Impacts of petroleum exposure on DNA integrity and gene expression in the Pacific oyster, *Crassostrea gigas*

by

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Abstract

The increase in petroleum use and proposed petroleum expansion projects on the Pacific west coast of British Columbia will increase the risk of oil spills in the marine environment. Advancements in molecular assays have propelled their use in identifying sublethal biomarkers. Molecular biomarkers aid in the early detection of exposure and toxic effects of oil in organisms. Adult Pacific oysters (*Crassostrea gigas)* were exposed to 3 different oils (marine diesel, crude oil, and diluted bitumen) for 7 d *via* water accommodated fractions ([WAFs] 1:30 oil/water ratio) diluted at 0 %, 25 %, 50 % and 100 % with seawater. Microsomal CYP1A enzyme activity (EROD) and the mRNA expression of 8 detoxification-related genes (*ARNT, CYP2C50, CYP2C23, GST-theta-1, mGST-2, MDR1, CuZnSOD and Hsc-70*) were measured in gill tissue at 7 d (exposure), 21 d and 35 d (recovery). DNA damage using the COMET assay in hemocytes was measured under the same exposure and recovery conditions. Individual polycyclic aromatic compound (PAC) concentrations were measured in water (at 0 d, 3 d, 7 d) and in whole soft tissue samples (7 d, 21 d, 35 d) for each oil exposure. The predominant PACs in both water and tissue samples consisted of C_1-C_4 chain napthalenes, fluorenes, and phenanthrene for all oil types, with crude exhibiting the highest total concentration of PACs in both media. Total PAC concentrations in water samples decreased exponentially from 0 d – 7 d and were rapidly depurated from oyster tissues between 7 d – 21 d. EROD activity was not detected in any oyster tissues, likely due to very low basal enzyme levels and lack of CYP-mediated induction. No significant temporal or concentration-dependent trends were observed in the expression of detoxification-related genes in gills , suggesting that biotransformation is not the primary elimination method for water-soluble PACs in this tissue. Similarly, no significant DNA damage was observed following exposure, suggesting that PACs accumulated did not intercalate with the DNA, or that strand-breaks were not accumulated at a rate that exceeded DNA repair mechanisms.

Keywords: Pacific Oyster; oil ; gene expression ; DNA integrity ; cytochrome P450; PACs

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List of Acronyms

AhR	Aryl hydrocarbon receptor
AOP	Adverse outcome pathway
API	American Petroleum Institute
BPD	Barrels per day
BTEX	Benzene, Toluene, Ethylbenzene, Xylene
CCME	Canadian Council of Ministers of the Environment
CYP	Cytochrome P450
DBT	Dibenzothiophenes
DNA	Deoxyribonucleic acid
GST	Glutathione-S-Transferase
EPA	Environmental Protection Agency
EROD	Ethoxy-resorufin-O-deethylase
HMW	High molecular weight
LMW	Low molecular weight
MDR	Multi-drug resistance gene
mRNA	Messenger RNA
NOAA MWP	National Oceanic and Atmospheric Administration Mussel Watch Program
OECD	Organization for Economic Cooperation and Development
PAC	Polycyclic aromatic compound
Pgp	P-glycoprotein
RNA	Ribonucleic Acid
[TPAC]	Total concentration of polycyclic aromatic compounds
WAF	Water Accommodated Fraction
qPCR	Quantitative polymerase chain reaction

Chapter 1.

General Introduction

1.1. Canadian petroleum industry

Canada is the world's 4th largest producer of oil and holds the 3rd largest proven oil reserves, estimated to contain 171 billion barrels (NRC, 2019). Of this total, 97% comes from Alberta's oil sands, while the remaining 3% account for conventional crude, offshore and shale sources (NRC, 2019). Production and supply forecasts are projected to increase from 4.9 million barrels per day (BPD) produced in 2018 to 5.8 million BPD by 2035, where 75% of projected production is expected to be from the oil sands region. This region is made up of 3 major deposits: the Athabasca, Cold Lake and Peace River deposits (CAPP, 2019). The bulk of oil produced in Canada is exported internationally for further processing. Of the 4.5 million BPD produced in 2020, 3.6 million BPD were exported, 97% of which went to the U.S, while 1.6 million BPD were allocated to domestic refineries (NRC, 2022).

Oil sands crude, also known as bitumen, is extracted by either surface mining or in situ (i.e., in place) recovery (CAPP, 2019). For bitumen extracted by surface mining, deposits must be within 70 m of earth's surface, where raw oil sand is shoveled into haul trucks for transportation to crushers that break down large pieces, and then the crushed oil sand is mixed with hot water for further upgrading into light or synthetic crude oil at refineries (CAPP, 2019). In 2020, 47% of raw bitumen produced in the oil sand was sent for upgrading in Alberta (NRC, 2022). In situ recovery is used for deeper deposits by either steam assisted gravity drainage (SAGD) or Cyclic Steam Stimulation (CSS), both of which involve injecting steam into drill wells to liquify the bitumen and pump it to the surface (CAPP, 2019). Bitumen extracted by in situ methods are blended with various diluents such as synthetic crude oil, natural gas condensates, or a mixture of other light hydrocarbons (Dupuis and Ucan-Marin, 2015). Bitumen diluted with synthetic crude (typically at a 1:1 ratio) is referred to as "SynBit", while bitumen mixed with condensates or other diluents (typically at a 1:3 ratio) is called "Dilbit" (CAPP, 2014).

Once transported to refineries, conventional and unconventional crudes are further processed into petroleum-based fuels and gasoline. Canada has a total of 17 refineries

capable of processing 2.0 million BPD of crude and 40% of this total capacity is attributed to Western Canada (CAPP, 2019).

Crudes are generally classified as conventional or unconventional based on their extraction method. Conventional crudes are extracted from underground reservoirs using traditional land-based and offshore drilling techniques since they are liquid at atmospheric temperature and pressure (CAPP, 2019). Unconventional crude requires advanced extraction techniques including horizontal drilling, hydraulic fracturing and in-situ recovery (CAPP, 2019). As mentioned, crude petroleum in Canada is produced by upgrading and refining raw bitumen from the oil sands with condensates and diluents, therefore are originally sourced via unconventional methods. Crudes are then further classified based on their API gravity value (discussed in Section 1.2) into light, medium and heavy blends. Export of heavy crudes increased by an annual average rate of 12.5% between 2010-2015 due to increased production in the oil sands region (CER, 2022). Light crude exports also increased within the same period by 2.9% due to offshore development in Newfoundland and Labrador, expansions in oil sands upgrading facilities, and advancements in light oil production technology in western Canada (CER, 2022).

All fuel oils are derived from further refinement of crude (CER, 2022). Diesel fuel has a greater power density and is designed for compression ignition engines (locomotives, marine vessels, trucks and farm equipment), while gasoline is made up of lighter hydrocarbons and additives for everyday use or personal vehicles (Canadian Fuels Association, 2022). Of the refined petroleum products processed in Canada between 2011-2016, the largest portion comprised of motor gasoline at 36% of the total, followed closely behind by diesel and middle distillate products at 33% (Statistics Canada, 2017).

