Biological effects of Cook Inlet crude oil degradation products and suspect screening of oxidized PAHs

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December 2023
Final Report
OCS Study BOEM 2023-031

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This study was funded by the University of Alaska Coastal Marine Institute and the U.S. Department of the Interior, Bureau of Ocean Energy Management Alaska OCS Region (cooperative agreement MXXAC00017). This report, OCS Study BOEM 2023-031, is available electronically from https://www.boem.gov/akpubs

Citation:

Tomco, P.L, Burkhead, J., Redman, Z.C., Robine, S.E. 2023. Biological effects of Cook Inlet crude oil degradation products and suspect screening of oxidized PAHs. Fairbanks (AK): University of Alaska Coastal Marine Institute and U.S. Department of the Interior, Bureau of Ocean Energy Management, Alaska OCS Region. 38 p. Report No.: OCS Study BOEM 2023-031. Contract No.: M21AC00017.

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LIST OF ACRONYMS

AhR	aryl hydrocarbon receptor
AI_{mod}	modified aromaticity index
ASET	Applied Science, Engineering, and Technology
ASW	artificial seawater
CALUX	chemically activated luciferase gene expression
DRO	diesel range organics
FEP	fluorinated ethylene propylene
HCA	hierarchical clustering analysis
HOPs	hydrocarbon oxidation products
KMD	Kendrick mass defect
oxyPAHs	oxidized polycyclic aromatic hydrocarbons
PCA	principal component analysis
PLSDA	partial least squares discriminant analysis
SFM	serum free media
SPE	solid phase extraction
TPH	total petroleum hydrocarbons
TPH_d	total petroleum hydrocarbons as diesel
UHPLC	ultra-high performance liquid chromatography
VIP	variable importance in the projection
WSF	water soluble fraction

ABSTRACT

Due to the nature of the Cook Inlet within south-central Alaska, natural oil seeps, oil spills from vessels, municipal and industrial permitted discharges, and recent oil and gas lease sales, there exists a need to further understand the chemical fate of spilled oil and its effects on biological life. We used non-targeted analysis of water soluble hydrocarbon oxidation products (HOPs), particularly oxidized polycyclic aromatic hydrocarbons (oxyPAHs), in combination with a luciferase bioassay to determine the composition and resulting toxicity of photooxidized crude oil. Cook Inlet crude oil was loaded onto artificial seawater and incubated under simulated sunlight for up to 14 days. Dissolved organic carbons (DOCs) were recovered from the water soluble fraction and isolated by solid phase extraction for non-targeted analysis by ultra-high performance liquid chromatography tandem quadrupole high resolution orbitrap mass spectrometry (UHPLC-HRMS) and were loaded onto rat hepatocytes transfected with an aryl hydrocarbon receptor (AhR) activated luciferase gene reporter construct. UHPLC-HRMS spectra indicated that light-treated HOP pools were more oxidized than dark samples with statistically significant HOPs (VIP score >1) containing 1–3 oxygens and being C2-3 alkylated. Cells showed increased AhR activity in the presence of light-incubated photooxidized crude oil across all 14 days of the study, while samples incubated in the dark had less AhR activity until days 10–14 at which point dark and light incubated samples were similar. Taken together, these data indicate that current targeted screening methods are limited as there is a diverse pool of HOPs that play a role in the toxicity of Cook Inlet crude oil as it is photooxidized. Since these intermediates are water soluble, they can travel large distances in the water column and affect larger areas of the ecosystem compared to parent oil. Thus, prompt clean-up of oil and dispersal of HOPs should be considered to determine the appropriate response were an oil spill to occur in the Cook Inlet.

INTRODUCTION

Background

With the recent Cook Inlet oil and gas lease sales 244 and 258, oil and gas exploration or development could increase drilling activity and vessel traffic. Oil spills have the potential to occur from oil and gas activities. As water soluble components in spilled oil dissolve into seawater, the bioavailability to marine aquatic life increases, creating a potential for bioaccumulation and bioconcentration of petroleum-derived residues. Since dissolution is a dominant pathway that poses substantial risks to ecosystem and human health, there exists a need to further understand the chemical fate of spilled oil and its effects on biological life in the Cook Inlet. While oil weathering model predictions for marine diesel spills result in 0% oil remaining after 30 days and crude oil spill predictions result in 24% oil remaining after 30 days during summer months (BOEM, 2016), the dissolved hydrocarbon oxidation products (HOPs) produced due to the weathering of crude oil and diesel remain overlooked . The Cook Inlet is particularly critical to investigate because it supports commercial and subsistence fisheries as well as seabird and marine mammal populations, including the critically endangered Cook Inlet population of beluga whales (Spies, 2006; Goetz et al., 2012). Due to its length, strong currents and large tidal changes, nutrients necessary for a rich ecosystem can rapidly disseminate across the Cook Inlet. For these same reasons, an oil spill in the summer could result in rapid dispersal of HOPs and pose a potential threat to marine life (Spies, 2006; Hood and Zimmerman, 1987; Royer and Grosch, 2006; Weingartner et al., 2002; Whitney, 2002).

HOPs are a class of chemical compounds that are formed as fuels derived from petroleum weather in the environment (Aeppli, 2012; Figure 1). Photochemical degradation processes produce HOPs in oxic environments by reacting with aromatic compounds (e.g. naphthalene, phenanthrene, anthracene, etc.) that absorb light in the solar spectrum; microbes can also produce HOPs through both aerobic and anaerobic biotransformation processes (Hazen et al., 2016; Townsend et al., 2003; Amos, 2012; McFarlin, 2014). Both of these pathways result in the production of an entire range of compounds that are chemically distinct from background seawater and have higher water solubility and bioavailability than parent oil (Bekins et al., 2020; Essaid et al., 2011; Zito et al., 2019a). Examples of these compounds include polar components such as ketones, aldehydes, and alcohols associated with polycyclic aromatic hydrocarbons (PAHs). These compounds are readily water soluble, may further degrade into potentially toxic compounds and can diffuse large distances in the water column, thus affecting a much larger area than predicted for parent oil (D'Auria et al., 2008; Garrett et al., 1998). Once mobilized as a result of dissolution, HOPs can rapidly diffuse both vertically and laterally in the water column, potentially traveling undetected vast distances ahead of any signs of a visible oil front. This mobility increases bioavailability and impacts both marine and freshwater ecosystems. HOPs represent a "regulatory blind spot" as they fall outside the window of many standardized "compliance-based" analytical techniques that are used for measuring total petroleum hydrocarbons (TPH). Quantification of total petroleum hydrocarbons (TPH) as diesel (TPH_d), diesel-range organics (DRO), and/or PAHs in water is performed by solvent extraction with dichloromethane followed by gas chromatography (USEPA, 1996a, 1996b and 2000). This method is used by regulators to monitor petroleum-contaminated waters and make regulatory decisions. However, neither of the aforementioned methods are specific for HOPs; rather, the extraction method only captures the relatively non-polar analytes and the boiling point

of these analytes must be between 170-430°C (Zemo et al., 2013). Recent studies (Mohler et al., 2020; Zito et al., 2019b) have shown that this "analytical window" misses the more-polar oxidized fraction of water soluble petroleum, which is what we have assessed in this project.

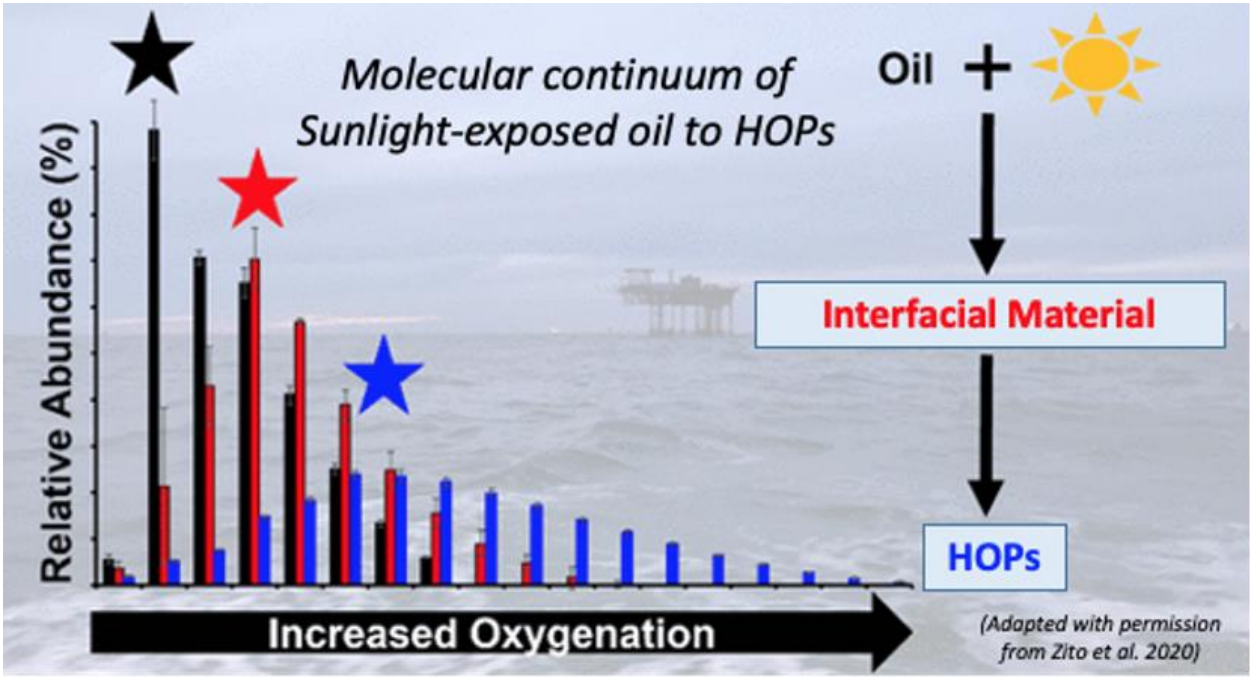

Figure 1. Schematic representation of HOP production (Zito et al., 2020), stars mark the oxygenation level with the greatest relative abundance among oil (black), colloidal interfacial material (red), and HOPs (blue).

Photo-enhanced oxidation of PAHs particularly poses a potential biological threat due to the solubility and toxicity of the resulting compounds (Barron, 2017; Barron and Ka'aihue, 2001; Lampi et al., 2006; Knecht et al., 2013; Figure 2). In more northern latitudes, such as the Cook Inlet, exposure time to sunlight is much greater in the summer months (up to 16 hours per day) which leads to the accelerated formation of photooxidized PAHs (oxyPAHs; Harsha, 2023). In other more temperate regions, such as the Gulf of Mexico region, this fate process is becoming a factor in the decision-making process regarding the deployment of oil spill response chemicals that aim to mitigate ecosystem damage, including deploying dispersants and surface cleaning agents, conducting *in-situ* burning, or collecting oil with chemical herder prior to *in-situ* burning, because oxidized oil may not be as easily recovered as fresh oil (Ward et al., 2018). The extreme tidal fluctuations in the Cook Inlet would cause rapid movement of spilled oil and create a challenging scenario for response logistics, so responses should include careful consideration of the hydrocarbon characteristics, environmental factors, and remediation tools available. Unfortunately, very little information specific to the Cook Inlet region on the chemical character of oxidized residues and to what extent these degradation intermediates have observable biological effects is known. Therefore, our project seeks to address BOEM-relevant needs to better understand their effects on biological systems and enable future efforts to determine their fate, transport, and monitoring.

