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Impacts of dibenzopyrenes on bacterial community isolated from Gulf of Mexico sediment

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Abstract

The presence of polycyclic aromatic hydrocarbons (PAHs) in marine environments as a result of contamination is an environmental concern, especially in regions where oil spills such as the *Deepwater Horizon* have occurred. While numerous PAHs have been studied for their effects on microbes, the family of dibenzopyrenes has yet to be investigated. In this preliminary study, the impacts of these molecules on the community structure of a bacterial consortium isolated from oil-impacted Gulf of Mexico sediment were examined using high-throughput sequencing, demonstrating intriguing negative impacts on species diversity and abundance. While no measurable degradation of the dibenzopyrenes was observed after 28-day incubation, the abundance of known oil-degrading bacteria from orders such as Oceanospirillales, Caulobacterales, Sphingomonadales, and Nitrosococcales were shown to be enhanced. Of the five isomers of dibenzopyrene studied, dibenzo[*a*,*h*]pyrene supported the fewer number of microbial species suggesting the isomer was more toxic compared to the other isomers.

KEYWORDS

bacterial consortium, bioremediation, *Deepwater Horizon* oil spill, dibenzopyrene, high-throughput sequencing, polycyclic aromatic hydrocarbon

1 | INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are composed of carbon and hydrogen atoms arranged in multiple rings such that the overall structure is aromatic. These molecules are components of crude oil that have been under great scrutiny in recent years (Aeppli et al., 2018; Ates & Argun, 2018; Bagby, Reddy, Aeppli, Fisher, & Valentine, 2017; Chukwura, Ojiegbu, & Nwankwegu, 2016; Ghosal, Ghosh, Dutta, & Ahn, 2016; Oaikhena, Makaije, Denwe, Namadi, & Fatanmi, 2016). PAHs include numerous toxins and carcinogens, such as 16 members of the United States Environmental Protection Agency Priority Pollutants List (Richter-Brockmann & Achten, 2018), as well as the first identified chemical carcinogen benzo[*a*]pyrene (B[*a*]P) (Osborne & Crosby, 1987).

Of greater concern than these known and regulated compounds are the larger high molecular weight (HMW) PAHs. These are molecules having molecular weights ≥302 g/mol like the dibenzopyrene (DBP) family, comprised of isomers formed from a central pyrene moiety with two additional fused benzene rings. This particular family includes five isomers that differ primarily in the location of the substituent benzenes: dibenzo[*a*,*e*]pyrene (DB[*a*,*e*]P), dibenzo[*a*,*h*]pyrene (DB[*a*,*h*]P), dibenzo[*a*,*i*]pyrene (DB[*a*,*i*]P), dibenzo[*a*,*l*]pyrene (DB[*a*,*l*]P), and dibenzo[*e*,*l*]pyrene (DB[*e*,*l*]P) as shown in Figure 1.

While advances have been made in the isolation and quantitation of these isomers from known mixtures and standard reference materials (Hayes, Wilson, Sander, Wise, & Campiglia, 2018; Santana, Comas, Wise, Wilson, & Campiglia, 2019), these HMW-PAHs are challenging