The transport of both raw petroleum and its refined products are primarily via pipeline, rail, and ship. Often several methods of transportation are used to distribute oil from ports, terminals, and refineries to markets across the country. In Western Canada, petroleum distribution from refineries to terminals is done by pipeline and excess volumes depend on rail (NRC, 2016; CAPP, 2019). Currently, three major pipeline projects in Western Canada remain under active construction to meet anticipated crude supply growth: the Enbridge 3 Line Replacement, the Transmountain Expansion Project, and the Keystone XL Project (CAPP, 2019). The Transmountain Expansion Project specifically will increase its current capacity of 300,000 BPD to 900,000 BPD (CAPP, 2019). Marine

transport in Eastern Canada is more popular than pipeline or rail transport compared to the Pacific Coast, however, in BC, barges carry product from Vancouver to terminals on Vancouver Island and along the Northern coast (NRC, 2016). In the Salish sea, the offshore over-water Roberts Bank Terminal 2 is estimated to increase petroleum container shipments by 50% (Georgia Straight Alliance, 2022). On BC's North Coast, there is approximately 860 BPD of petroleum products shipped between or to, more remote communities using dedicated fuel barges or general cargo barges carrying fuel trucks (Transport Canada, 2016).

Transport-related oil spills are primarily monitored and reported by Provincial governments (Dupuis and Ucan-Marin, 2015). The BC Ministry of Environment's Environmental Emergency Program (EEP) receives an average of 4000-5000 hazardous spill calls per year and a significant portion come from business sectors associated with petroleum usage (EEP, 2021). Of the environments impacted (air, land, and water pollution) , marine based spills accounted for 1100-1300 of reports in the period between 2017-2021, 500 of which were attributed to the oil and gas sector (EEP, 2017; EEP, 2021). The Canada Energy Regulator (formerly the National Energy Board) is responsible for the federal regulation of 10% (71,126 km total) of Canada's pipeline network, with the remaining 90% falling under provincial jurisdiction (CER, 2022). The provincial pipeline regulator in BC is the British Columbia Oil and Gas Commission, which encompasses 118 companies responsible for 42, 681 km of pipeline (NRC, 2017). Incidents from CER-regulated pipelines are reported to the Transportation Safety Board of Canada (TSB). In BC, spill incidents regulated by the province are required to be reported immediately to Emergency Management BC and the commission (NRC, 2017). Of the CER pipeline-related incidents from the period between 2010-2019, 88 involved unintentional release of hydrocarbons: 15% natural gas, 4% crude oil, and 1% condensate/diluents (TSB, 2021).

Spills from marine vessels are investigated by the Canadian Coast Guard which oversees the Marine Pollution Incident Reporting System (MPIRS) (Dupuis and Ucan-Marin, 2015). The largest tanker spill in Canadian waters occurred in 1970 off the coast of Nova Scotia, when the *M/T tanker Arrow* spilled over 10, 000 tonnes of Bunker C fuel oil (Transport Canada, 2020). The largest spill in Western Canada was due to the sinking of the *M/V The Queen of the North* ferry in 2006, which released 240 tonnes of diesel fuel (Transport Canada, 2020). The total volume of marine hydrocarbon spills detected in Canadian waters from 2010-2020 ranged from 1000-9000 L (ECCC, 2021). A record high

number of spills was reported in 2021, accounting for spillage of 17, 651 L (ECCC, 2021). Increased demand for petroleum production, coupled with transport expansion plans through marine ecosystems along the Pacific West Coast of BC warrants further investigation into the fate, toxicity, and impact of these complex mixtures on the environment.

1.2. Composition and environmental fate of petroleum products

Chemical composition of conventional and unconventional crudes is made up of hydrocarbons , elemental components (sulfur, oxygen, nitrogen) and some metals (nickel, vanadium, chromium) (Dupuis and Ucan-Marin, 2015). Of these categories, the hydrocarbon fraction is the most abundant, accounting for 50-97% of the petroleum formulation (Dupuis and Ucan-Marin, 2015). Hydrocarbons are first characterized by their size, which is categorized by the number of carbon atoms into low range (≥C5–<C9), mid-range (≥C9–<C19) and high range (≥C19–<C35) (Kuppusamy et al., 2020). They are then further grouped by structure into aliphatics (alkanes/alkenes), aromatics, resins and asphaltenes (Kuppusamy et al., 2020; Dupuis and Ucan-Marin, 2015). The aromatics are the most studied in terms of environmental toxicity due to their persistence and contain at least one benzene ring, these include monoaromatics (benzene, toluene, ethylbenzene, and xylene – referred to together as BTEX) and polycyclic aromatic compounds (PACs) (Dupuis and Ucan-Marin, 2015). Diesel fuel is generally made up of mid-range hydrocarbons and typical composition by volume is as follows : 66% aliphatics and 35% aromatics (BTEX and 2-ringed PACs) (Kuppusamy et al., 2020). Conventional crudes contain a higher percentage of PACs than refined distillates, particularly 2-3 ringed PACs (Dupuis and Ucan-Marin, 2015). In oil sands products, Synbit and Dilbit blends have greater abundance of 3-5 ringed alkylated PACs (DFO, 2013). Analytical analyses of petroleum mixtures for toxicity studies typically target saturated hydrocarbons (aliphatics C_8-C_{40}), alkylated PACs (C_1-C_4) and the 16 U.S Environmental Protection Agency (U.S EPA) priority parent PACs (Figure 1.1, Dupuis and Ucan-Marin, 2015).