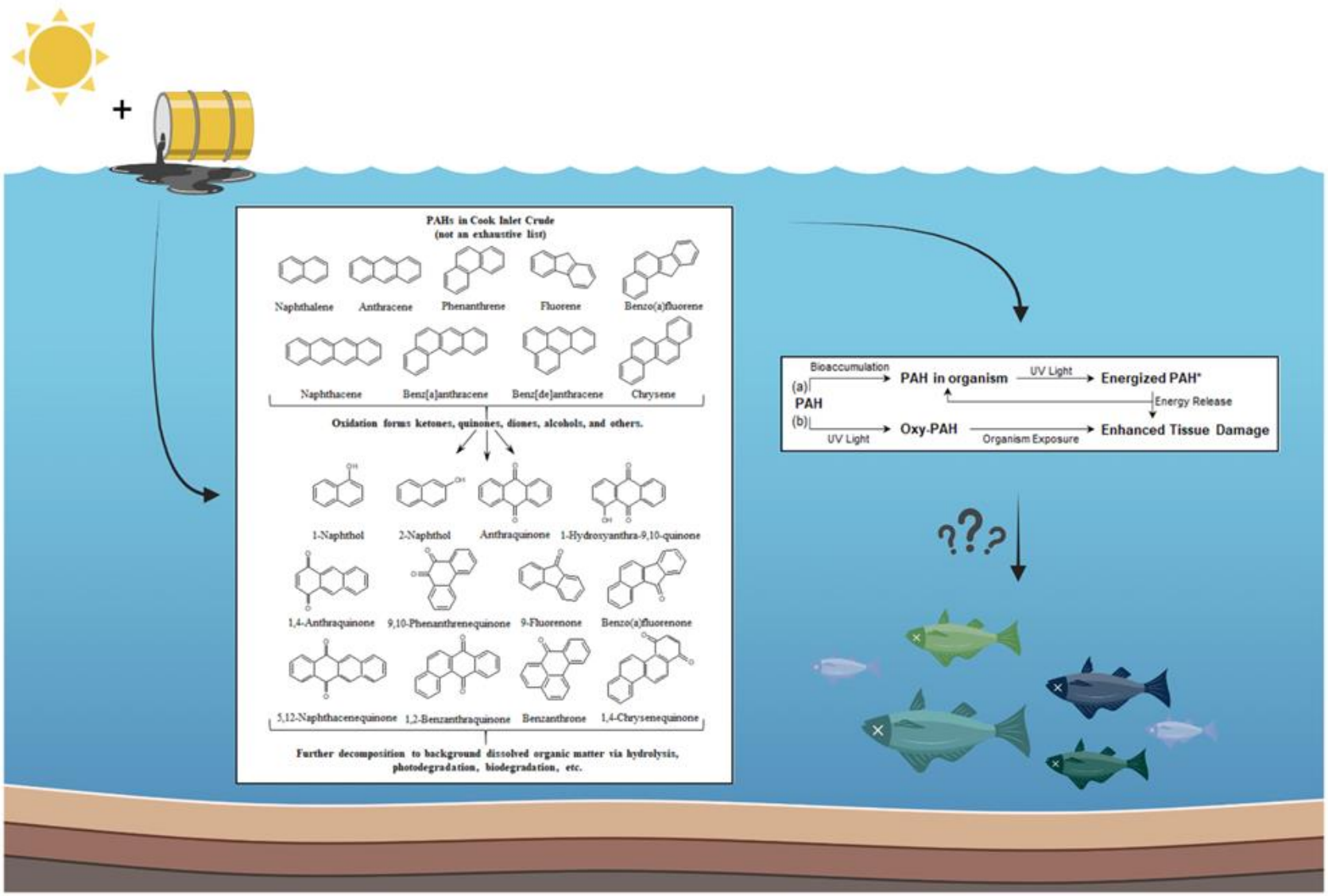

Figure 2. Schematic representation of oxyPAH production and potential biological effects through both (a) photosensitization and (b) photomodification.

Production of these degradation intermediates via photooxidation occurs when solar radiation, particularly ultraviolet (UV) light, excites chromophores in the oil to initiate a series of reactions that result in more polar and water soluble oxidized products. PAHs are particularly sensitive to the process of photooxidation and have been shown to be genotoxic when present in a mixture of both parent and oxidized compounds (McCarrick et al., 2019). Toxicity could also rise due to bioaccumulation and photosensitization of PAHs in translucent tissue *in vivo* (Ankley et al., 1994; Figure 2a). In fact, oxyPAHs have caused mortality in aquatic organisms and induced malformations and oxidative stress in fish embryos (Barron et al., 2017; Lampi et al., 2006; Knecht et al., 2013). Due to the demonstrated negative effects and the seasonally high potential for the formation of oxyPAHs in the Cook Inlet in the event of a summertime oil spill, the importance of the formation rates, persistence, and biological effects of this class of compounds is especially relevant to assess for ecosystem health (Harsha et al., 2023). While the majority of recent studies focus on photosensitization (initial hydrocarbon bioaccumulation, followed by UV activation of residues in translucent tissue), the pathway of photomodification (exposure to the oxidized hydrocarbons formed upon exposure of oil to sunlight) is well-established (Barron, 2017; Figure 2b) and the occurrence and persistence of these compounds is relevant to identifying appropriate post-spill monitoring plans. Since mixtures containing both parent and oxidized PAHs have been shown to exhibit higher genotoxicity relative to their binary components (McCarrick et al., 2019), synergistic toxicological response to multiple contaminant stressors is feasible. This study focuses on photomodification of Cook Inlet crude oil components by oxidizing oil with simulated sunlight for up to 14 days, detecting and characterizing HOPs and quantifying the cytotoxicity of the compounds using a reporter bioassay.

To assess the chemical structures of HOPs and the production of oxyPAHs by non-target screening, we used an emerging approach of tandem quadrupole orbitrap mass spectrometry with data-dependent ion fragmentation (MS/MS or MS^2) (Avagyan and Westerholm, 2016 and 2017). In contrast, recent methods of petroleum fingerprinting to characterize HOPs in aquatic systems identify “chemical features” (e.g., condensed aromatic, aliphatic, polyphenolic, etc.) and molecular formula rather than specific compounds and associated isomers (Bianchi et al., 2014; Zhou et al., 2013; Dvorski et al., 2016; Mirnaghi et al., 2019). In MS^2, complex chemical mixtures are separated with ultra-high performance liquid chromatography prior to the acquisition of full-scan precursor ion spectra via high resolution mass spectrometry (HRMS). During acquisition, real-time data analysis “triggers" simultaneous collection of MS^2 fragmentation spectra using user-established ion abundance thresholds. This data-dependent acquisition allows for comparison of both full-scan precursor and MS^2 fragmentation ion spectra to identify compounds. This powerful approach is complementary to other HRMS methods such as ion cyclotron resonance (ICR) and can provide insight into the chemical structures present in HOPs that may warrant inclusion into target screening lists for future monitoring efforts. To be clear, this method does not attempt to quantify the compounds present, which would require analytical-grade reference standards, but rather to detect and identify. By investigating the specific chemical structures formed when Cook Inlet crude oil is exposed to Southcentral Alaska-relevant doses of sunlight during summer, we are able to leverage and extend our current targeted screening approaches using available reference standards and lead to further evaluation of new targeted quantitative methods (Avagyan and Westerholm, 2017).

Bioassays have recently been developed as tools to analyze toxic contaminants in environmental samples. Cell-based assays have been used as screening tools for dioxin-like compounds (DLCs) as well as other contaminant classes such as halogenated aromatic hydrocarbons (HAHs) and PAHs (Pieterse et al., 2013; Largot et al., 2018). The bioassay used in this study is the Aryl hydrocarbon Receptor (AhR)

third-generation Chemically Activated LUciferase gene eXpression (CALUX) assay (Figure 3). This cell-based assay has been established as a reliable system for quantifying AhR activation *in vitro* by a wide range of AhR agonists (Pierterse et al., 2013; Brennan et al., 2015; Brennan et al., 2018; Baston and Denison, 2011; Bekins et al., 2020). AhR is a ligand-activated transcription factor (TF) that has wide ranging roles in activating genes related to xenobiotic metabolism (i.e. detoxification), cell proliferation, adhesion and motility, as well as numerous physiological functions (Largot et al., 2018). These target genes include cytochrome P450 family 1 subfamily A member 1 (CYP1A1) and cytochrome P450 family 1 subfamily B member 1 (CYP1B1) (Go et al., 2015), which have downstream toxicological effects. Well-described exogenous activators (DLCs, HAHs, PAHs) have been used to optimize cell lines and detection limits. However, the diversity of activating chemicals from environmental or other sources that function as agonists makes AhR activity appealing as a broad toxicity screening tool. Reporter gene methods that link AhR transactivation activity to production of a fluorescent or luciferase protein in mammalian cultured cells allow for high throughput screening (HTS) of dioxin-like halogenated compounds and PAHs (Figure 3). These assays can be performed in microplate formats (up to 384 wells/plate) and allow for fast screening with relatively simple instrumentation (Brennan and Tillit, 2018). The scalability of bioassays presents an opportunity for detection of a wide range of both known and unknown AhR-activating compounds in samples with diverse matrices that are applicable to a wide range of aquatic life. These HTS bioassay methods surpass instrumental methods in screening capacity for large numbers of samples and allow a first pass to preserve instrument time for validation assays.

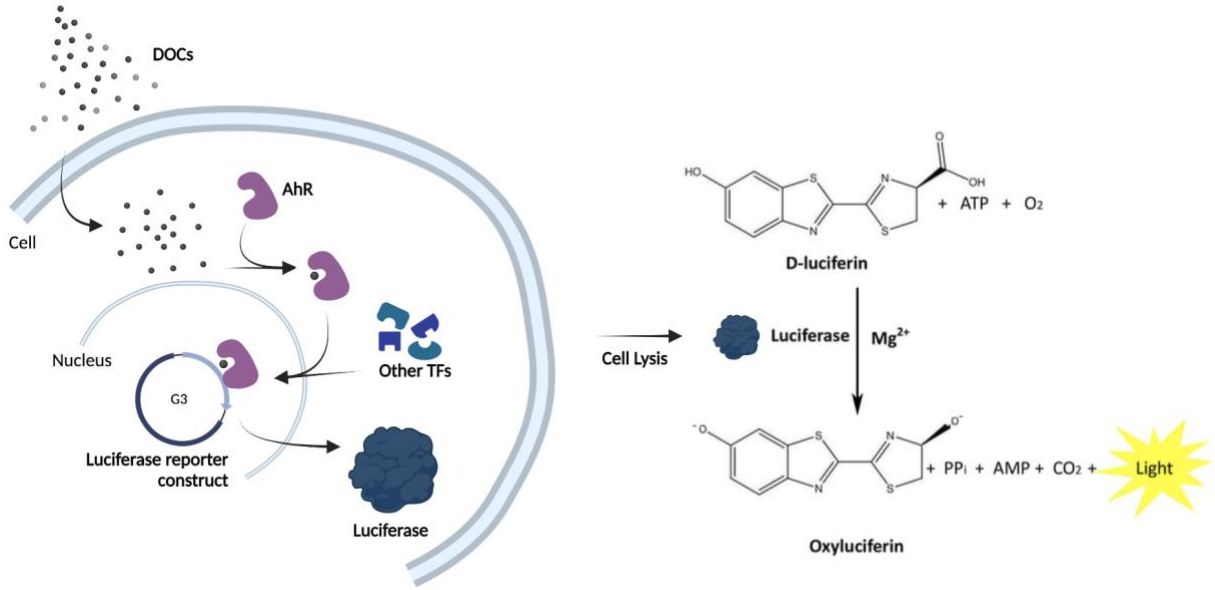

Figure 3. Schematic representation of the AhR CALUX bioassay.