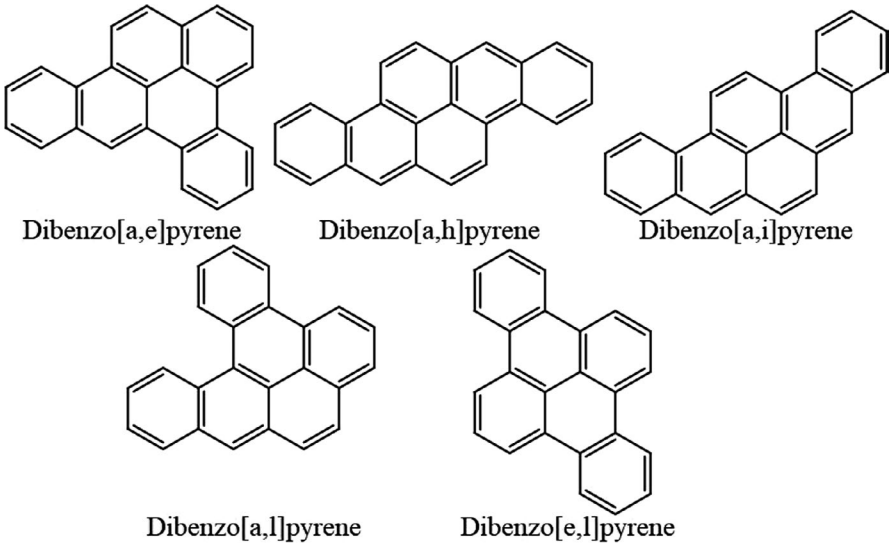

FIGURE 1 The dibenzopyrene family of PAHs

to distinguish without specialized analytical instrumentation unavailable to the typical environmental laboratory; therefore, little is known about their distribution in nature. PAHs up to the five-ringed B[*a*]P and dibenz[*a,h*]anthracene are degraded by bacteria isolated from the soil within two weeks, with measurable degradation in as little as 16 hr (Kanaly & Harayama, 2000). Because water solubility is a key factor in PAH bioavailability (Abdel-Shafy & Mansour, 2016) the significantly less soluble DBPs will likely require more time to be consumed, so a period of 28 days was selected for this preliminary study.

PAHs are particularly important in the context of oil spills, comprising up to 7% of crude oil by mass (Albers, 2003), and are recalcitrant and bioaccumulative (Sekar & DiChristina, 2017). While numerous bacterial and fungal species are known to degrade PAHs, the toxicity of PAHs to other microbes have had profound impacts on the microbial communities where they are deposited, as with other soil pollutants (Obaroh, Yahaya, & Ibrahim, 2016).

Studies have shown the significance of individual microbial species, even at low abundance, to the overall health of the microbiome and oil degradation capability (Dyksterhouse, Gray, Herwig, Lara, & Staley, 1995; Mason et al., 2012; McFarlin, Perkins, Field, & Leigh, 2018; Zozaya-Valdes, Egan, & Thomas, 2015). Bacteria known to degrade crude oil, including Sphingomonads, Caulobacters, Oceanospirilla, Nitrosococci, Colwelliae, and Cycloclasticus, each serve a purpose in the long process of oil biodegradation. By comparing the relative abundance of these species in the presence of various DBPs, conclusions may be drawn as to the toxicity of individual isomers and overall impacts. One species, *Mycobacterium vanbaalenii* strain PYR-1, has been reported (Moody, Freeman, Fu, & Cerniglia, 2004) to degrade PAHs as large as B[*a*]P, and although other Mycobacteria have yet to be fully characterized, their ubiquity in the environment makes them an important factor for consideration (Sabine et al., 2016).

The effects of various PAHs on individual and commingled microorganisms have been examined for decades (Heitkamp & Cerniglia, 1988; Laflamme & Hites, 1978; National Research Council, 1983), yet to our knowledge, the dibenzopyrene family has not been investigated as such. This paper presents a novel study wherein a bacterial consortium obtained from an oil-impacted marsh located in the Gulf of Mexico was exposed to individual and combined DBP isomers in a series of preliminary experiments to determine the ability of the consortium to degrade these HMW-PAHs. High-performance liquid chromatography (HPLC) was used to analyze DBP isomers, while the effects of the PAHs on the microbial community structure were observed using high-throughput sequencing (HTS).

2 | MATERIALS AND METHODS

2.1 | PAH standards and stock preparations

Standard reference materials DB[*a,i*]P and DB[*e,l*]P were purchased from Toronto Research Chemicals, Ontario, Canada; certified reference materials DB[*a,e*]P, DB[*a,l*]P, and DB[*a,h*]P were purchased from the European Commission Joint Research Centre, Institute for Reference Materials and Measurements, Community Bureau of Reference, Geel, Belgium. Analytical grade B[*a*]P was obtained from Millipore Sigma, St. Louis, Missouri. HPLC-grade solvents and trace-metal-grade acids were purchased from Fisher Scientific, Hampton, New Hampshire. Molecular biology grade water was obtained from Corning, Corning, New York.

PAH standards were prepared in dimethylsulfoxide (DMSO) at concentrations appropriate to their solubility and working conditions. Each standard was filter-sterilized with a 0.2 μm polytetrafluoroethylene (PTFE) syringe filter before use.

2.2 | Sediment collection and processing

Sediment cores were collected from the western shore of the Chandeleur Islands (29.895448°, −88.827780°), a chain of uninhabited islands located southeast of the Mississippi River adjacent to the coast of Louisiana in the Gulf of Mexico. This site was selected because it was directly impacted by the *Deepwater Horizon* oil spill in

2010 and, to this day, shows evidence of oil contamination. Sediment cores were collected by hand from the marsh zone of the black mangrove-dominated shoreline and transported on ice before processing.