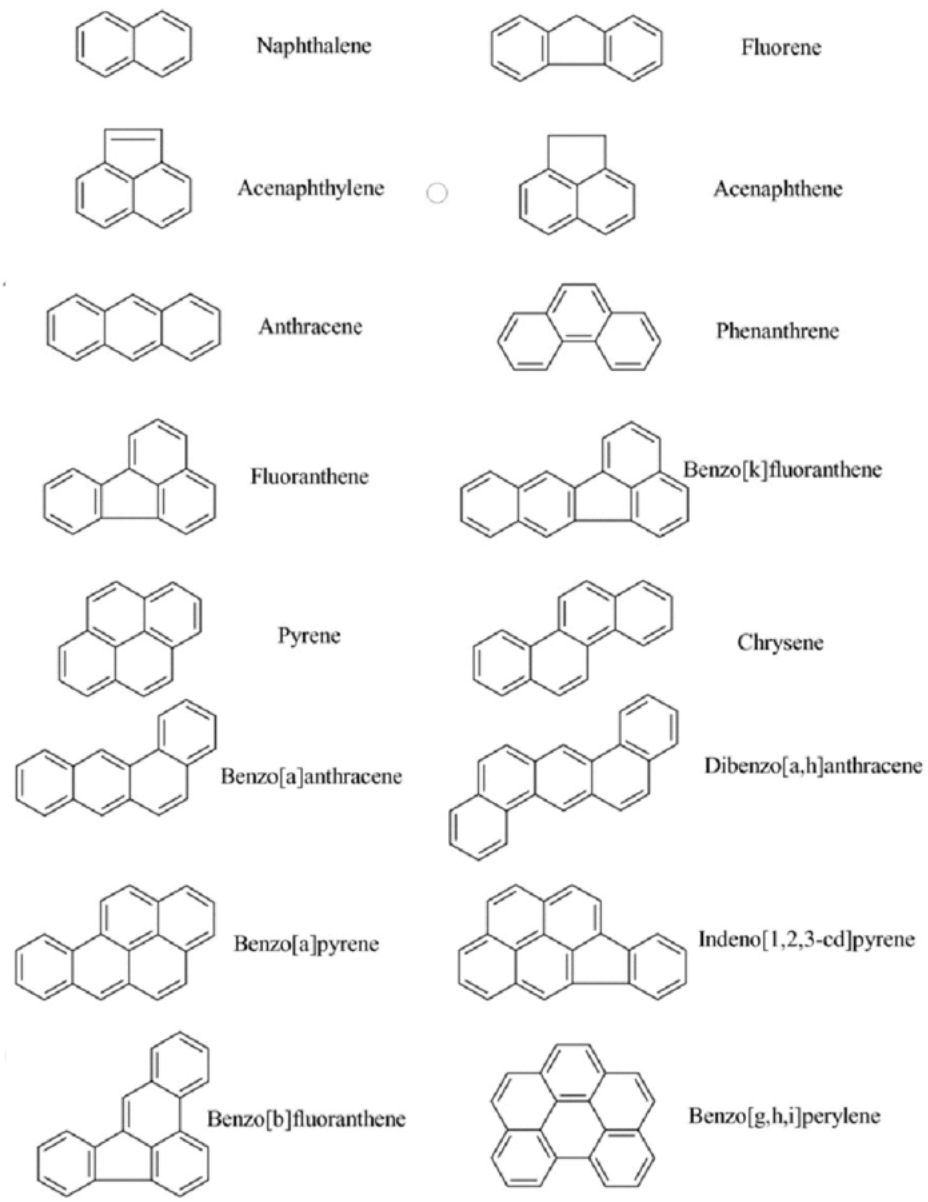

Figure 1.1. The 16 U.S EPA priority PACs for environmental evaluation
Adapted from: Mastanjević et al. (2020) under CC BY.

The environmental fate of specific compounds in petroleum products must be considered in the event of a spill or seepage into the marine environment. Both physical and chemical properties are highly variable across constituents in petroleum products due to their complex and unique formulations; these can encompass thousands of individual compounds (Dupuis and Ucan-Marin, 2015). Important physical properties include American Petroleum Institute (API) gravity values, density, viscosity, surface tension, flash point, pour point, predicted evaporation and emulsion formation (Dupuis and Ucan-Marin, 2015). Density is a key property to determine if the spilled oil will float or sink in the water

column; typically oils with an API gravity below 6 (>1.035 g/mL) will sink in seawater (DFO, 2013). Most conventional oils have an API gravity >10, except for oil sands bitumen, which ranges from API 7-13 in its raw form (Dupuis and Ucan-Marin, 2015). However, since raw bitumen is regularly mixed with lighter diluents for transport, SynBit and Dilbit blends have API values ranging from 18-19 (Dupuis and Ucan-Marin, 2015). In a marine spill, evaporation of lighter constituents, adherence to sediments and emulsion formation due to wave activity can alter the density of oils and thus its persistence in marine environment (Dupuis and Ucan-Marin, 2015). Temperature is also an important factor regarding the variation of the physical properties mentioned. In a colder-temperate marine environment, the evaporation rate will be slower, which in turn reduces the volatilization rate of lighter hydrocarbons (Schmutz, 2018).

The solubility and volatility of PACs in the marine environment result in a trade-off in terms of bioavailability and potential toxicity to organisms. Low molecular weight (LMW) hydrocarbons (e.g., BTEX and other monoaromatics) have higher solubility, however, they generally volatilize within hours to days following a spill. These characteristics generally lead to more acute toxic effects in marine organisms (Dupuis and Ucan-Marin, 2015). Conversely, high molecular weight (HMW) PACs have reduced solubility and volatility but are more lipophilic; meaning they have higher affinity for fatty tissues and will readily adsorb to particulate matter in the water-column, which are ingested by sediment-dwelling organisms (Kuppusamy et al., 2020). For these reasons, HMW PACs tend to be more associated with sub-chronic and chronic toxic effects. Alkylated PACs can also be present at higher proportions in petroleum products than their associated parent compounds. The addition of alkyl groups increases molecular weight, further reducing water solubility and volatility, which increases their ability to accumulate if taken up by tissues (Dupuis and Ucan-Marin, 2015).

In the event of an oil spill, weathering processes that take place both at and below the surface of the water cause changes in the composition of the oil and thus the bioavailability and potential toxicity of the oil to aquatic organisms. Evaporation is usually the most immediate and rapid weathering process that occurs on the surface of the water column, and has greater effects on the spilled mass of light fuels and crudes (Lee et al., 2015). Evaporation accounts for nearly 75% of mass lost from condensates and ultra-light oils like marine diesel; 20-30% for light fuel oils and crudes, and ≤ 10% for heavy oils (NRC, 2003; Kuppusamy et al., 2020). These evaporation rates largely reflect

the proportion of volatile hydrocarbons present in the oil, which typically include BTEX and ≤ C_{16} PACs (AOSRT, 2014). Diluted bitumen blends generally exhibit similar evaporation rates as heavy crudes, AWB and CLB dilbits lost only 15-18% of mass in an open-air flume tank over a 13-day period (King et al., 2014) and lost 17.4% of mass under laboratory conditions (Waterman and Garcia, 2015). Dilbits however are unique in their evaporation behaviour because they tend to follow a bimodal distribution, where diluent components are lost rapidly (like light fuel oils) and this rate then slows with time as the residual oil becomes more viscous, reducing losses of volatile hydrocarbons (Lee et al., 2015). The higher viscosity of dilbit also causes formation of thick oil slicks rather than thin sheens as the spill spreads, further reducing evaporation rates of PACs (Fingas, 2013).

Another weathering process that occurs at the surface of the water is photooxidation of aromatic hydrocarbons. The reaction of aromatics with oxygen in the presence of sunlight yields oxygenated products that are more water soluble than the parent compounds (Lee et al., 2015). C_3-C_5 PACs are particularly susceptible to photooxidation (Kuppusamy et al., 2020). Therefore, petroleum products or diluents with a higher proportion of these PACs, along with the degree of UV exposure, would be affected more significantly by photooxidation. The formation of water-soluble products can make them more resistant to biodegradation and increase their bioavailability to aquatic organisms (Lee et al., 2015).