The AhR CALUX bioassay used in this study allows for use of rat or human cell lines that are stably transfected with a tandem AhR reporter system driving luciferase gene expression. The cell lines were a gift from Dr. Michael S. Denison (UC Davis). These cells have a limit of detection of 0.1 pM for 2.3.7.8-tetrachlorodibenzo-*p*-dioxin (TCDD) and about 10 nM for β-Naphthoflavone. They detect a range of related chemicals in environmental extracts, including HOPs (Brennan et al., 2015; Bekins et al., 2020). We implemented this assay to identify and characterize AhR agonists in our experimental samples

of artificial seawater (ASW) exposed to Cook Inlet crude oil and sunlight. This approach allowed for the integration of multiple compounds and the detection of toxins that were not previously described or may not have been defined by LC- or GC-MS assays. Thus, it serves as a good system for testing the cytotoxicity of HOPs, PAHs, and their degradation products produced by photooxidation over the 14-day course of this study.

To investigate the fate of photooxidized Cook Inlet crude oil and its biological effects, we used the MS^2 technique to characterize both polar and non-polar oxyPAHs and measure their potential toxicity using the AhR CALUX bioassay. This approach is particularly relevant when considering the variability introduced by local environmental properties and region-specific oil types in developing novel methods to assess toxicity. By combining non-target screening of oxyPAHs and determining their AhR activity, this data can assist the BOEM in determining the fate and potential environmental impact of water soluble petroleum products following an oil spill.

Objectives and Hypotheses

The overall goal of the project is to establish data to correlate a) the weathering patterns of Cook Inlet crude oil when spilled in the marine environment to b) toxicological impacts that may be indicative of potential effects to both marine aquatic life and humans. The specific objectives of this project focus on suspect screening and toxic effects as revealed from cell bioassays:

Objective 1: Conduct non-target suspect screening of oxyPAH residues in HOPs from water-solubilized Cook Inlet crude oil during progressive photooxidation.

Objective 2: Define the relative Aryl hydrocarbon receptor (AhR) activation of water-solubilized Cook Inlet crude oil during progressive photooxidation.

We *hypothesize* the following:

Hypothesis 1: With light exposure, oxyPAHs will accumulate with predominantly carbonyl-containing PAHs at earlier time points followed by a progressive increase in oxygenation levels as the duration of sunlight exposure increases.

Hypothesis 2: With increased duration of light exposure, AhR activity in response to crude oil HOPs will increase followed by a decrease in activity as toxic oxyPAHs are further photooxidized to non-toxic compounds.

METHODS

Laboratory Simulated Oil Spill

Cook Inlet crude oil was obtained from Blue Crest Energy (Anchor Point, AK) in July 2020. All glassware was acid-washed and combusted at 500°C for 5 hours. Water used to make artificial seawater (36.86 g/L, ASW) from Instant Ocean® (Blacksburg, VA) for experiments and for all chemical analyses was filtered to 18.2 MΩ·cm. Films of crude oil at a load of 10 mg/L, previously determined to produce a thin film (~100 μm), were added over ASW in thermostatically controlled 100 mL jacketed beakers (Chemglass USA), similar to described previously (Zito et al., 2019b; Figure 4). Jacketed beakers were

placed, 12 samples at a time, in an Atlas Suntest XLS+, which contains a xenon-arc lamp and previously documented by our group to mimic the natural solar spectrum of Southcentral Alaska (Redman et al., 2021). Solar irradiance was programmed at 250 W/m^2, equivalent to the peak mean monthly radiation of Southcentral Alaska summers (Dissing and Wendler, 1998). The seawater temperature in each jacketed beaker was thermostatically controlled at 12°C (typical of Cook Inlet summers) and represents a single time period of 1, 4, 7, 10, or 14 days. Beakers were covered with quartz lids to allow for light transmittance and secured with teflon tape to reduce evaporation. A UV-transparent fluorinated ethylene propylene (FEP) liner (Welch fluoropolymers) was placed in each beaker to minimize adhesion of aromatic residues to each reaction flask (Krüger et al., 2014). Dark controls with the same thermostatically-controlled flasks were prepared identically and incubated in an aluminum foil-wrapped box enclosure free of light. All treatments (light and dark at each time point) were conducted in triplicate. After incubation, each sample was sacrificed and the FEP liner containing the sample was removed from the jacketed flask. The water soluble fraction (WSF) was collected by separating undissolved Cook Inlet crude oil from WSF by cutting a slit in the base and allowing the WSF to drain into a 125-mL acid-washed HDPE bottle. Samples were stored at -20°C in the dark until analyzed.

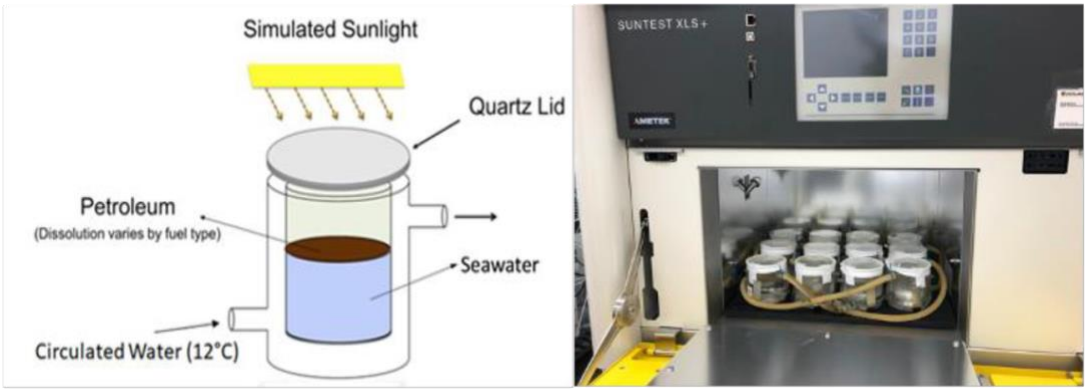

Figure 4. Experimental set-up of jacketed reaction beakers in Atlas Suntest XLS+.

Solid Phase Extraction of HOPs

HOPs were extracted from the aqueous samples using established methods for solid phase extraction (SPE) of organic matter from seawater, both natural and petroleum-derived (Zito et al., 2019b; Dittmar et al., 2008), consistent with our concurrent efforts with other petroleum studies using Agilent PPL® cartridges. To determine the volume of sample to be loaded onto the cartridges for recovery of consistent concentrations of HOPs across samples, non-purgeable organic carbon (NPOC) analysis in mg/L was conducted courtesy of Maxwell Harsha (University of New Orleans). For SPE, cartridges were pre-washed with methanol, then equilibrated with one column of water, one column of LC-MS grade methanol and one column of acidified water (0.01N HCl) prior to loading the acidified samples (pH 1.8-2.2, ~0.1N HCl) at the appropriate volume to achieve a final concentration of 1000 ppm DOC. If there was insufficient sample volume to reach 1000 ppm, the entirety of the sample was loaded onto the cartridge. Cartridges were rinsed with three column volumes of acidified water (0.01N HCl) to remove salts then eluted in two fractions: 3 mL of methanol to extract polar HOPs and 15 mL of 9:1

chloroform:tetrahydrofuran to extract non-polar HOPs. Samples were then dried under nitrogen and eluted in 100 uL of methanol and transferred to autosampler vials with 200 uL glass inserts for analysis by ultra-high performance liquid chromatography tandem quadrupole high resolution orbitrap mass spectrometry (UHPLC-HRMS). This method was previously shown to have good affinity for photochemically derived oxidized petroleum residues compared to liquid extraction methods (Zito et al., 2019a; Zito et al., 2019b). To prepare samples for the AhR CALUX bioassay, samples were dried under nitrogen and reconstituted in the methanol (final concentration 1000 ppm DOC.) Samples were stored at -20°C before use in the bioassay.

Non-target UHPLC-Orbitrap Mass Spectrometry Analysis

Ultra-High Performance Liquid Chromatography (UHPLC) with high resolution orbitrap mass spectrometry analysis of solid-phase extracted HOPs was performed at the University of Alaska Anchorage ASET lab using a Thermo Vanquish UHPLC system coupled to an Orbitrap Exploris 120. Briefly, 5 μL of extract was injected onto a Phenomenex Kinetix C18 column (150 x 2.1mm; 1.7μm) and eluted using a gradient of acetonitrile and water with 0.1% formic acid. Eluted compounds were ionized with positive mode atmospheric pressure chemical ionization, previously shown to effectively ionize oxyPAHs (Gross and Letzel, 2007), for detection via high resolution orbitrap mass spectrometry (full scan 120-800 *m/z*, 120,000 resolution). Data-dependent acquisition (DDA) of product ion spectra (30,000 resolution) were collected for oxidized PAHs in the in-house library and the top three most abundant precursor ions using step normalized collision energies of 40, 60, and 100 eV. Raw data files were processed using Compound Discoverer (Thermo Fisher Scientific), including chromatogram alignment, compounds detection and grouping, background removal, chemical formula assignment, and in-house library comparisons. The in-house library was developed from a mixture of thirteen available standards to provide retention times and mass spectra for oxidized PAHs of varying ring abundance and configuration, oxidation level, and functional group (carbonyl and alcohol). This simultaneously detected a range of targeted compounds and established a retention index to aid the identification of other compounds during suspect screening. Following initial assignments, detected UHPLC peaks were further filtered to ensure only compounds with high peak rating (>7 in at least two replicates) and mass accuracy (±1 ppm) paired with a product ion spectrum containing neutral mass losses indicative of oxidized functional groups (alcohols, carbonyls, and carboxylic acids). Double bond equivalents (DBE), H/C, and O/C ratios were calculated from molecular formulae to monitor the overall aromaticity and oxidation level of extracted Cook Inlet crude oil dissolved residues.

Data were log transformed and mean-centered prior to statistical analysis using Metaboanalyst v5.0. Statistical methods included hierarchical clustering analysis (HCA), principal components analysis (PCA), and partial least squares discriminant analysis (PLSDA).

AhR CALUX Bioassay

An initial pilot experiment was conducted in the spring of 2022 using archived samples from Harsha et al., 2023, to determine effects of photo-oxidized Cook Inlet crude oil compared to diesel on AhR activation (Appendix I). HepG27.5 and H4IIe7.5 cells seeded to 96-well tissue culture plates were treated with a concentration gradient of polar DOC (0.001, 0.01, 0.1, 1, 5, 10, 15, 20 and 30 ppm) to determine minimal and maximal AhR activation by HOPs from Cook Inlet crude oil/diesel photooxidation. The concentrations selected were in accordance with previous work and because cells

began to die at DOC concentrations above 30 ppm (Bekins et al., 2020). Samples used for the pilot experiments were light-treated Cook Inlet crude oil and diesel at 4, 7 and 10 days of exposure.