Each core was extruded and sectioned in centimeter (cm) increments for the first 4 cm, then 2-cm sections to 14 cm depth. Sectioned sediment was homogenized according to depth, and the presence of oil in each core section was visually and analytically confirmed in the 4–8 cm depth range.

2.3 | Consortium isolation and growth conditions

Sediment (1.06 g) from the 6 to 8 cm depth (within the oil layer) was suspended in 10 ml of sterile 0.85% sodium chloride (NaCl), gently vortexed, and incubated at 28°C and 110 rpm for 1 hr. A total of 5 ml of this suspension were then transferred to a brackish marine medium (M10b), and this consortium culture was maintained at 28°C and 110 rpm. The M10b medium consisted of 4.0 g tryptone, 2.5 g yeast extract, 10.53 g NaCl, 0.45 g KCl, 7.41 g $MgSO_4 \cdot 7H_2O$, and 0.87 g $CaCl_2 \cdot 2H_2O$ per liter with a salinity of ~15 ppt, replicating the brackish conditions of the site.

Duplicate autoclaved 250-ml screw-top flasks containing 50 ml sterile M10b medium amended with 1 ppm of a single PAH isomer (either B[*a*]P, DB[*a*,*e*]P, DB[*a*,*i*]P, DB[*a*,*h*]P, DB[*a*,*l*]P, or DB[*e*,*l*]P), a mixture containing 1 ppm of each DBP isomer, or left unamended for biotic controls were inoculated with 1 ml of the consortium culture. Abiotic controls contained the exact medium and PAH but lacked consortium culture inoculation. All assays were conducted at room temperature in the dark under constant stirring for 28 days. After incubation, each of the flasks was sampled for colony counts, DNA analysis, and PAH extraction.

2.4 | PAH extraction and HPLC analysis

Each culture (50 ml) was extracted using an equal volume (50 ml) of HPLC-grade ethyl acetate three times to obtain the neutral PAH fraction as described by Moody and Cerniglia (Moody et al., 2004). The three extracts were pooled and evaporated to dryness using a Buchi Rotary Evaporator, then resuspended in 2 ml of HPLC-grade acetonitrile (ACN). The remaining water fractions were then acidified (pH < 2) with concentrated trace metal-grade hydrochloric acid, and the extraction process repeated to obtain the acidic fraction. The acidic fractions were also evaporated and reconstituted in ACN.

Reconstituted extracts were filtered with glass syringes through 0.2 µm PTFE syringe filters then introduced to a Thermo Scientific UHPLC + Dionex UltiMate 3000 with a Hypersil® Green PAH column (25 cm length, 0.4 cm inner diameter, 5 µm particle size) and diode array detector. HPLC-grade solvents (ACN and H_2O) were used as the mobile phase. All samples were run with the following HPLC method: The column compartment was set to 30°C with a flow rate of 2.00 ml/min, initially 50:50 ACN:H_2O ramped to 90:10 ACN:H_2O over 15 min, held at 90:10 ACN:H_2O for 10 min, then ramped to 100% ACN over 2 min, held at 100% ACN for 5 min, then ramped to 50:50 ACN:H_2O for 1 min, and held at 50:50 ACN:H_2O for 5 min. All sample injection volumes were set to 10 µl.

TABLE 1 Primer sequences for HTS

Primer	Sequence
Arch 349F	5′-GYGCASCAGKCGMGAAW
Arch 806R	5′-GGACTACVSGGGTATCTAAT
V4 515F	5′-GTGYCAGCMGCCGCGGTAA
V4 806R	5′-GGACTACNVGGGTWTCTAAT

2.5 | DNA extraction

Culture aliquots (1.8 ml) from the initial consortium culture and the 28-day assays were collected for DNA extraction. Total genomic DNA was extracted using the Qiagen® UltraClean DNA Extraction Kit (Qiagen) using an Omni Bead Ruptor 24 (Omni International, Inc.). Extracted DNA was quantified using the Qubit 3.0 dsDNA HS fluorescence assay; samples with high concentrations were diluted to ~50 ng/µl with sterile molecular biology grade water. Blank controls (molecular biology grade water) were run with each set of extractions to verify the absence of contaminants.

2.6 | HTS and data analysis

DNA extracts were shipped overnight on dry ice to the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana–Champaign for Illumina MiSeq 2 × 250 V2 amplicon sequencing using primer pairs 16S V4 515F-806R and Arch 349F-806R targeting the bacterial 16S V4 hypervariable region and the archaeal 16S V4 hypervariable region, respectively (Table 1). Raw reads of the amplicon sequencing data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under the study submission identification SUB6333356.