The last major weathering process that can happen at the water surface following an oil spill is emulsification. Emulsion formation can be classified as either water-in-oil (w/o) or oil-in-water (o/w), each of which can be considered stable or unstable (Lee et al. 2015). Stable emulsions persist indefinitely while unstable emulsions rapidly separate into immiscible layers (Wang et al., 2003). Stable emulsions can increase the effective volume of spilled oil by 2-5-fold and increase the viscosity of the oil by 1000-fold (Fingas, 2013), which in turn reduces the evaporation rate of volatile PACs. Conventional crudes tend to form w/o emulsions rapidly but then revert to separate oil and water phases, while heavy fuel oils are less susceptible to w/o emulsion formation (Fingas, 2013). An EPA study on the chemical and physical properties of a suite of petroleum products found that diesel fuel (Fuel Oil No. 2) and conventional crude (Alberta Sweet Mixed Blend) formed unstable emulsions with high water content (Wang et al., 2003). Controlled studies using dilbit blends also found that unstable emulsions formed despite

the high viscosity of heavy oil that would be expected to prevent phase separation (Zhou et al., 2015). The Royal Society of Canada Expert Panel highlighted the need for more laboratory and field trials to assess the conditions under which dilbit blends will form stable or unstable emulsions in seawater (Lee et al., 2015).

Natural dispersion of oil occurs both at and below the surface of the water column, primarily due to turbulence introduced by wave activity (Lee et al., 2015). This process causes droplets of oil to detach from the slick and either become suspended in the water column, combine with other droplets, or resurface (Lee et al., 2015). Stable dispersions remove oil from the surface slick, which reduces the potential for evaporation. Oil droplets suspended in the water column can also interact with sediment and other particulate matter, which can increase the droplet density and promote sinking (Lee et al., 2015). The dispersion behaviour of conventional crudes and fuels are generally well understood and amenable to droplet formation (Wang et al., 2003). Studies using dilbit exhibited poor natural dispersion with wave-tank simulations (King et al., 2014) and the U.S EPA standardized Baffled Flask Test (BFT) (Comny et al., 2017).

Another natural weathering process that occurs in the water column is biodegradation of oil components by bacteria and microbes. Several species have been identified as obligate hydrocarbon-degrading bacteria (OHCB) in marine ecosystems (Lee et al., 2015). Biodegradation affects the composition of oil in a similar way as the physical processes (evaporation and photooxidation); microorganisms' breakdown LMW alkane and aromatic fractions of the oil (Liao et al., 2009). Therefore, heavy crudes and bitumen that have greater proportions of HMW PACs are naturally more resistant to biodegradation (Lee et al., 2015). Production and application of biosurfactants using OHCB and other generalist hydrocarbon-degrading microbes has been an increasingly useful bioremediation method by spill responders.

1.3. Marine Bivalves and Biomonitoring

Given the degree of complexity regarding the fate of petroleum products following a spill and the suite of environmental factors that influence the bioavailability of PACs, it is important to assess toxicity in aquatic species that are relevant to a particular ecosystem. Marine bivalves are often used in environmental monitoring programs to assess the degree of exposure and accumulation of contaminants (Gerhardt, 2002).

Adults are sedentary, which is helpful for assessing localized contamination (Gerhardt, 2002). Adults also settle on hard substrates to create dense beds or reefs that provide a multitude of ecosystem services for higher level taxa (Padilla, 2010). As suspension feeders, they have a substantial effect on the water column in which they reside. At high densities, large volumes of filtered water can alter biogeochemical cycles in terms of organic content and sediment particulate size (Padilla, 2010). Marine bivalves are also considered to accumulate xenobiotics due to their slow biotransformation rates, leading to the bioaccumulation of compounds in tissues that can be measured for evidence of exposure (NAP, 2003). Marine bivalves have been readily used as exposure indicators for PACs and oil spills (NAP, 2003). Arguably, the most well-known and longest-running monitoring study involving marine bivalves is the National Oceanic and Atmospheric Administration Mussel Watch Program (NOAA MWP). The NOAA MWP has measured the concentration of trace chemicals including PACs along the coastal U.S since 1986 and has more recently added oysters to their annual sampling effort (Kasiotis and Emmanouil, 2015). The NOAA Mussel Watch Program established threshold concentrations to group PAC contamination into three categories: (1) low contamination (47-828 ng/g), medium contamination (828-2511 ng/g), and (3) high contamination (>2512 ng/g) (Kasiotis and Emmanouil, 2015). These concentrations are based on bivalve uptake and depuration rates to assess the potential for toxic effects, and to provide spatiotemporal trends of coastal contamination across North America.

In the present study, the Pacific oyster, *Crassostrea gigas,* was used as a model bivalve species. The Pacific oyster is originally native to regions in Sakhalin Island and coastal Russia, Japan, China, Southeast Asia and Pakistan. They were first introduced to the west coast of North America, including BC, in the early 1900s for aquaculture purposes (DFO, 2017). Reproductively successful populations were transplanted to the west coast of Vancouver Island, where they are now established in sustainable habitats, primarily south of the Brooks Peninsula (DFO, 2020). Established oyster populations also currently exist in the Skidegate Inlet, Haida Gwaii (DFO, 2020). Pacific oysters are protandric hermaphrodites, meaning they initially spawn as males and become females during the winter season (DFO, 2020). They have extremely high fecundity and longevity, females can spawn hundreds of millions of eggs each year and individuals can live for decades (Padilla, 2010). The natural distribution of oysters in BC is generally limited to waters with warm enough temperatures (between 16-34°C) for spawning, gonadal development, and

larvae metamorphosis (DFO, 2020). The larval period lasts approximately 15-25 d depending on temperature, allowing for distant dispersal from natal populations (Padilla, 2010). Adults prefer to settle on hard substrates in mid to high intertidal zones (DFO, 2020). The Pacific oyster is the most widely cultivated shellfish in B.C and the Pacific Northwest, making up a significant proportion of the ~$1.0 billion Canadian Aquaculture industry (DFO, 2017). Pacific oysters are also heavily incorporated into the diets of Indigenous populations along the northwest coast, including the T'aaq-wiihak First Nations peoples (DFO, 2020).

1.4. Toxicity of Petroleum in Marine Bivalves

Lethal concentrations of hydrocarbons in bivalves have mostly been determined or modeled using single-chemical exposures in controlled laboratory conditions (ECOTOXicology Knowledgebase, U.S EPA). A large portion of the toxicity research has focused on carcinogenic PACs such as benzo[a]pyrene (BaP) because of the focus on human health outcomes related to consumption of contaminated shellfish (Hyland, 2006). Comparatively, there is less information about non-carcinogenic PACs (Hyland, 2006). The Canadian Council of Ministers of the Environment (CCME) currently only has a water quality guideline for the protection of all marine life for the non-carcinogenic PAC naphthalene (1.4 $\mu g.L^{-1}$) (CCME, 2010). A comprehensive review on acute lethal endpoints (i.e., mortality or immobility) in benthic invertebrates was conducted to determine LC_{50} values for the 16 EPA priority PACs (Jesus et al., 2022). Of the ~300 laboratory studies compiled ranging from 1-28 days in length, median LC_{50} values were below 1.0 $mg.L^{-1}$, apart from naphthalene and chrysene (Jesus et al., 2022). For other HMW PACs, multiple median LC_{50} values exceeded their water solubility limit at room temperature, therefore the authors concluded they would be unlikely cause acute toxic effects in the natural environment. Species sensitivity distributions generated from the study found that anthracene and pyrene were most potent in terms of acute toxicity (Jesus et al., 2022). Fewer studies have identified lethal concentrations for whole-oil or dissolved fractions of oil due to the complexity of evaluating chemical mixtures, however, mass mortality events in bivalves have been recorded shortly following major oil spills, including the *Exxon Valdez (*40 % mortality in blue mussels) and *Deep-water Horizon* (77% mortality in pacific oysters) crude spills (Soon and Ransangan, 2019). Regarding diesel, mortality

studies conducted using fractions of No. 2 Fuel Oil yielded 96-h LC_{50} values between 0.4-6 mg/L in multiple invertebrate species including molluscs (Wang et al., 2003).