Using the information gained from the pilot data, we repeated the bioassay experiment in the spring of 2023 using samples from light- and dark-treated Cook Inlet crude oil after 1, 4, 7, 10 and 14 days of exposure. H4IIe7.5 cells were treated at 10 ppm DOC, which was the most suitable concentration for demonstrating differences in AhR activation between samples according to our pilot data.

Third generation stably transfected pGudLuc7.5 (an AhR-responsive luciferase reporter plasmid) rat (H4IIe7.5) and human (HepG27.5) hepatoma cell lines were generously donated by Dr. Michael S. Denison (University of California, Davis). Cells were grown in α-MEM and 400 mg/L G418 before being seeded in non-selective media in white 96-well plates at $7.5x10^4$ cells/well (for H4IIe7.5's) or $1.0x10^5$ cells/well (for HepG27.5's). Cells were grown to 100% confluence over 24-hours for H4IIe7.5's and 72-hours for HepG27.5's, then treated with the appropriate concentration of polar DOC extracts from either parent oil or diesel (for pilot data) stored at 1000 ppm in methanol. DOCs were diluted in serum-free media (SFM) for treatment. With each set of plates, a β-Naphthoflavone standard curve (Figure 5), method control (i.e. artificial seawater ran through the extraction process), solvent control (i.e. MeOH or DMSO only in SFM), no treatment control (i.e. SFM only) and blank control (i.e. no cells) were also incorporated to ensure bioluminescence was caused by DOC samples inducing AhR activity in the cells. Cells were incubated with treatments for 4h at 37°C, 5% CO_2 to maximize AhR activity (Bekins et al., 2020). After incubation, cells were lysed and luciferase activity was measured as described previously, but by hand pipetting using a multichannel pipet rather than an auto-injector for pilot data (Brennan and Tillit, 2018). An auto-injector was installed and used for the bioassay data collected in spring 2023. Luciferase activity of lysates were normalized to protein concentrations of each well using the Pierce Rapid Gold BCA Protein Assay Kit. Luciferase activity per µg protein was then converted to percentage of the maximal induction by β-Naphthoflavone (% max induction).

Light treatments were run in triplicate and each resulting extract was run in triplicate for the AhR CALUX bioassay (n = 9). Data processing for the AhR CALUX bioassay was performed in Excel. R Studio was used for the construction of protein standard curves. Statistical analysis using one-way ANOVA and Tukey's HSD for pairwise comparison was performed in JMP 16. Plots were constructed using JMP 16, Microsoft Excel and PowerPoint.

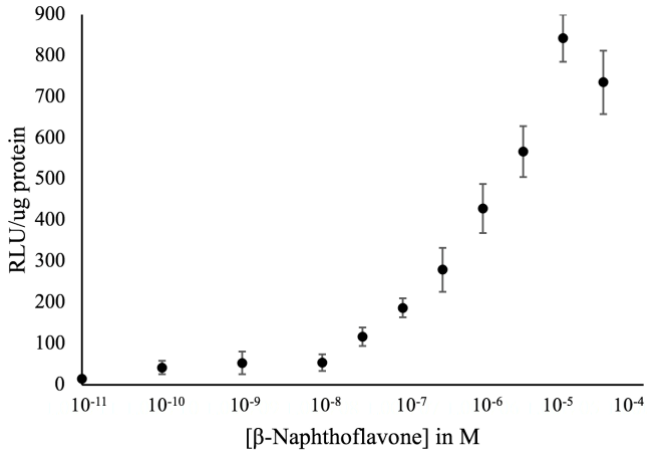

Figure 5. β-Naphthoflavone standard curve on rat hepatocytes (H4IIe7.5 cells). Error bars represent ± one standard deviation.

RESULTS AND DISCUSSION

Characterization of oxyPAHs in Photooxidized Cook Inlet Crude Oil

An increase in total DOC was observed over the 14-day duration of the experiments in light exposed samples, indicative of the photoproduction of HOPs from Cook Inlet crude oil (Figure 6A). Overall, 2,633 chemical features were detected: primarily consisting of condensed aromatic hydrocarbons (18%), aromatic hydrocarbons (33%), and unsaturated low oxygen containing hydrocarbons (48%). A minor portion of aliphatic compounds were detected (1%); however, this class of compounds was intentionally filtered from the data set in order to focus our screening efforts on oxidized aromatic compounds. Light exposed samples contained a greater abundance of more oxidized compounds than those incubated in the dark at every time period, further confirming the photoproduction of unique aromatic and unsaturated HOPs from Cook Inlet crude oil (Figure 6B). Finally, 9-fluorenone, anthraquinone, 1,4-anthraquinone, and 1-hydroxy-9,10-anthraquinone were positively identified against the in-house library (minimum of two replicates) in the light exposed samples. These findings corroborate our previously reported characterization of Cook Inlet crude oil in which three-ring oxidized PAHs (anthraquinone and phenanthrenequinone) were positively identified via triple-quadrupole mass spectrometry while non-targeted analysis of HOPs showed the majority of chemical features unique to light exposed samples were oxidized condensed aromatics, aromatics, and unsaturated hydrocarbons (Harsha et al., 2023).

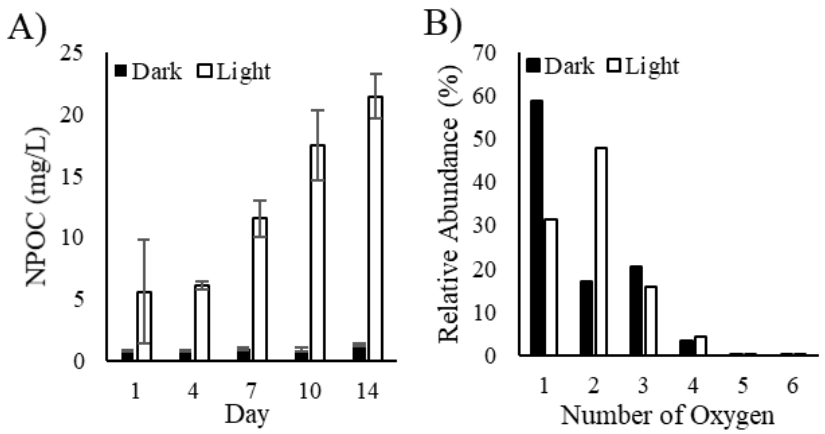

Figure 6. Changes in DOC quantity and oxygenation. (**A**) Concentration of dissolved Non-Purgeable Organic Carbon (NPOC) with increasing oxidation time for both light and dark treated samples. (**B**) Number of oxygen in HOP formulae identified in samples incubated in the light and dark for all time periods.

Our previous targeted analysis (Harsha, 2023) showed methylated PAHs to be in high abundance, indicating that the targeted analysis of oxidized PAHs was limited by both the availability of analytical standards and the inherent complexity of the HOP mixture formed during the photooxidation of Cook Inlet crude oil. To investigate the presence of alkylated congeners further, Kendrick mass defects (KMD) were calculated in order to identify homologous series of compounds differentiated by CH_2 and calculate the relative abundances of non-alkylated (C0) and potentially alkylated aromatic hydrocarbons (C1-C10)

in light and dark samples (Figure 7). Initial KMD analysis shows that alkylated formulae, in particular C_3H_6 (C3), are in much greater abundance than predicted non-alkylated formulae (C0) in both light exposed samples and dark controls. Follow-on principal components analysis (PCA, Figure 8A), partial least squares discriminant analysis (PLSDA, Figure 8B), and hierarchical clustering analysis (HCA, Figure 9) showed clear separation of both polar and non-polar fractions of the light exposed and dark controlled samples. From these analyses we see that light exposed samples rapidly become chemically distinct from the dark controls with time, in particular we observe that the variability between groups is primarily due to the increased abundance of Kendrick series containing one to three oxygens within the polar fraction of sample extracts (Figure 8C & 8D) that become present within 1 day of light exposure. This is consistent with the hypothesis that aromatic components of crude oil are oxidized to more polar, and therefore more water soluble, and potentially bioavailable, products when weathered by sunlight.

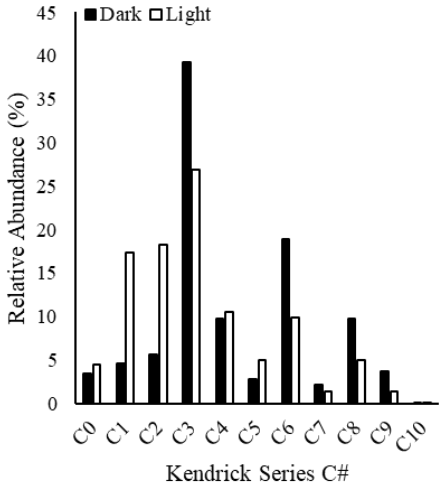

Figure 7. Relative abundance of non-alkylated (C0) and alkylated (C1-C10) hydrocarbon oxidation products in light and dark incubated samples.

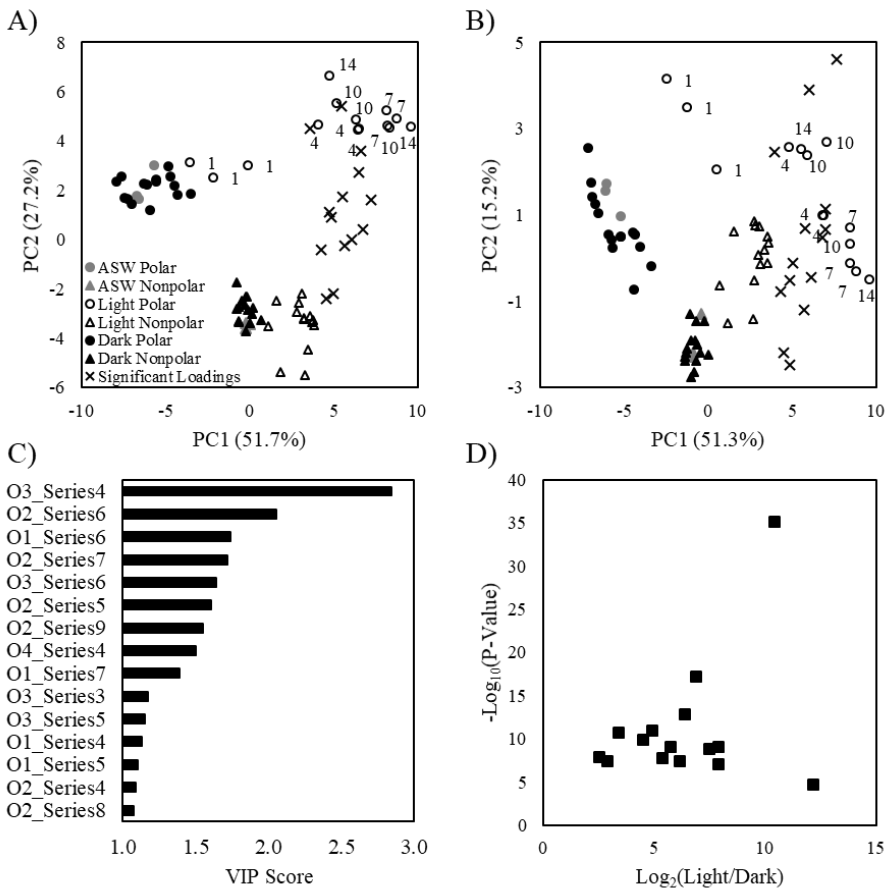

Figure 8. (**A**) PCA and (**B**) PLSDA biplots show clear separation of light (open symbols) and dark (filled symbols) incubated samples, as well as polar (circle) and nonpolar (triangle) extracts, based upon loadings from identified Kendrick series (x's). Artificial seawater controls (ASW, gray symbols) group with samples incubated with crude oil in the dark. Numerical labels for polar extracts of light exposed samples (open circles) indicate incubation time (days). (**C**) Variable importance in the projection (VIP) scores for the significant (VIP score > 1) Kendrick series and (**D**) volcano plot show that HOP series responsible for the variation between samples are primarily composed of photooxidation products containing 1-3 oxygen.

