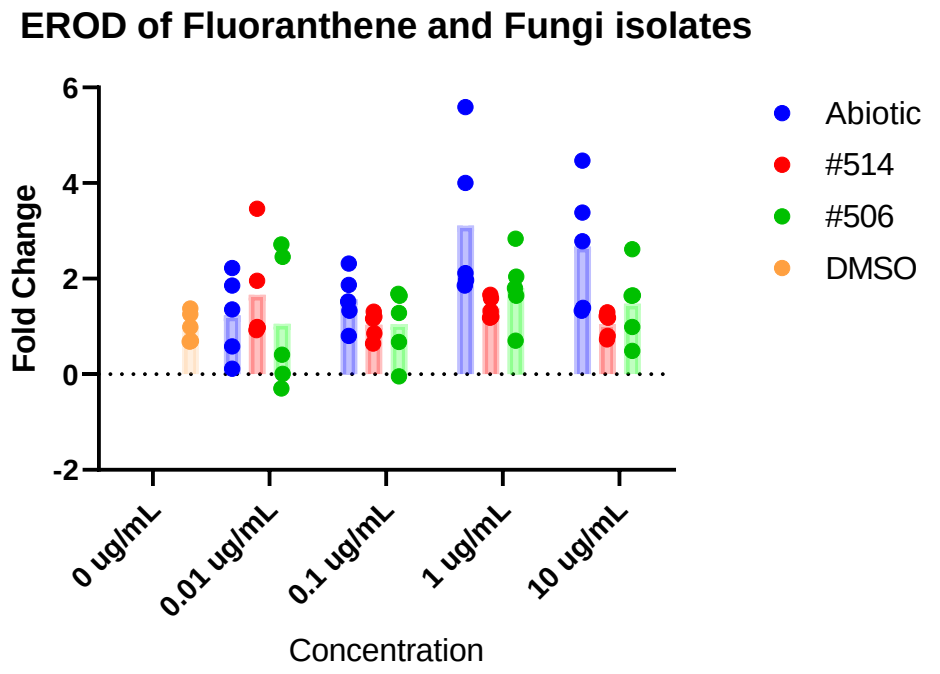




Top graph represents bacteria isolates and bottom graph represents fungi isolates Bars are means for each group

Figure 14. EROD assay results for fluoranthene

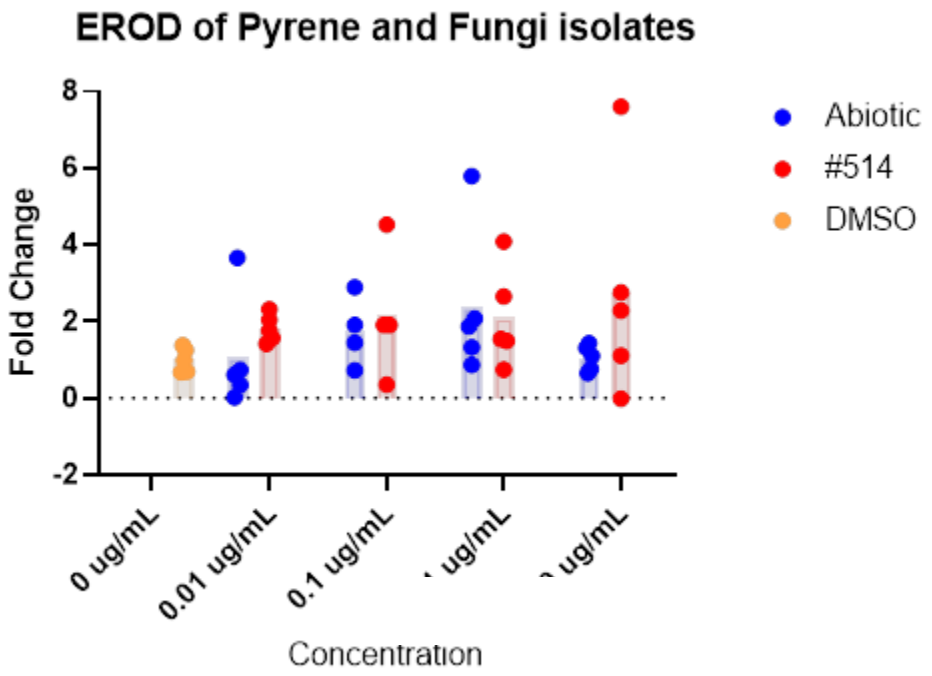




Top graph represents bacteria isolates and bottom graph represents fungi isolates