Sublethal effects induced by PACs occur at concentrations much lower than concentrations that are associated with acute effects (NAP, 2003). Sublethal effects are usually detected by various biomarkers (King et al., 2021). The term biomarker can generally be defined as any functional measure of exposure or effect that is characterized at a sub-organism level of biological organization (Adams et al., 2001). Biomarkers are particularly useful for evaluating exposure to complex mixtures such as petroleum products because they can integrate effects of multiple chemicals and their metabolites that represent the bioavailable components of the mixture (Ricketts et al., 2003).

Sub-lethal effects at the physiological level in bivalves are often determined using endpoints related to shell-growth, feeding and respiration (i.e., filtration) rates, and condition index (CI). Many of these studies were conducted on mussels, primarily the blue mussel, *Mytulis Edulis.* As the shift towards using ecoystem sentinels has become more prevalent, studies using various species of clams and oysters have increased in number. Mussels and clams exposed to residual oil from spills showed reduced clearance (filtration) rates compared to samples collected from designated reference sites (Culbertson et al., 2008; Aarab et al., 2011). Other studies have also observed a dose-dependent effect between declining clearance rate and increasing concentration of PACs in mussel tissues (Widdows and Jonhson, 1988; Park et al., 2009; Toro et al., 2003). Morphological effects in bivalves are determined using histopathology to measure the degree of visible tissue abnormalities. Pathologies assessed often include but are not limited to: presence of adipogranular (ADG) tissue, epithelial tubule cell height, melanization and degeneration of kidney/liver tissue, and non-specific inflammatory lesions (Brooks et al., 2009). Multiple tissue atrophies in these categories have been observed following PAC exposure in bivalve tissues such as gill, digestive gland, mantle, adductor muscle and gonad (Yifei et al., 2019, Aarab et al., 2011).

Sublethal effects at the biochemical level have also been assessed in bivalves following exposure to PACs by measuring changes in activities of enzymes related to immune response, antioxidant defence and metabolism (Hampel et al., 2016). Quantifying enzyme activities related to biotransformation (CYP1A , GSTs, SULTs) and oxidative stress (catalases, peroxidases, heat shock proteins etc.) , are the most common targets

in bivalve biomonitoring studies. PACs and studies using various oil mixtures have been shown to induce the activity of these enzymes (Zacchi et al., 2019; dos Reis et al., 2020; Liu et al., 2010; Boutet et al., 2004; Yao et al., 2017). Reduction in lysosome membrane stability has also been observed in circulating hemocytes of bivalves following PAC exposure, which is used as an index of immune function (Bado-Nilles et al., 2008).

Research efforts in developing molecular biomarkers using “omics” approaches (transcriptomics, proteomics and metabolomics) for evaluating sub-lethal effects have greatly increased in recent years due to advancement in high-throughput technologies (Veldhoen et al., 2012). Alterations in the expression of genes, proteins and metabolites induced by contaminant exposure can aid in elucidating the mechanism of action and predicting adverse effects at the physiological level (Veldhoen et al., 2012). Candidate genes involved in physiological processes such as antioxidant defence, detoxification, cell protection, immunity and apoptosis have been analyzed in bivalves following PAC exposure (Ballachey et al., 2015). Multiple genes within the mentioned categories have been up-regulated in studies using mussels and oysters that were exposed to individual PACs and whole-oil fractions (Ballachey et al., 2015).

Genotoxicity is another sub-lethal endpoint at the molecular level that refers to effects associated with the structural integrity of DNA (Ballachey et al., 2015). DNA damage accumulation can serve as an early warning biomarker for susceptibility to mutations and carcinogenesis (Bandi et al., 2014). Quantification of DNA damage with various bioassays has been used to assess genotoxicity following oil spills in bivalve species (Hose and Brown, 1998; Bolognesi et al., 2006). Specific PACs have been classified as carcinogenic partly because of their tendencies to induce DNA damage (U.S EPA, 2010). Petroleum mixture exposure studies that assessed DNA damage using bivalves have primarily been performed with conventional crudes (Hamoutene et al., 2002; Taban et al., 2004; Laffon et al., 2006; Large et al., 2002). Results from these studies produced mixed results, suggesting that the DNA damage accumulation does not follow a typical dose-response relationship.

Although a substantial amount of research has been conducted to elucidate toxic effects following PAC and oil exposure in bivalves, the wide range of experimental conditions and biomarkers used makes it difficult to draw conclusions about the relative toxicity of different oil types, particularly at the biochemical and molecular levels. For this

reason, the purpose of the current study was to determine how Pacific oysters were affected by exposure to three different petroleum products: marine diesel , conventional crude and diluted bitumen. This was tested by measuring if the oil exposure caused induction of (1) Cytochrome P450A (CYP1A) enzyme activity, (2) mRNA expression of selected biotransformation related genes and (3) haemocyte DNA damage.

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Chapter 2.

Effects of Petroleum WAF exposure on Gene Expression and CYP1A Enzyme Activity

2.1. Introduction

The bioavailability of oil constituents to marine organisms varies significantly between different types of oil, such as marine diesel, conventional crude oil, and diluted bitumen (dilbit). Marine diesel, being a refined and lighter petroleum product, tends to have relatively higher bioavailability to marine organisms due to its lower viscosity and higher water solubility of most of its constituents. Marine diesel and other light fuel oils contain a greater percentage (~35%) of low molecular weight (LMW) PACs and monoaromatics, which are more water soluble and therefore initially more bioavailable to aquatic species (Kuppusamy et al., 2020). Conventional crude oil, while still relatively bioavailable compared to dilbit, is less so than marine diesel. Conventional crudes contain a similar proportion of aromatics while diblit blends have higher concentrations of asphaltenes (~10%) in addition to PACs and lower concentrations of saturates (40%) (Lee et al., 2015). Crude oil contains a mixture of hydrocarbons, including both lighter and heavier fractions. Some of the lighter fractions can evaporate relatively quickly upon exposure to the environment, reducing their bioavailability. However, the heavier fractions, which contain more complex and less soluble hydrocarbons, tend to persist in the environment longer (Wang et al., 2003). Diluted bitumen, or dilbit, poses unique challenges regarding bioavailability. Dilbit is composed of heavy bitumen mixed with diluents to facilitate transportation through pipelines. In the event of a spill, these diluents can evaporate quickly, leaving behind the heavier bitumen components (Comny et al., 2017). These components are less soluble and may form into dense, sticky residues that can sink in water (Dupuis and Ucan-Marin, 2015). This behavior can reduce the immediate bioavailability of dilbit to surface-dwelling marine organisms. However, over time, weathering processes can alter dilbit's properties, potentially increasing its bioavailability as it weathers and undergoes physical and chemical changes (Comny et al., 2017). In summary, marine diesel generally has higher bioavailability due to its lighter and more soluble nature, while conventional crude oil has intermediate bioavailability. Diluted bitumen, on the other hand, may have lower immediate bioavailability, but its complex

behavior during weathering can make its long-term impact on marine organisms more challenging to predict and assess.