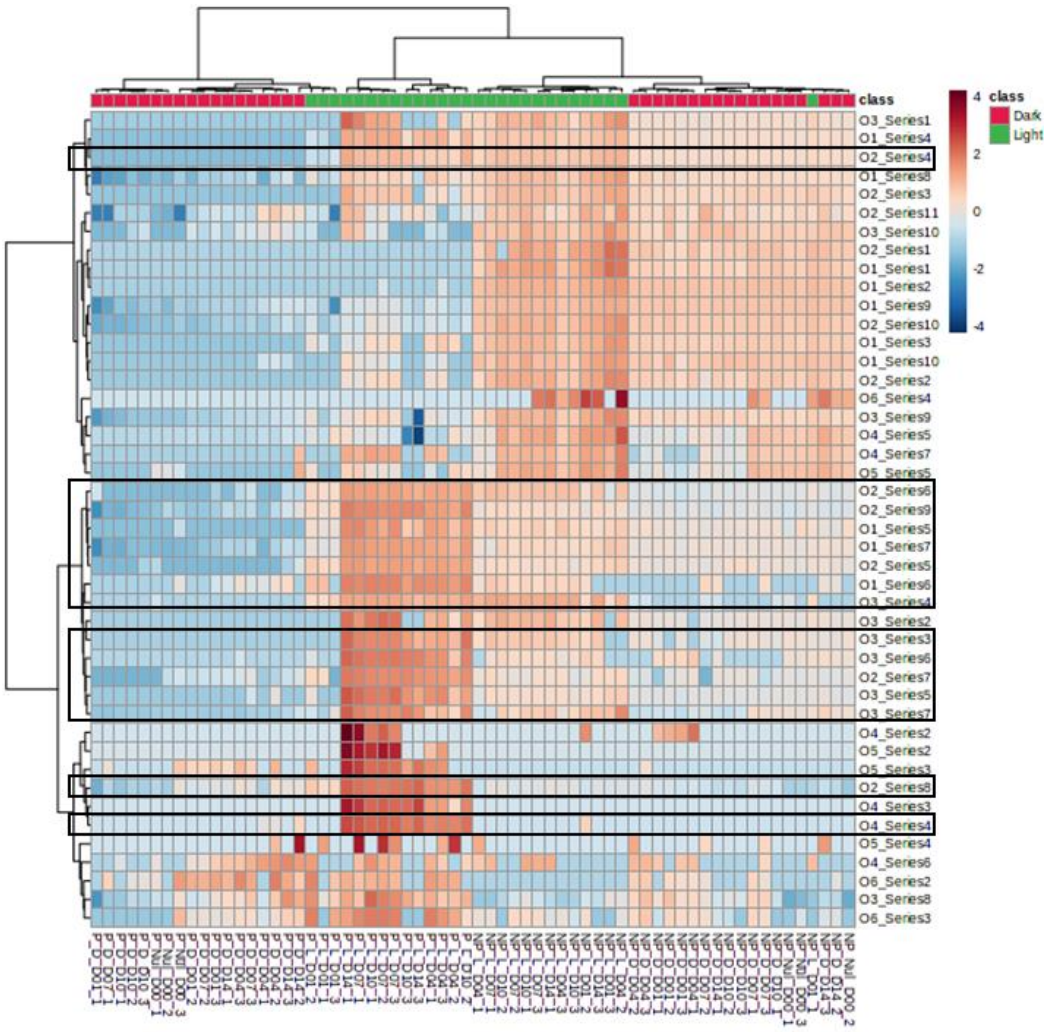

Figure 9. HCA shows clustering of samples based upon extract polarity (e.g. polar-P vs nonpolar-NP), light exposure (e.g. light-L vs dark-D), and illumination time (e.g. day 1-D01 vs day 10-D10); artificial seawater controls are labeled Nul_D00. Boxes highlight Kendrick series with VIP scores greater than 1. Significant Kendrick series contain features characteristic of HOP photochemical production containing one to three oxygen that are more prevalent in the polar extracts of light exposed samples.

Statistical analyses were repeated to include all 2,633 compounds, while the trends remain unchanged (Figure 10A) we observe that 79% (201/255) of individual compounds with a PLSDA VIP (Variable Importance in the Projection) score greater than or equal to one fall within a Kendrick series with the top VIP scores. The majority of significant compounds (VIP score > 1) were prevalent in light exposed samples (Figure 10B), contained two oxygen (76% vs 48% relative abundance in the top VIP scores and all 2,633 compounds, respectively; Figure 10C), and were normally distributed around Kendrick group C2 (Wilks-Shapiro, W=0.935) while the darks typically contained very low abundance compounds in the highly alkylated C8 and C9 Kendrick groups (Figure 10D).

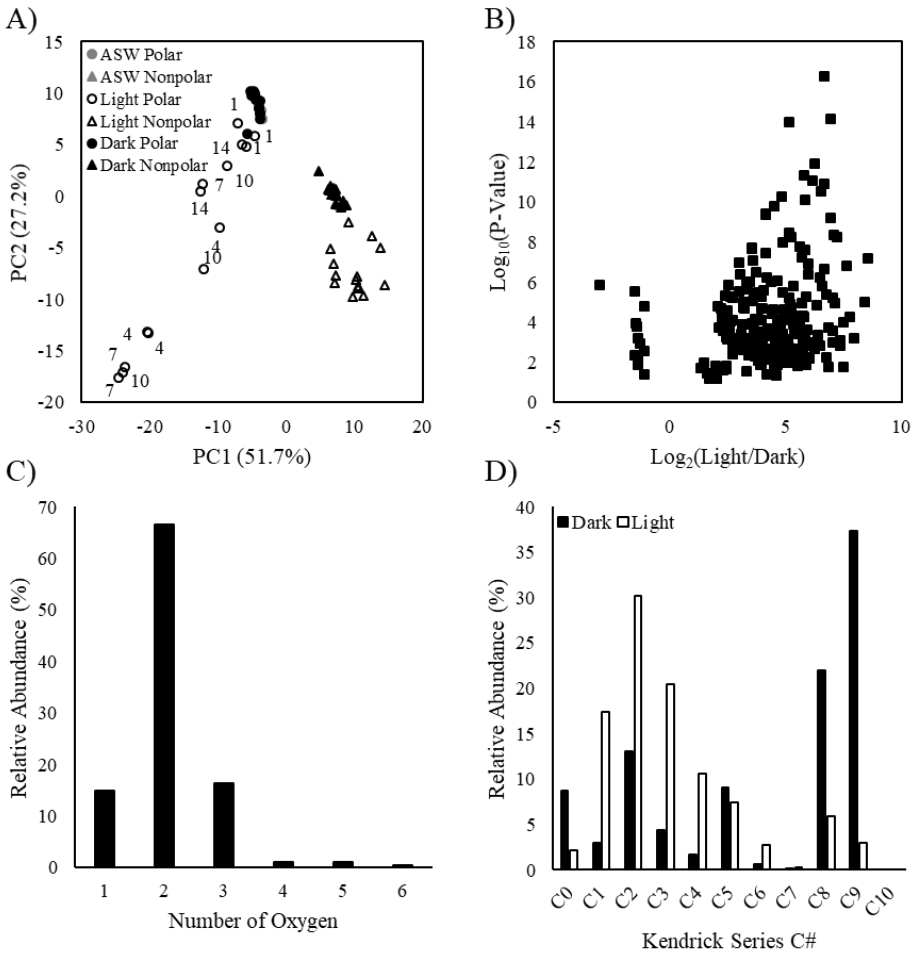

Figure 10. (**A**) PLSDA scores plot for samples incorporating loadings from all 2,633 detected compounds shows similar separation of light (open symbol) and dark (closed symbol) incubated samples, as well as polar (circle) and nonpolar (triangle) extracts. Artificial seawater controls (ASW, gray symbols) group with samples incubated in the dark. Numerical labels for polar extracts of light exposed samples (open circles) indicate incubation time (days). (**B**) Volcano plot of significant HOPs (VIP score > 1) shows the majority are more abundant in light exposed samples. (**C**) Relative abundance of significant HOP formulae containing one to six oxygen. (**D**) Total relative abundance of non-alkylated (C0) and alkylated (C1-C10) congeners of significant HOPs in light and dark incubated samples.

The significant chemical compounds (VIP score >1), identified primarily in the light exposed samples, were consistent with the photochemical oxidation products of two to four ring PAHS and their alkylated derivatives (Figure 11) (Fan et al., 2022; Nguyen et al., 2020; Luo et al., 2021). Among the most significant compounds, 51% (130/255) belonged to two O2 Kendrick series with base formula C9H6O2 (O2 Series 8, n=73) and C8H6O2 (O2 Series 9, n=54) with the majority of features corresponding to Kendrick groups C0-C3 and C1-C3, respectively (Figure 12A). The prevalence of formulae in these series are consistent with the anticipated formation of small, oxidized ring opening products (Figure 11 Boxes 19, 20, and 23) with a high degree of alkylated isomers (Figure 13) (Luo et al., 2021). In light exposed samples, the abundance of significant HOPs (VIP score > 1) peaked at ten days in the polar extracts (Figure 12B). After ten days, production of HOPs from crude oil likely diminishes due

to a combination of limited remnant unweathered parent petroleum compounds and further degradation of HOPs to smaller products (Figure 11 Box 45, further degradation structures not shown), as evidenced by the continuous increase in total dissolved organic carbon (Figure 6A).