Amplicon sequences were demultiplexed and processed using a typical Qiime2 (Bolyen et al., 2019) pipeline, including quality filtering, denoising, and chimera removal with DADA2 (Callahan et al., 2016) through the BIOM format (McDonald et al., 2012), alignment and phylogeny construction with mafft (Katoh, Misawa, Kuma, & Miyata, 2002) and fasttree2 (Price, Dehal, & Arkin, 2010), and naïve-Bayes classification (McKinney, 2010; Pedregosa et al., 2011) against the Silva (Quast et al., 2013) database.

3 | RESULTS AND DISCUSSION

3.1 | HPLC results

Reconstituted culture extracts analyzed by HPLC showed no measurable decrease in the concentration of dibenzo[*a*,*e*]pyrene, dibenzo[*a*,*i*]

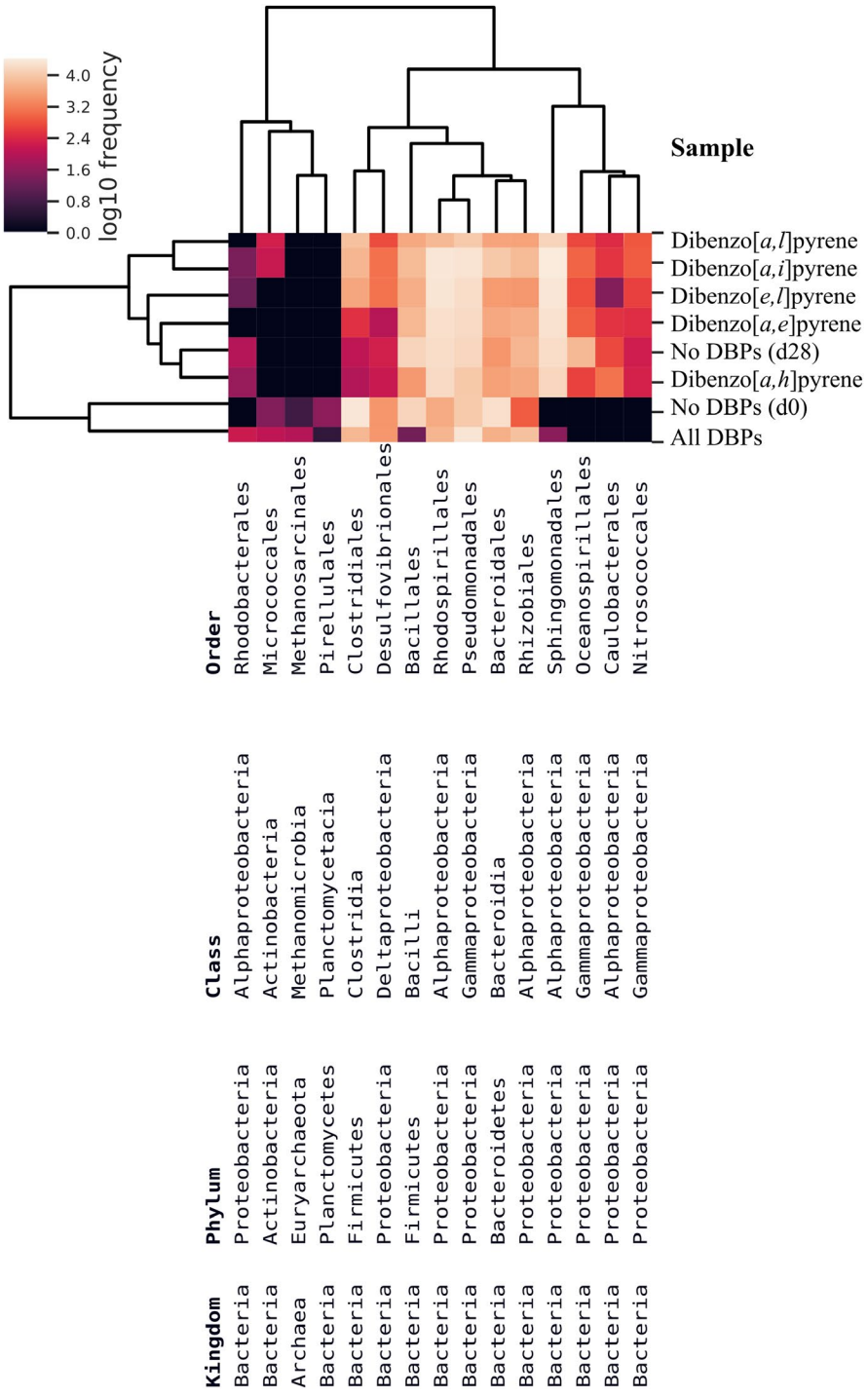

FIGURE 2 Heatmap of identified microorganisms collapsed to taxonomic level 4 (order)