Bivalves including the Pacific oyster have been used extensively as indicators of exposure to PACs due to their sessile nature; allowing for site specific monitoring, and their ability to accumulate contaminants quickly because of their high filtering feeding capacity (0.35-0.74 L per hour) (Wheat and Ruesink, 2018). Research efforts towards using bivalves for contaminant biomonitoring programs has led to a wide range of studies measuring the effects associated with exposure to single PACs, PAC mixtures and whole-petroleum products.

Sublethal effects of PAC exposure can be analyzed using a suite of available biomarkers (King et al., 2021). Biomarkers can be broadly defined as signals or biological responses at the sub-organismal level that are related to xenobiotic exposure (von der Oost et al., 2003). In toxicology, biomarkers can fall into three categories: 1) exposure biomarkers, which indicate uptake and accumulation of the contaminant into the target organism, 2) effect biomarkers which measure the nature and magnitude of functional response to the exposure, and 3) susceptibility biomarkers, which indicate heightened sensitivity of a sub-population to the effects of contaminant exposure (Banks et al., 2008). Advancements in molecular techniques have increased the utility of biomarkers that can be used as initial screening in monitoring programs and serve as early warning signals for toxicity (Hampel et al., 2014). Biochemical and molecular markers often target steps in metabolism and detoxification pathways following contaminant exposure, which include quantifying biotransformation enzyme or catalytic activity, protein abundance, and mRNA expression (Hampel et al., 2014).

2.1.1. Ethoxy-resorufin O-deethylase

Xenobiotic defence response following contaminant exposure is known to involve a series of biotransformation reactions to reduce the accumulation of hydrophobic constituents. Arguably, the most well studied mode of action regarding PACs in aquatic vertebrates is mediated by the aryl hydrocarbon receptor (AhR) and its several targets (Dupuis and Ucan-Marin, 2015). The AhR induction pathway is directly related to the ability of both vertebrate and invertebrate organisms to effectively biotransform and excrete xenobiotic metabolites via a series of Phase I and Phase II biotransformation reactions.

Phase I biotransformation reactions reduce the hydrophobicity of foreign compounds through either hydrolysis, reduction, or oxidation reactions (Siroka and Drastichova, 2003). Metabolites that are not excreted following Phase I reactions can be subjected to Phase II reactions, which further reduce the hydrophobicity of target compounds *via* conjugation reactions (Kennedy, 1995). Cytochrome P450 (CYP) mono-oxygenase systems are responsible for the majority of Phase I oxidation reactions (Siebert et al., 2017). CYP1A proteins are usually found at high concentrations in excretory tissues and those with higher lipid contents (e.g., liver, hepatopancreas). The vertebrate CYP450 1 (CYP1) enzyme family comprises of CYP1A, CYP1B, CYP1C, CYP1C subfamilies, where CYP1A and CYP1B are universally found in all vertebrates (Siebert et al., 2017). To date, evolutionarily related CYP1A genes in molluscs have not been confirmed, however, several studies have found CYP1-*like* genes in molluscan species such as *M. Edulis, C. brasiliana and C. gigas* that warrant further investigation for evidence of an AhR-related metabolic pathway in bivalves (Siebert et al., 2017). The AhR induction pathway can be activated by xenobiotics (e.g., PACs) binding to the AhR receptor in the cytosol, forming a ligand-receptor complex. It translocates to the nucleus, where gene regulatory elements are then activated to increase synthesis of CYP1A proteins (Lester et al., 1993). Glutathione S-transferases (GSTs) and sulfotransferases (SULTs) are key phase II enzymes that facilitate conjugation reactions and have been identified in bivalves (dos Reis et al., 2020). GSTs and SULTs act as a further line of cellular defence by increasing hydrophilicity and excretion of reactive metabolites produced by phase I biotransformation (Baird et al., 2005). These enzymes also assist in detoxification by recycling cell components that become damaged from oxidative stress (Van Veld et al., 1992).

PACs can act as AhR-agonists, stimulating the transcription of CYP1A along with several other genes known as the AhR gene battery (Nebert et al., 1994). Therefore, measuring CYP1A activity is a suitable biomarker of exposure and uptake of PACs by an organism (Jesus et al., 2022). High CYP1A enzyme activity (above basal levels) is recognized as an early warning sign for active phase I metabolism that can lead to cellular stress and other downstream effects associated with toxicity (Hodson et al., 1991). The most widely used assay to quantify CYP1A protein activity is the ethoxy resorufin-O-deethylase (EROD) assay. In this assay, the substrate 7-ethoxyresorufin-O-deethylase (EROD) is converted to resorufin (a fluorescent compound) through oxidation by CYP1A

(Sarkar et al., 2006). Resorufin fluorescence is quantified and used as to assess CYP1A activity.

Several studies have examined the total microsomal CYP450 content, levels of CYP1A enzyme activity or CYP1A protein activity levels using the EROD assay (Canova et al., 1998; Livingstone, 1998; Monari et al., 2007; Shaw et al., 2002; Snyder et al., 2001; Sole and Livingstone, 2005; Wootton et al., 2003). The results of these studies have rendered somewhat inconclusive data due to the low catalytic activity of bivalve CYP enzymes using EROD and BPH (benzo[a]pyrene hydroxylase) substrates and weak interference with CYP1A polyclonal antibodies (Chaty et al., 2004; Jonsson et al., 2004). These observations highlight the need for further characterization of CYP-like proteins that are sensitive to PAC induction and if they respond differently to various petroleum formulations (marine diesel, conventional crude and diluted bitumen).