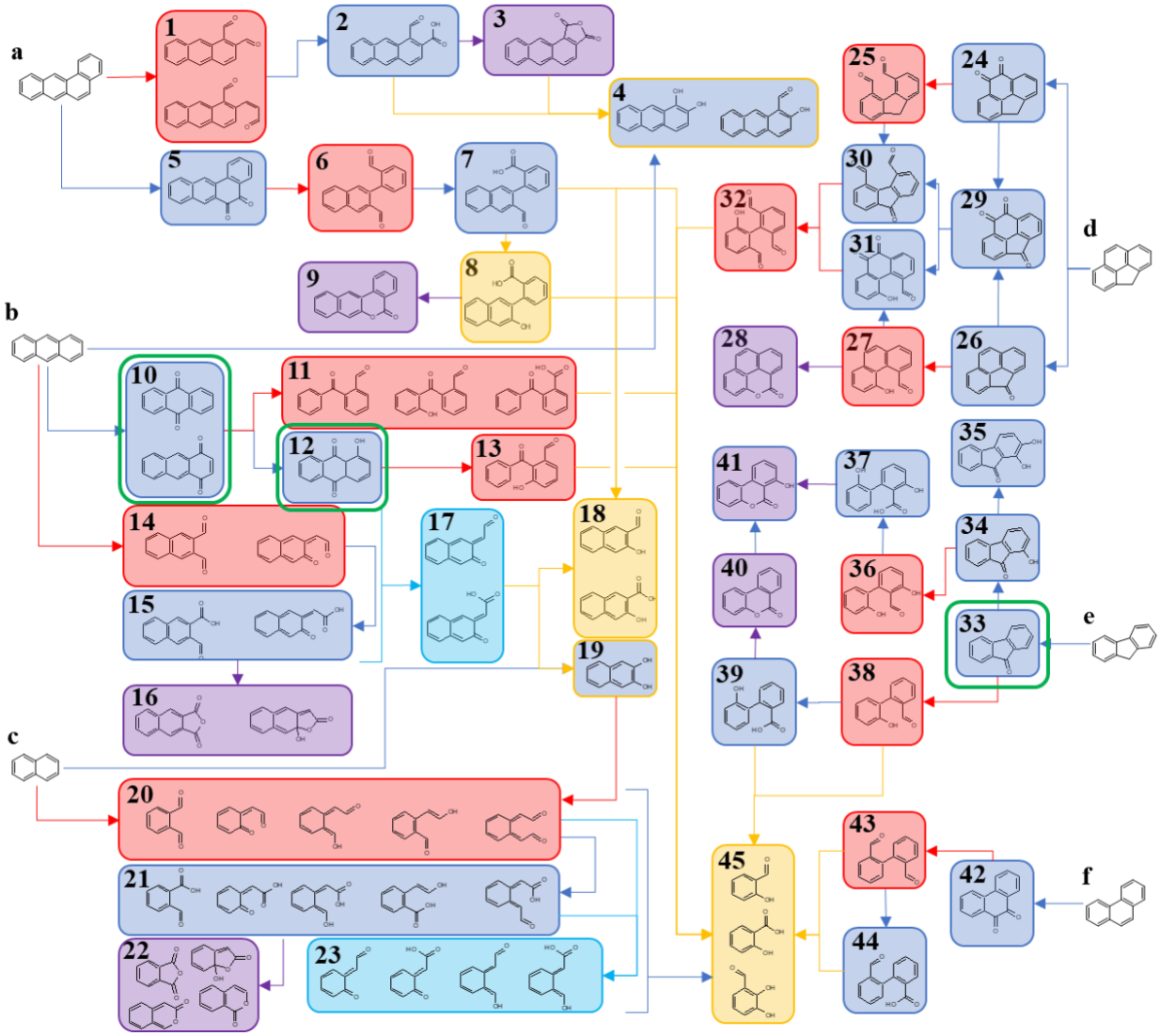

Figure 11. Proposed HOP formation pathways (arrows) from model parent petroleum compounds benzanthracene (compound a), anthracene (compound b), naphthalene (compound c), 4H-cyclopenta[def]phenanthrene (compound d), fluorene (compound e), and phenanthrene (compound f). Bold green outlines indicate detected compounds that were matched to the in-house library. Colors indicate different chemical processes including ring opening (red), oxidation (blue), bond cleavage (gold), photoisomerization (aqua), and ring closing (purple) that may account for the production of numerous HOPs from crude oil.

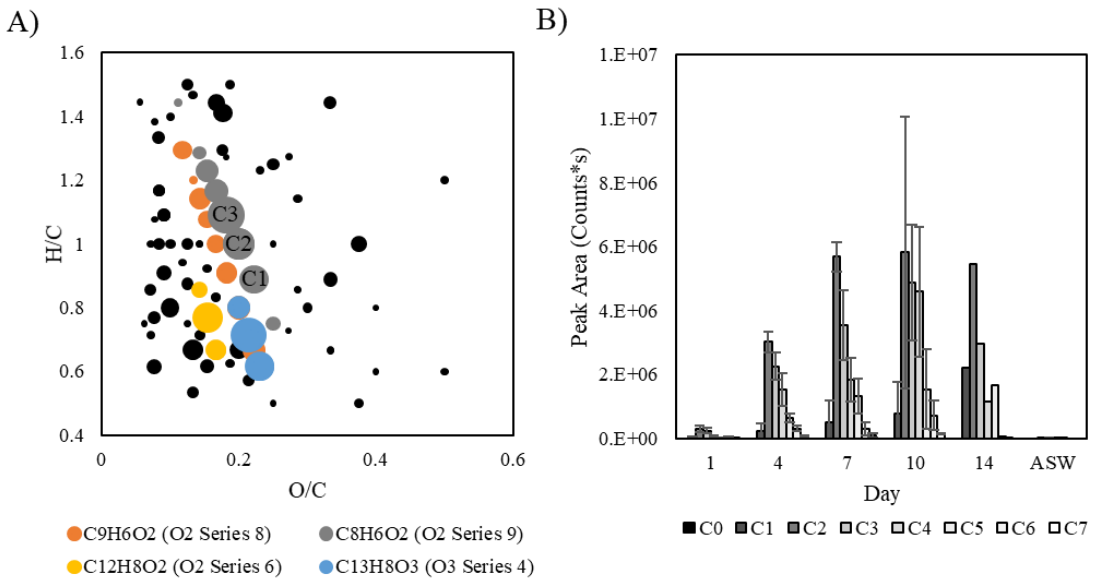

Figure 12. (**A**) Van Krevlen diagram for significant compounds (VIP score > 1) with circle size scaled to compound VIP scores. Prevalent Kendrick series are highlighted and C1-C3 labels corresponding to the alkylated Kendrick groups for O2 Series 9, base formula C8H6O2 (see Figure 11 Boxes 19, 20, and 23 for representative structures), are provided. (**B**) Average abundance (n=3, Day 14 n=2) of detected compounds within O2 Series 9 over time further shows the accumulation of significant HOPs peaks at ten days, with the majority of detected features present as alkylated congeners. Artificial seawater controls (ASW) confirm that significant HOP features were not detected in the absence of crude oil. Error bars represent one standard deviation.

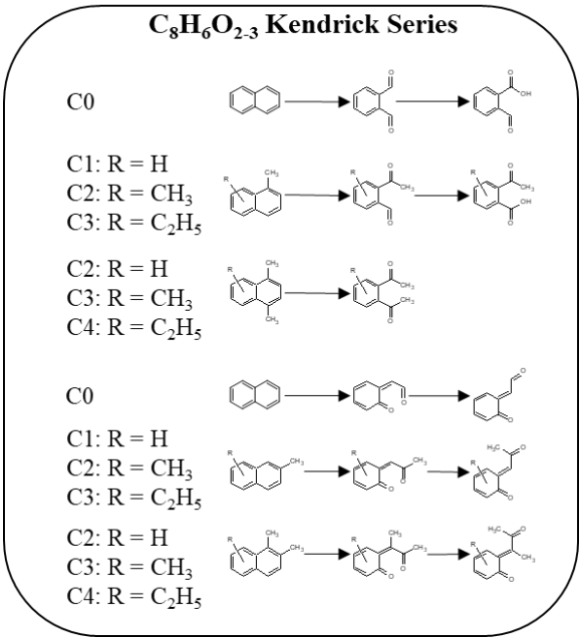

Figure 13. Proposed HOP formation pathways from model parent petroleum compound naphthalene and its methylated congeners.

The next most abundant series contained larger compounds containing one to three oxygen with Kendrick groups C0-C4 with base formulae C13H8O3 (O3 Series 4, n=17, Figure 11 Boxes 35 and 41), C12H8O2 (O2 Series 6, n=12, Figure 11 Boxes 14 and 15), C13H8O2 (O2 Series 5, n=10, Figure 11 Boxes 11 and 43), and C10H8O (O1 Series 7, n=9, naphthalene-4H-one, structure not shown); accounting for 19% (48/255) of significant features. Additional series containing compounds of higher molecular weight were also observed (6%, 15/255), including C13H8O (O1 Series 4, n=3, Figure 11 Box 33), C1310O (O1 Series 5, n=6, Figure 11 Box 34), C14H8O3 (O3 Series 3, n=2, Box 12), and C15H10O2 (O2 Series 4, n=3, Figure 11 Boxes 4, 25, and 27). These larger, less diverse chemical species are consistent with intermediate photooxidation products of three and four ring PAHs and alkyl-PAHs including benzanthracene (Figure 11, Compound a), 4H-cyclopenta[def]phenanthrene (Figure 11 Compound d), anthracene (Figure 11, Compound b), fluorene (Figure 11, Compound e), and phenanthrene (Figure 11, Compound f). Previous work has shown that alkylated derivatives of PAHs, particularly C1-C3, are in high abundance in crude oil and degrade quickly when subject to photooxidation (Garrett et al., 1998; Al Darouich et al., 2005). While our previous targeted efforts support this observation through the identification of oxidized anthracene and phenanthrene products, targeted analyses were limited by the complexity of the crude oil mixture stemming from the prevalence of alkylated PAH isomers and limited or unavailability of analytical standards for alkylated oxyPAHs (Harsha et al., 2023). Overall, this non-targeted approach provides the most comprehensive analysis of hydrocarbon oxidation products to date, highlighting the diversity of the complex mixture resulting from the photooxidation of Cook Inlet crude oil and the limitations of targeted analyses for adequately monitoring HOPs.

Toxicological Effects of Photooxidized Cook Inlet Crude Oil

Across the 14-day period of this study we see a trend towards increased AhR activity after one day exposure to simulated sunlight (Figure 14 and 15). This AhR activation persists throughout the study period, indicating that degradation of Cook Inlet crude oil by sunlight results in HOPs that persist in inducing a xenobiotic response *in vitro*. Toxicity response is likely initially driven by HOPs that significantly (VIP > 1) drive the separation of light vs. dark HOP pools; largely compounds with two oxygens normally distributed around Kendrick series C2 (Figure 10C and 10D). These compounds are consistent with oxidation products from the most common and acutely toxic PAHs in petroleum products, naphthalenes (two-ring) and phenanthrenes (three-ring) (Neff, 1988; Fan et al., 2022; Nguyen et al., 2020). Since these significant HOPs contribute most to the differences between light and dark samples and less AhR activation is observed in the dark samples, it's possible that these compounds result in increased AhR activity and thus toxicity. After 10 days, AhR activity persists as the relative distribution of alkylation of significant HOPs remains unchanged up to 14 days (Figure 15). While further degradation of HOPs results in a small decrease in the absolute abundance of significant HOPs, all samples with which cells were treated were concentrated to the same DOC concentration to determine AhR activity based upon sample composition rather than sample concentration. Thus, the relative distribution and AhR activation by significant HOPs remains unchanged within14 days.