Bars are means for each group

Figure 15. EROD assay results for Pyrene



Bars are means for each group

Discussion

The capability of each of the tested bacterial and fungal isolates to degrade any of phenanthrene, pyrene, or fluoranthene was tested or confirmed in this study. The significant degradation of fluoranthene was observed in this study by Rep 36 and Rep 43, previously identified as *Novosphingobium indicum* and *Alcaligenes faecalis*, respectively. The bacterial strains Rep 38, Rep 36, and Rep 42 were isolated on media with phenanthrene as the sole carbon source while Rep 2, Rep 4, and Rep 43 were isolated on media with fluoranthene as the sole carbon source (Volkoff 2019). As each of the bacterial strains in this experiment were isolated on one of the PAHs tested in this experiment, and can therefore grow on them, the lack of statistically significant removal for most strains can be partially attributed to large variance in the measured residual PAH concentrations of the experimental and control incubations. Other possible explanations for low removal rates are sorption of PAHs to the lid of the storage container or that inconsistent amounts of the stock PAHs were added to the bottles before incubation. However, the observed turbidity of cultures, color changes in media containing PAHs indicating the formation of oxidized metabolites, lower (if not significant) measured PAH concentrations, and historical data clearly indicate that each of these strains are capable of metabolizing at least one of the tested PAHs.

Significant degradation of all three PAHs by the fungal isolate 508 and two PAHs each by strains 506, 507, 503, and 502 were observed in this work, the first to directly measure PAH degradative capability by any of the isolated strains. Interestingly, all five of these fungal strains were identified as members of the *Trichoderma* genus. *Trichoderma* are known to be able to PAHs such as phenanthrene, benzo[*a*]pyrene, and pyrene (Zafra et al. 2015; Yao et al. 2015). Degradation of all three PAHs were also accomplished by fungal isolates 514 and 518. Isolate

514 is a member of the *Paraphaeosphaeria* genus and 518 is a member of the *Septoriella* genus. *Paraphaeospaeria* is one of the genera that is able to degrade benzo[*a*]pyrene and pyrene (Passarini et al. 2011). *Saptoriella* has been a potential PAH degrader (Gałązka et al. 2020) but not know much if it can truly degrade PAHs. It is interesting to note this may the one of the first studies to show PAH degradation from *Septoriella.* It should be noted that while some of these fungal strains generally are shown in the literature to harbor pyrene-degrading capabilities, experiments involving pyrene in this work were not emphasized due to difficulties in performing those experiments due to the presence of pyrene crystals in stock solutions, making reproducible conditions over multiple tubes difficult if not impossible.

To test for potential mutagenicity of bacteria and fungi metabolites of PAHs, an AMES test using three *E. coli* strains harboring different mutations and either inactivated or S9-activated extracts of PAHs and PAH metabolites were used. Based on the results, it is evident that bacterial degradation of phenanthrene and fluoranthene without S9 activation did not produce a significant difference in mutagenicity compared to the parent compound in the AMES test for either strains. However, for Ames strain YG1041 the parent compound fluoranthene and extracts of fluoranthene incubated with Rep 43 and Rep 36 did produce mutagenicity when S9 activation occurred. These results are consistent with a study by Pagnout et al. that found that the products of the bacteria, *Mycobacterium sp.* from the PAH compounds: phenanthrene, fluoranthene, pyrene, and benzo[a]pyrene were less toxic than the parent compound. The Ames test in this study proved the metabolites to be mutagenic with S9 activation with phenanthrene and fluoranthene as opposed to the study (Pagnout et al. 2006).

Similar trends were observed concerning the mutagenicity of PAHs and PAH metabolites exposed to fungi when S9 activation occurred. However, extracts from fungal isolate 506

incubated with phenanthrene and fluoranthene caused higher mutations in Ames strains TA 98 and YG1041 and extracts from isolate 514 incubated with either pyrene or fluoranthene caused a higher mutagenicity in Ames strain YG1041, but only after activation of the extracts with S9. The observed increased mutagenicity with the addition of S9 chemical is not consistent with another study that did not find mutagenicity with S9 chemical added (Cerniglia et al. 1985)

In this study, metabolites from fungi isolate 514 and 506; bacteria isolate Rep 43 and 36; and the abiotic controls did require metabolic activation for mutagenicity. The results are consistent with prior studies that PAHs and epoxide metabolites require metabolic activation to become mutagenic (Xue and Warshawsky 2005). We also observed that the fact that the abiotic controls incubated without exposure to either bacterial or fungal strains were mutagenic with S9 activation as well as when those compounds were incubated with organisms demonstrates that the addition of S9 alone regardless of microbial biotic influences can cause higher mutation rates.