2.1.2. Gene Expression

Advances in molecular technologies in recent years have led to the emerging field of toxicogenomics and its use in contaminant exposure and effects assessments (Hayes et al., 2014). Genotoxicants are characterized by their ability to induce mutations, alter chromosome structure (clastogenicity) and chromosome number (aneuploidy) (Hayes et al., 2014). The initial catalyst for these genotoxic effects is often related to a change in gene expression induced by contaminant exposure (Hayes et al., 2014). Analyzing genes affected by contaminant exposure provides a foundation when developing adverse outcome pathways (AOPs) for different species. An AOP can be described as a series of events, typically beginning with a molecular event, that are linked to toxic effects at the individual or population level (Ankley and Edwards, 2018). Once an AOP is established, the molecular initiating event can serve as a consistent molecular biomarker for potential downstream toxic effects (Ankley and Edwards, 2018). The AhR metabolic pathway described in section 2.1.1 illustrates an ideal example of an AOP. As mentioned previously, induction of the AhR initiates the expression of a battery of genes in addition to *CYP1A* that may be continuously or inappropriately expressed during development, leading to physiological abnormalities, as demonstrated in early life-stage fish (Dupuis and Ucan-Marin, 2015; Carls et al., 1999; Barron et al., 2004; Wu et al., 2012).

Although biotransformation pathways for xenobiotics in bivalves are not as clearly elucidated as in fish, several studies have observed changes at the transcript level for biotransformation-associated genes following PAC exposure (Lüchmann et al., 2015; Rewitz et al., 2006; Rust et al., 2004; Schlenk and Buhler, 1989; Siebert et al., 2017; Solé and Livingstone, 2005; Zhang et al., 2012). Recent sequencing and transcriptomic analysis of the Pacific oyster genome has also provided insight into candidate genes sensitive to stress induced by contaminant exposure. Genome expansions of heat shock proteins, phase I biotransformation genes (CYPs) and phase II biotransformation genes (*SODs, GSTs, SULTs*, *MDRs*) were observed in the sequencing of the Pacific oyster genome (Zhang et al., 2012).

Validation of candidate genes in bivalves can assist monitoring programs by designing a PAC-specific gene battery using emerging technologies such as the ToxChip PCR array, which has already been developed for model avian (Japanese Quail and Double Crested Cormorant), amphibian (African clawed frog), and fish (rainbow trout and fathead minnow) species (Zahaby et al., 2021). To contribute to establishing reliable genes as transcript biomarkers for PAC exposure and further elucidating if these genes are induced by oil exposure, the second objective of this study was to analyze mRNA transcript levels of 8 genes likely involved in PAC biotransformation (*ARNT, CYP2C50, CYP2C23, CuZnSOD, GST-theta-1, mGST-2, MDR1, hsc-70*) over time and at varying concentrations of each oil product (marine diesel, crude, and diluted bitumen).

2.2. Methods

2.2.1. Oyster Maintenance

Pacific oysters (approximately 2 y old) were obtained from Deep Bay Marine Field Station (Vancouver Island University, Centre for Shellfish Research, BC) and transported dry in a cooler to Simon Fraser University (<24 h). Oysters were acclimated for at least 7 d in 60 L aerated tanks with filtered seawater at 10°C. Oysters were fed algae: *Isocrysis galbana* (University of British Columbia) daily up to 24 h before the exposure (1 L of algae per 60 L tank at a concentration of 100, 000 cells/mL). Algae stock cultures were prepared and maintained in a Guillard's f/2 enrichment (GE) solution at 16°C under a 12/12 h light-dark cycle according to the natural seawater medium (HESNW) protocol described by the Canadian Centre for the Culture of Microorganisms (University of British Columbia). Algae

stock cultures were diluted every 2-3 days to maintain the desired cell density and health. The density of both the stock culture and diluted aliquots for oyster feeding were determined with a fluorescence spectrophotometer (Varian Cary Eclipse, Agilent Technologies) using a 12-point standard curve. Both a low-density curve ($15x10^3$–$350x10^3$ cells/mL) and high-density curve ($1.2x10^6$ – $7.0x10^6$ cells/mL) were generated based on haemocytometer cell counts to encompass the range of algae concentrations in stocks and diluted aliquots. The spectrophotometer was set to ex. λ = 341 nm and em. λ = 682 nm for both standard curves. Slit lengths and reading times differed between low-density (20 nm, 3 sec) and high-density (10 nm, 0.3 sec) curves to adjust for limits of fluorescence detection. Fluorescence readings were obtained using PS 4-sided polystyrene BRANDO macro-cuvettes (Sigma, Cat No. BR759053).

2.2.2. WAF preparation, exposure, and sampling

Oysters were exposed to the water accommodated fractions (WAFs) of each of the petroleum products in 6 L glass aquaria (15 oysters per tank) submerged in 10°C water baths for 7 d. There was a total of sixteen 6 L tanks for all exposures, resulting in four replicate tanks per WAF concentration. The three different unweathered oil products were marine diesel, crude oil, and diluted bitumen (obtained from Centre of Offshore Oil and Gas, Nova Scotia). WAFs were prepared in a 500 L distribution tank (Singer, 2001). Eight liters of an individual oil was added to 240 L of filtered sweater to generate a 1:30 oil/water ratio mixture (Singer, 2001). The prepared WAF was then sealed and mixed at 92 rpm with a stainless-steel stir rod for 24 h to encourage oil components to partition to the water phase. After mixing, the oil was allowed to settle for 1 h with the lid remaining sealed. Once settled, the dissolved fraction of the WAF was siphoned into the 6 L oyster exposure tanks at either 25, 50 or 100 % dilutions (plus a control group containing regular sea water). Oysters and water parameters (temperature and salinity) were monitored for 7 d until termination of the exposure. Oysters were not fed during the exposure.

Water samples were taken from each treatment group (control, 25 %, 50 %, 100 %) from the 6 L exposure tanks on d 0, 3 and 7 of the exposure. Water (1 L) from each treatment group was collected in amber bottles and treated with sodium azide (50 mg) as a preservative and stored at 4 °C for a maximum of 2 d before being sent to SGS Axys Analytical (Sydney, BC) for quantification of PACs by low resolution GC/MS (Mundy et al., 2019). Approximately 10 g of whole-oyster tissue samples were also collected for each

treatment group (approximately 2 oysters per tank) immediately after exposure termination on d 7, as well as d 21 and d 35 recovery timepoints, put on dry ice and stored at -80°C in 60 mL amber glass vials. Tissue samples were shipped to SGS Axys Analytical on dry ice for chemical analysis quantification of PACs by low resolution GC/MS (Mundy et al., 2019). A complete list of analytes for WAF and whole tissue samples is displayed in Appendix Figures A9-A26.

A subset of 2 oysters were sampled from each replicate tank associated with each WAF concentration (resulting in biological replicates of n = 8 oysters per treatment group). Sampled oysters were removed from their tanks, shucked and gills were dissected, weighed and immediately flash frozen using liquid nitrogen and stored in plastic vials at -80 °C for EROD and gene expression analyses. 30 mg of gill tissue was preserved for gene expression analysis and 300-500 mg of gill tissue was preserved for EROD analysis. The remaining live oysters in each tank were sorted into mesh bags corresponding to the WAF concentration and transferred back to the 60 L acclimation tanks. Sampling was repeated at 21 d and 35 d post-exposure to measure endpoints after a 14 d and 28 d recovery in uncontaminated seawater.