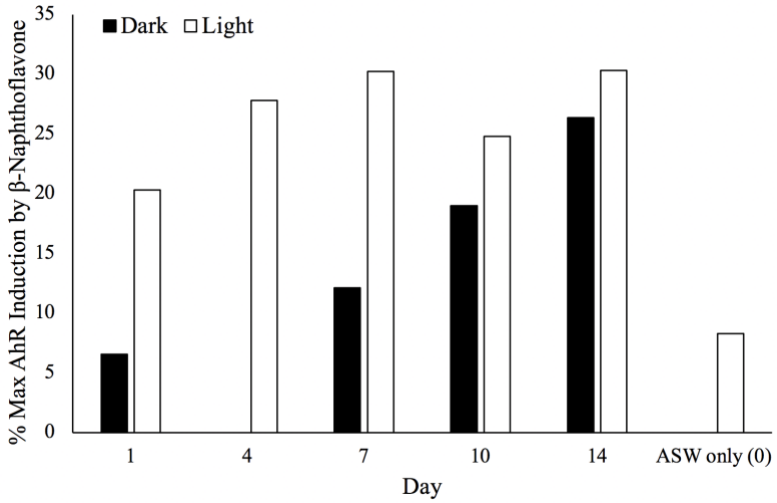

Figure 14. Activation of AhR in H4IIe7.5 cells by photooxidized Cook Inlet crude oil polar extracts with increasing oxidation time. The day 4 dark data set was discarded due to limited sample volume resulting in n = 1. The ASW only (i.e. day 0) control samples were run at <10 ppm NPOC also due to limited sample volume. Bars represent the geometric mean.

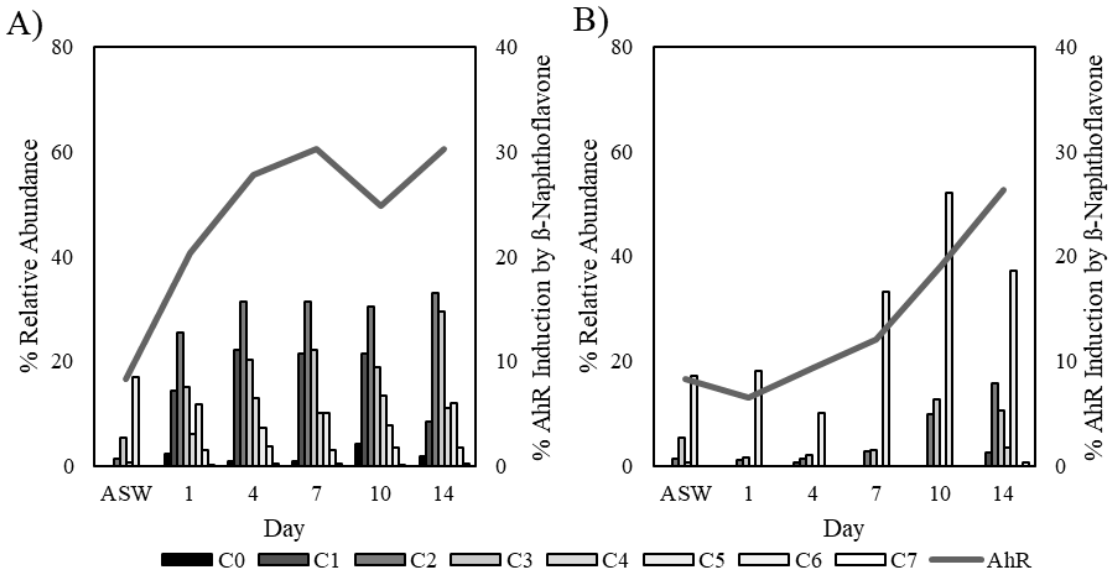

Figure 15. Average (n=3, (A) Day 14 n=2) relative abundance of HOPs in Cook Inlet crude oil polar extracts incubated in the (**A**) light and (**B**) dark at each time point and day 0 ASW (bars) and the percent AhR induction in H4IIe7.5 cells relative to ß-naphthoflavone of extracts concentrated to 10 ppm C (line).

Further photooxidation of HOPs may result in compounds such as aldehydes, ketones, alcohols, peroxides and fatty acids, which could contribute to the persistence in toxicity of the day 14 samples (Neff, 1988; Fan et al., 2022; Yang et al., 2018; Nguyen et al., 2020; Lee, 2003). These compounds may also accumulate in dark-treated samples over time and result in AhR activity as indicated by day 14 of the study. However, as supported by our NPOC data (Figure 6A), the naturally occurring abundance of compounds resulting in AhR activity in dark-treated samples may not be high enough to result in toxic

effects. According to pilot data, a minimum concentration of about 5 ppm DOC is required for AhR activation (Figure 16, Appendix I), but the concentration of DOC in dark-treated samples in this work never exceeded 1.5 ppm DOC. Similarly, a study that examined the relationship between non-volatile dissolved organic carbon (NVDOC) concentration and sample toxicity in terms of distance from a crude oil source found that low levels of NVDOC (<5 ppm) induced minimal AhR activity and that background NVDOC (1.42 ppm) did not induce AhR activity (Bekins et al., 2020). The light samples, on the other hand, had up to 21.4 ppm DOC on average after 14 days of light exposure which would likely be enough to activate AhR *in vitro*. This concentration of carbon also matches levels of DOC found at naturally occurring spill sites (Bekins et al., 2020; Zito et al., 2020; Bianchi et al., 2014; Lunnel, 1998).

In addition to polar compounds, we treated cells with non-polar DOC extracts for both light and dark treatments. Unfortunately, concentrating the polar compounds to 10 ppm resulted in cell death for most treatment wells. We did not run non-polar extracts during the pilot study of the AhR CALUX bioassay (Appendix I), so we were unaware of the increased toxicity of these samples. Due to limited sample volume, troubleshooting and redoing these samples at the appropriate DOC concentration will need to be completed in future work. The composition of non-polar extracts likely contains compounds that are more acutely toxic to cells in comparison with polar compounds. Similarly, marine sediment extracts tested on H4IIe cells resulted in only 50-60% cell viability after treatment with non-polar compounds vs. >80% with polar compounds at the same concentration (Mennilo et al., 2020). AhR activation was also found to be strongest amongst non-polar aromatic substances, including PAHs, extracted from suspended particulate matter from flood events in Germany (Wölz et al., 2010). In particular, substances with more than 16 aromatic C-atoms drove the highest AhR activity. Likely the decreased availability of oxygen and exposure to sunlight of the compounds stored in the sediment of this study result in preservation of higher molecular weight PAHs which resulted in high toxicity. In comparison, our dataset focuses on oxyPAHs which may have fewer aromatic carbons, but their transformation to oxidized species still poses a potential threat as indicated by increased AhR activity after just 4 days of light exposure.

These potential toxicological effects are of concern because the DOC pool resulting from photooxidation of Cook Inlet crude oil persists in activating AhR for up to fourteen days during summer. AhR plays a role in xenobiotic metabolism including the cytochrome P450 (CYP450) system which serves as a biomarker for exposure to certain environmental contaminants in addition to endogenous and exogenous drug metabolism (McDonnell and Dang, 2013; Silva et al., 2020). For example, increased concentrations of alkylated PAHs are correlated with an increase in CYP4501A catalytic activity (including EROD: ethoxyresorufin-O-deethylase and MROD: methoxyresorufin-O-demethylase activity) in greenling fish close to oil-spill sites (Bak et al., 2019). These same PAHs also activated AhR *in vitro* at concentrations lower than those found in fish tissues *in vivo*. Similarly, bioaccumulation of PAHs in *S. rivulatus* gill and liver tissue is associated with CYP450 induction (Gaber et al., 2021). AhR bound to PAHs combines with AhR Nuclear Translocator (ARNT) in the nucleus to activate genes such as CYP1A1 which play a role in metabolically activating aromatic hydrocarbons into reactive metabolites (Androutsopoulos et al., 2009; Nakano et al., 2020). These metabolites can be biologically harmful leading to DNA and tissue damage (Androutsopoulos et al., 2009; Coelho et al., 2022). Thus, the AhR activation by HOPs from this study may have downstream effects that result in biologically harmful reactions. While showing trends of increased AhR activity with photooxidation, high variation in luciferase activity between treatment replicates limited our findings of significant differences. It's possible that by highly concentrating all samples to 1000 ppm DOC, the fluctuation in background DOC amongst samples resulted in high variation. Since samples with dark exposure and ASW controls had very little DOC present in the water soluble fraction, samples had to be concentrated by eluting in very small volumes (~6 uL). Limited sample volume meant that we did not have enough of the ASW controls and several of the dark samples to reach 10 ppm. Because of this issue, the day 4 dark data was removed from the dataset. It would also be useful to extend the experimental timeline up to 21 days or more to determine when toxicity of DOCs begins to decline as HOPs are eventually transformed to CO_2 and H_2O. This type of study would help shape the picture of what timeline and oxyPAH concentrations would result in a toxicity response in conditions comparable to an actual crude oil spill in the Cook Inlet. AhR activation, although strongly associated with toxicity response, is also not a guarantee of adverse health impacts. There have also been several other studies that demonstrate the toxicity of oxyPAHs by mortality, malformations, and oxidative stress in aquatic organisms (Barron, 2017; Lampi et al., 2006; Knecht et al., 2013).

CONCLUSIONS

UPLC-Orbitrap mass spectrometry analysis revealed a total of 2,633 unique oxidized chemical features in the complex mixture; with follow-on multivariate statistical analysis identifying 255 HOPs, including known OPAHs anthraquinone, phenanthrenequinone, hydroxyanthraquinone, and 9-fluorenone, that describe the variance between light exposed samples and dark controls. Compounds from the light-treated samples had higher levels of oxidation compared to dark-treated controls with differences between the two treatment groups being primarily driven by compounds containing 1-3 oxygens and being C2-3 alkylated. These compounds are also likely driving the observed toxicity of the samples indicated by an increase in AhR activity in rat hepatocytes treated with crude oil DOCs. Since this toxicity response persists across all 14 days of the study and is specific to light-treated samples, photooxidation of crude oil is particularly concerning for bioremediation and spill mitigation efforts. This non-targeted analysis of HOPs is the most comprehensive to date and highlights the diversity of the complex mixture resulting from the photooxidation of crude oil and reveals the limitations of targeted analyses for adequately

from the photooxidation of crude oil and reveals the limitations of targeted analyses for adequately monitoring HOPs in the environment. With long daylight hours during summer months in the Cook Inlet, these toxic products could rapidly accumulate at high enough concentrations in the water column to result in harmful biological effects. Thus, prompt response to an oil spill in the Cook Inlet would likely be important to minimize the transport and bioconcentration of petroleum-derived residues that could result in distress on aquatic life.

RECOMMENDATIONS

Future research should test AhR activation and downstream toxicity biomarkers of photooxidized Cook Inlet crude oil at concentrations closer to those that occur during an actual spill, rather than concentrated extracts, to provide an environmentally relevant picture of the biological effects of the studied HOPs. Future work should further consider the non-polar compounds of the mixture which appear to have greater toxic effects on cells and extend the study period to further characterize crude oil breakdown intermediates and their toxicological effects. Finally, future studies should investigate the effects of photooxidized Cook Inlet crude oil on other CYP450 biomarkers in vitro and in vivo to more specifically determine its toxicological impact.

ACKNOWLEDGEMENTS

Funding for this project was provided by U.S. Department of Interior, Bureau of Ocean Energy Management Environmental Studies Program (BOEM Cooperative Agreement M21AC10017) to P. Tomco through the University of Alaska Coastal Marine Institute. Additional support was provided to P. Tomco by the ConocoPhillips Arctic Science and Engineering Endowment and the National Science Foundation Award numbers 1929173 and 2019123.