Extracts of selected bacteria and fungi isolates were also tested for teratogenicity in zebrafish embryos. In this study, the zebrafish morphology of the yolk sac area increased with exposure to abiotic phenanthrene. Increased yolk sac and pericardial areas are consistent with a study that looked at effects of embryos exposed to phenanthrene (Sogbanmu et al. 2016). The majority of the findings of this study showed no significant changes in zebrafish development that might be attributed to microbial metabolites (except for the yolk sac area of embryos treated with fungal isolate 514 incubated with phenanthrene). This finding does not agree with a study that found higher developmental and genotoxicity in zebrafish from aerobic remediated PAHs in which the greatest effects were seen in the yolk sac and swim bladder from phenanthrene and fluoranthene present in soil mixtures (Chibwe et al. 2015). The discrepancy in this study arises

from the fact that the PAHs used were from pure culture (98% purity) as opposed to fractionated mixtures in the soils. Other compounds may have been present in soil including quinones (Chibwe et al. 2015).

The EROD results were inconclusive in this study, with the effect of CYP1A activity seemingly unaffected in the zebrafish embryos. This is consistent with other studies that found no EROD activity with exposure to phenanthrene. Fluoranthene had weak EROD activity at the highest concentrations (Bosveld et al. 2002; Pathiratne and Hemachandra 2010). Even though we did not find significant results, there was a general trend of higher CYP1A induction in abiotic controls and the PAHs incubated with bacterial isolates Rep 36, Rep 43, and fungi isolates 514 and 516. It is important to note that phenanthrene and fluoranthene are non-agonists of the AhR pathway and pyrene is a weak agonist (Barron et al. 2004).

Apart from the abiotic phenanthrene causing teratogenic effects, there was not a significant effect of PAHs nor the extracts containing PAH metabolites on the embryos. In terms of potential bioremediation products, as this indicates that at least for the combination of these organisms and PAHs tested, this could be considered a positive outcome of the study since there is not strong evidence for the generation of toxic byproducts to form while these microorganisms degrade pollutants in the environment. Overall, most of the observed mutagenicity came from the addition of S9 to both extracts from the isolates and abiotic controls, but there was not an organismal effect of the extracts from bacterial and fungal isolates.

Future directions and recommendations

Due to the constraint of time, the fungi isolates could not be tested for benzo[*a*]pyrene (BaP) degradation and for transformation products. BaP is a suspected carcinogen and as a 5-

ringed compound tends to remain in the environment longer that the other PAHs tested here as most microorganisms have difficulty transforming high molecular weight (HMW) PAHs of 5+ rings. The significant degradation of both phenanthrene and fluoranthene by the fungal isolates suggest they may be adept in degrading a wider variety of PAH compounds, including HMW PAHs such as BaP. The next steps for this project might be to test a mixture of bacteria and fungi to degrade all PAHs up to and including benzo[*a*]pyrene. The isolates of both bacteria and fungi could also be incubated with actual Elizabeth River estuarine sediment to measure degradation of residual, weathered PAHs in that inoculum. The estuarine sediment is especially imperative due to the mixture of PAHs present and is more environmentally relevant than pure single compound studies.

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Supplemental Section

Table 1. HPLC methods

PAH (Bacteria)	**Flow Rate**	**Time window**	**Excitation**	**Emission**
Phenanthrene	1.20 mL/min	0.0 minutes	252 nm	370 nm
Fluoranthene	1.20 mL/min	11.0 minutes	281 nm	453 nm
PAH (Fungi)	**Flow Rate**	**Time window**	**Excitation**	**Emission**
Phenanthrene	1.20 mL/min	0.0 minutes	252 nm	370 nm
Fluoranthene	1.20 mL/min	7.3 minutes	288 nm	440 nm
Pyrene	1.20 mL/min	9.4 minutes	336 nm	398 nm