pyrene, or dibenzo[*a*,*l*]pyrene throughout this study. Analysis of dibenzo[*a*,*h*]pyrene and dibenzo[*e*,*l*]pyrene proved inconclusive, as these isomers did not appear on any chromatograms, even as pure standards. Samples of these incubations have been stored for further analysis in future collaborations. Replicate extractions of the combined PAHs from the same M10b medium without consortium bacteria were highly reproducible with less than 10% carryover. This observation indicates that PAHs were possibly associated with the hydrophobic cell membranes, thus resisting extraction in the initial procedure. Upon acidification, cell membrane components may become protonated and therefore more hydrophilic, dissociating from hydrophobic PAHs, which are then more likely to dissolve in the hydrophobic ethyl acetate layer.

Even though the sediment was selected for its proximity and therefore acclimation to crude oil components (Ayodeji, 2018), this microbial consortium was unable to degrade any of the measurable PAH isomers in this study. Additionally, no additional peaks were observed in the extraction HPLC chromatograms to indicate the presence of potential degradation byproducts.

3.2 | Sequencing results

Analysis of amplicon HTS results indicated changes to the microbial community after exposure to the various PAHs, as shown in the following heatmap (Figure 2). This particular heatmap demonstrates both the prevalence of specific bacterial orders in samples and the Euclidean distance between those orders, as indicated by the length of the branches in the upper tree. The relationship between samples was also calculated, as indicated in the left-hand tree, and indicated that the initial consortium and the consortium exposed to a mixture of DBPs were most closely related. Conversely, the unamended control consortium was distinctly closer to the individual DBP isomers, especially DB[*a*,*h*]P. These relationships, along with the specific taxonomic orders indicated, help shed light on the community ecology of this microbial consortium. In this heatmap, darkened squares indicate fewer instances of the given microbial population, while brighter squares indicate a higher incidence of that population within a particular sample. The frequency of occurrence has been $\log_{10}$ transformed to more readily show large-scale differences.

The evidence of this heatmap shows a major increase over the 28 days of incubation in Sphingomonadales and Caulobacterales, both of class Alphaproteobacteria, as well as the Gammaproteobacteria orders Oceanospirillales and Nitrosococcales, which are all known oil-degraders (Dyksterhouse et al., 1995; Mason et al., 2012; McFarlin et al., 2018; Yang, 2014). Oceanospirillales, in particular, has been identified as a biological indicator of oil contamination, serving to initially degrade alkanes before being succeeded by populations more prone to complex hydrocarbon degradation, such as Colwellia and Cycloclasticus (Mason et al., 2012).

It is interesting to note the minimal observable presence of Oceanospirillales initially (No DBPs d0), while it shows a frequency approaching 10^4 after 28 days (No DBPs d28). This population explosion was inhibited slightly in each of the individual DBP incubations and greatly inhibited in the presence of the DBP mixture. Another order of note is the Alphaproteobacterium Caulobacterales, which is genetically similar to the PAH-degrading Sphingomonads (Leys, 2004). Like Oceanospirillales, the mixture of DBPs inhibited the population of Caulobacterales, as well as the isomer DB[*e*,*l*]P. Conversely, DB[*a*,*h*]P seems to slightly enhance the viability of this order.

The small population of Blastopirellula (the only detected genus of order Pirellulales) present initially (No DBPs d0), though it decreased slightly over time (No DBPs d28), was undetectable in all PAH incubations save DB[*a*,*l*]P. This particular genus is noteworthy as it is abundant in the microbiomes of healthy coral reefs and diminished in those of bleached reef communities (Zozaya-Valdes et al., 2015).

Comparing the bacterial communities formed in incubations amended with the five different isomers of DBP yielded further insight, indicating which species may be more tolerant of each PAH isomer. Higher tolerance of a bacterial species to a specific DBP is significant in that, even if a given species is capable of degrading DBPs, if the DBPs are too toxic to it, the cells will not survive long enough at higher DBP concentrations to accomplish any significant detoxification through degradation. DB[*a*,*h*]P supported the fewest unique species, while DB[*a*,*l*]P allowed the most.