STUDY PRODUCTS

Presentations

Redman, Z.C; Robine, S.; Burkhead, J.; Tomco, P.L. Non-target analysis and toxicity screening of photochemically formed hydrocarbon oxidation products from crude oil; implications for monitoring and high-latitude spills. 2023 Fall National ACS Meeting, Oral Presentation. Accepted April, 2023.

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APPENDIX I: Initial characterization of AhR response to photooxidized Cook Inlet crude oil and diesel

For the initial pilot experiments of the AhR CALUX bioassay, AhR activity of rat (H4IIe7.5) and human (HepG27.5) hepatoma cells in percent of the maximum activity induced by β-Naphthoflavone was plotted against a range of concentrations of DOCs extracted from artificial seawater (ASW) treated with either Cook Inlet crude oil or diesel after 4, 7 and 10 days of light exposure (Figure 16). For the H4IIe7.5 cells, increasing concentrations of DOCs caused an increased AhR response for both oil and diesel extracts. While diesel extracts seemed to induce a higher AhR response compared to oil after 4 and 7 days of light exposure, by day 10, induction of AhR activity by diesel extracts was minimal. Cook Inlet crude oil extracts, on the other hand, consistently induced AhR activity across all light treatments ranging from 23.4% ± 4.0% (day 7) and 32.6% ± 5.1% (day 10) to 45.4% ± 3.9% (day 4) max induction at 30 ppm DOC.

In the HepG27.5 cells, AhR induction was much less consistent. Increasing concentrations of DOCs did not always induce higher AhR activity. For example, in 10-day light treated oil extracts, the highest induction was caused by the 10 ppm DOC treatment. Nonetheless, day 4 oil and diesel extracts seem to induce higher AhR activity compared to day 7 and 10 extracts. Maximum AhR induction by day 4 oil was 113.4% ± 62.0% (30 ppm) versus only 27.0% ± 8.9% (10 ppm) after 10 days of light exposure. For diesel, maximum induction at day 4 was 238.8% ± 106.8% (30 ppm) whereas at day 10 induction was 17.3% ± 9.6% (20 ppm).

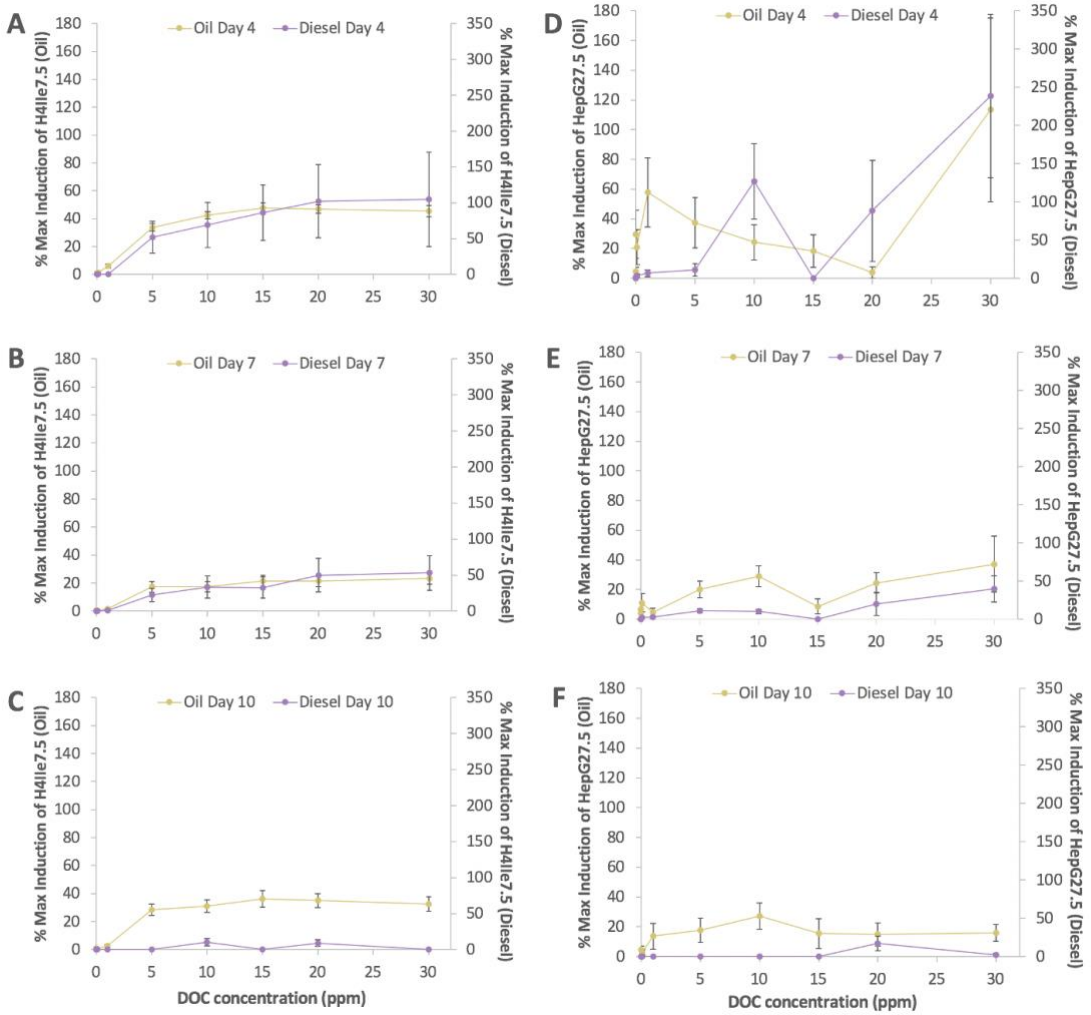

Figure 16. AhR activity of H4IIe7.5 and HepG27.5 cells exposed to Cook Inlet crude oil and diesel DOCs. (**A-C**) H4IIe7.5 and (**D-F**) HepG27.5 cells treated with a concentration range (0.001, 0.01, 0.1, 1, 5, 10, 15, 20 and 30 ppm) of oil and diesel samples after (**A, D**) 4, (**B, E**) 7 or (**C, F**) 10 days of light exposure. Error bars represent ± standard error.

To directly compare diesel and oil extracts across light treatments, percent max induction at 10 ppm was plotted against time exposed to simulated sunlight for H4IIe7.5 and HepG27.5 cells (Figure 17). In both cell types, day 4 diesel extracts induced significantly higher AhR activity compared to other extracts ($p < 0.05$). While there were no other significant differences due to lack of power in this data set, there is a trend where Cook Inlet crude oil extracts more consistently induce AhR activity across all light treatments. Diesel extracts, on the other hand, tend to have decreased AhR activity with increased light exposure.

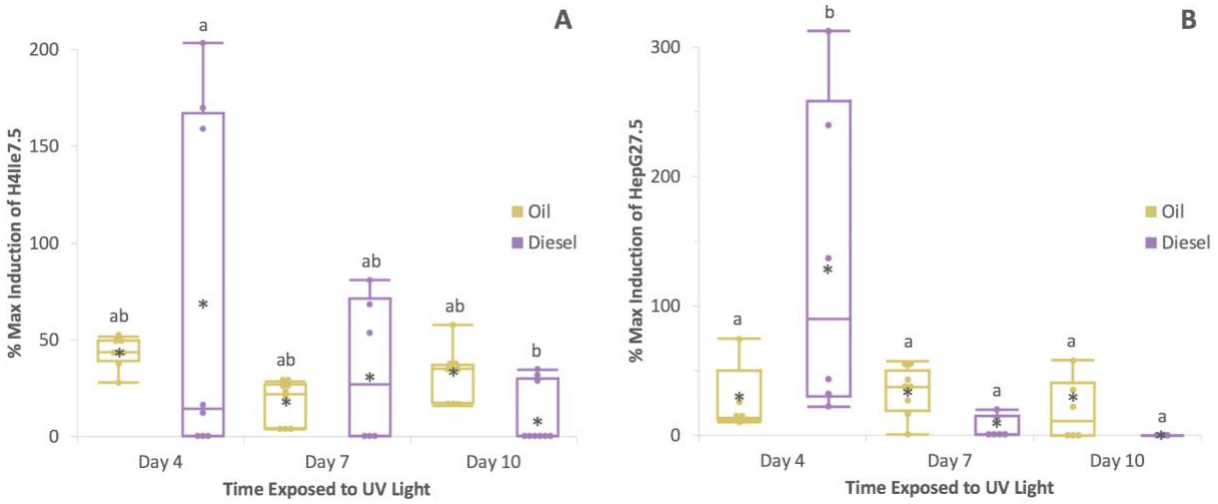

Figure 17. AhR activity of H4IIe7.5 and HepG27.5 cells exposed to oil and diesel DOCs across time. Boxplots of (**A**) H4IIe7.5 (**B**) and HepG27.5 cells treated DOCs diluted to 10 ppm after 4, 7 and 10 days of light treatment. Letters denote significant differences ($p < 0.05$). Asterisks (*) represent the mean.

This initial pilot data matches our findings that HOP toxicity is high early in the degradation process. In both the H4IIe7.5 and HepG27.5 cells, 4-day light oil and diesel samples tended to induce the highest AhR activity. Despite a lack of statistically significant differences between samples, it is apparent that both oil and diesel extracts contain dissolved organic carbons (DOCs) that activate AhR. The concentration range of activation was in the range of 10-30 ppm (Figure 16). With DOC concentrations higher than 30 ppm, cells began to die (data not shown; viewed during visualization under a microscope, pre-lysis). Specifically in diesel extracts, we saw increased AhR activation in the first 7 days of light exposure followed by a drop in activation. Since diesel is refined from crude oil, it's possible that the more consistent AhR induction by oil across all 10 days of the pilot study was due to the presence of higher molecular weight PAHs in crude oil. The toxicity of PAHs is inversely related to their molecular weight (Neff, 1988). These higher weight PAHs require more energy to break down into acutely toxic two- to three-ring PAHs and oxyPAHs, which are at higher abundance in diesel. Diesel is a fraction of crude oil containing compounds with boiling points between 163-357°C (Gad, 2005). PAHs with more than three-rings tend to have boiling points of 300°C and higher so diesel does not contain many PAHs with more than three rings (Achten and Anderson, 2015; Neff, 1988). Since naphthalenes (two-ringed PAHs) and phenanthrenes (three-ringed PAHs) tend to contribute most to the toxicity of petroleum products, diesel extracts have the potential to be more acutely toxic than crude oil as lighter weight PAHs are more quickly oxidized to toxic oxyPAHs.

The toxicity of oxidized and photooxidized petroleum products has a finite lifespan. In our pilot data, more than 7 days of simulated sunlight exposure resulted in decreased AhR activation by diesel DOCs. These results match previous data on the half-life of photo-modified PAHs and their toxicity. The maximum photooxidation half-life of the studied PAHs under simulated sunlight was about 4 days with the majority of PAHs having half-lives of less than 48 hours (Krylov et al., 1997; El-Alawi et al., 2002). Naphthalenes typically have half-lives of about 3 days when simulating mid-summer sunlight at 40° N latitude (Neff, 1988). Thus, it makes sense that AhR activation in diesel decreases with long-term exposure of parent oil to simulated sunlight (> 7 days) due to decreased availability of PAHs as they are broken down by photooxidation. Crude oil, on the other hand, has PAHs with higher molecular weight

that take longer to break down into toxic oxygenated products. Thus, toxicity of crude oil DOCs remains in effect for a longer period of time.

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