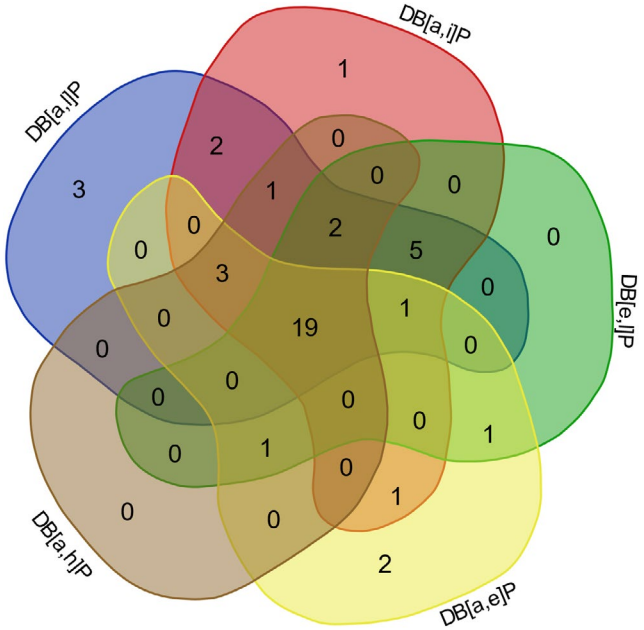

FIGURE 3 Venn diagram (Van de Peer Lab, 2019) showing the number of species unique to or shared by microbial consortia exposed to each of the DBP isomers for 28 days, as indicated by the isomer label

In the following Venn diagram (Figure 3), the number of shared and exclusive species in each consortium is portrayed, showing the similarities and differences among these communities. One species was only present in the DB[*a*,*i*]P incubation: the Clostridium *Oscillibacter* sp.; while two unique species were identified in the DB[*a*,*e*]P incubation: *Caulobacter* sp. (the major Caulobacterales representative being *Hyphomonas* sp.) and *Microbacterium* sp., an Actinobacterium. DB[*a*,*l*]P played host to the most unique species: the *Blastopirellula* sp. mentioned above, *Amorphus suaedae*, and an uncultured *Parvibaculum*. Shared among all isomers were a total of 19 identified genera, including *Prolixibacter*, *Bacillus*, *Paenibacillus*, *Lachnoclostridium* 5, *Tyzzerella*, *Hyphomonas*, 3 Rhizobiaceae (*Cohaesibacter*, *Martelella*, and an unclassified genus), *Labrenzia*, a *Rhodobacter*, *Candidatus Riegeria*, *Thalassospira*, and a Sphingomonad.

4 | CONCLUSION

Each DBP isomer had a clear impact on the composition of the sediment-associated microbial community in this exploratory study. While there were similarities in the nature and intensity of these effects, it appears that DB[*a*,*h*]P is likely more toxic than the other isomers, as fewer unique species were able to survive in its presence. The results of this study indicate that this broad and diverse community of microorganisms was incapable of degrading any DBP isomers within 28 days. While it is possible that longer exposure would allow greater acclimation, and therefore degradation, these

preliminary results support the recalcitrance and resistance of these molecules to biodegradation, along with the observed bioaccumulation of HMW-PAHs (Ghosal et al., 2016; Richter-Brockmann & Achten, 2018).

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTION

Charles G. Lewis: Conceptualization (supporting); Data curation (lead); Formal analysis (lead); Investigation (lead); Methodology (supporting); Writing-original draft (lead). **Melanie J. Beazley:** Conceptualization (lead); Data curation (supporting); Funding acquisition (lead); Methodology (lead); Project administration (lead); Supervision (lead); Writing-review & editing (supporting).

ETHICS STATEMENT

None required.

DATA AVAILABILITY STATEMENT

All data are publicly available through the Gulf of Mexico Research Initiative Information & Data Cooperative (GRIIDC) at https://data.gulfresearchinitiative.org (https://doi.org/10.7266/n7-0ywd-xm97; https://doi.org/10.7266/n7-2mb9-yh29; https://doi.org/10.7266/n7-618e-at55; https://doi.org/10.7266/n7-egx5-bz83; https://doi.org/10.7266/n7-t9zb-b771; https://doi.org/10.7266/n7-sq1m-n064; https://doi.org/10.7266/n7-hams-bb35). Raw reads of the amplicon sequencing data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under the study submission identification SUB6333356 (www.ncbi.nlm.nih.gov).

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