2.2.3. Gene expression

Primer design

Eight target genes were selected for qPCR analysis in gill tissue and are listed in Table 2.1. Primers with optimal annealing temperature of ~58-60ºC were designed using Primer3 and BLAST (National Center for Biotechnology Information Database). All primers were found for the target organism (*Crassostrea gigas,* GenBank Association No.29151) and set to amplify sequences of 80-150 base pairs. Maximum T_m difference was set to 1. For genes where an exon-exon junction could be spanned, this specification was also included. Optimal primer size was set to 20 nucleotides with a minimum GC content of 40% and a maximum GC content of 60%. Maximum GC in 3' end and Maximum Poly-X were both set to 3. Maximum 3'-end stability was set to 5 and Maximum self-complementarity was set to 3. For microsomal GST genes, these parameters were slightly modified as follows: T_m difference = 3, minimum GC content = 30%, maximum GC content = 70%, maximum Poly-X = 4, maximum GC in 3' end = 3, maximum self-complementarity = 4, maximum 3' end stability =5. All primer pairs were then checked for levels of hairpin

structure, self-dimerization and hetero-dimerization using Oligocalc Analyzer (Integrated DNA Technologies). Binding efficiency was considered acceptable if primer pairs did not exhibit ΔG values blow -9 kcal/mol. All primers were tested using a 5-point standard curve and deemed acceptable based on efficiency values (90-110%), a single melt-peak curve and goodness of fit to linear regression (R^2 >0.90). A total of 8 gene primer sets met these parameters and were used in subsequent qPCR experiments. A summary of validated primers and genes are listed in Table 2.1.

Table 2.1. List of validated primer sequences used for RT-qPCR experiments. For each gene, the National Center for Biotechnology and Information (http://www.ncbi.nlm.gov/) accession numbers, forward and reverse primer sequences (5'- 3'), annealing temperatures (T_m), efficiency values(%), and goodness of fit of linear regression (R^2) are provided for reference genes (EFU) and genes of interest (*ARNT, CYP2C50, CYP2C23, CuZnSOD, GST-theta-1, mGST-2, MDR1, hsc-70*).

Target Gene	Genbank Accession No.	Primer Sequence	Tm (°C)	Efficiency (%)	R^2
ARNT	XM_011451529	F: ACCAGTGTGGACGGTTCTTAC R: CACAAACAGGAAGCCATCGG	56.8	97.4	0.996
CYP2C50	XM_034474224	F: ACAGGACGAAGAGTGTGTTTAGG R: CATTCGGGATTCTCTGGTTGC	56.3	101.4	0.992
CYP2C23	XM_034474225	F: CGTCATTTGTTCCCTGTTGTTTGG R: CGAGGACCCACTTCCAGTTG	57.4	106.1	0.988
CuZnSOD	NM_001308888	F: GACGACCACGGAGAGGTGAATG R: GACCAATGACGCAGCAAGCG	59.7	92.2	0.995
GST-theta-1	XM_011443380	F: CGTGTCGGATTCTTGTGCTG R: TGTGTGGTGTGAACCTCGTC	57	93.7	0.972
mGST-2	XM_011415590	F: TTGGACATCTTGCCGTGGAG R:TGCATGTCTGAGGAGTTCTGC	56.4	93	0.992
MDR1	XM_011457017	F:GAAGTAGAAACTGAGGCGGAATG R:CTAAGCCCTCTCTGTATCTGGTC	55.9	126.5	0.946
hsc-70	XM_034445125	F: ATGCCAGAAAGACCCTGATGTG R: ATTGGGAGGTGGGACTGTGG	58	109.2	0.983
EFU (reference gene)	AB122066	F: GAGCGTGAACGTGGTATCAC R: ACAGCACAGTCAGCCTGTGA	57	109.4	0.990

RNA isolation and cDNA synthesis

RNA was extracted from gill tissue using the RNAeasy mini plus extraction kit (Qiagen © Cat No. 74034). Briefly, ~30 mg of tissue was homogenized using a mixer mill homogenizer (CMIX1, Qiagen ©, Mississauga, ON). The homogenized lysate was centrifuged at >8000 rpm and the supernatant was then transferred to a 2 mL gDNA eliminator collection tube. The gDNA flow through was collected and resuspended in 70% ethanol. The suspended flowthrough was then added to an RNAeasy spin column and subjected to 3 buffer wash steps and a final elution step in 50 µL of nuclease free water. Total RNA from the initial extraction was then further treated using TURBO-DNA-free™ kits (Invitrogen, ThermoFisher Scientific) to eliminate any remaining co-extracted genomic DNA. DNAse treated RNA samples were then stored at -80 °C until further analysis.

For quality assurance, RNA purity and total RNA concentrations (ng/µL) were measured using an Epoch BioTek Microplate Spectrophotometer (BioTek, Winooski, VT, USA). Total RNA concentrations were quantified using the optical density unit (OD260) and RNA purity was determined by the OD260/280 and OD260/230 ratios. Samples with OD260/280 ratios ranging between 1.8-2.1 were deemed as pure and therefore used in subsequent cDNA synthesis and RT-qPCR experiments. RNA integrity for 50% of samples was qualitatively assessed following the validation procedure described by (Dheilly et al., 2011) for absence of degradation using the Experion™ Automated Electrophoresis System and Experion software spectrophotometer readings (BioRad Technologies).

Reverse transcription cDNA synthesis was performed using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Mississauga, ON). DNase-treated RNA samples were briefly thawed on ice and appropriately aliquoted to obtain volumes for a 1 µg template. 4 µL of the 5x iScript Reaction mix, 1 µL of the iScript Reverse Transcriptase enzyme and the respective amount of nuclease free water were added to the 1 µg template to generate a 20µL reaction mix for each sample. Reverse transcription reactions resulted in cDNA samples with a final concentration of 1000 ng/µL for bitumen and 500 ng/µL for crude and marine diesel. cDNA samples were then stored at -20 °C until qPCR analysis.

Quantitative real-time polymerase chain reaction

All RT-qPCR experiments were performed using the Bio-Rad CFX384™ Real-Time PCR Detection System using hard-shell 384 well plates as per the manufacturer's instructions (Bio-Rad Technologies, Mississauga, ON). Each well contained the following components to make up a 10 µL master mix: 1 µL of each of the forward and reverse gene-specific primers; 5 µL of SsoFast™ EvaGreen® Supermix (Bio-Rad Technologies, Mississauga, ON); and 4 µL of template cDNA (1:40 dilution, 1000 ng/µL for bitumen samples and 1:80 dilution, 500 ng/µL for crude and marine diesel samples). cDNA samples were run in duplicate along with a 7-point standard curve and a no template control (NTC), both of which were run in triplicate. Thermocycler conditions were run as follows: activation at 95 °C for 30 sec followed by 45 cycles of (1) denaturation at 95 °C for 5 sec and (2) primer annealing at 60 °C for 5 sec. A melt curve analysis was conducted at an initial temperature of 60 °C and was increased by increments of 0.5 °C every 5 sec until a maximum temperature of 95 °C was obtained. The RT-qPCR data was normalized by applying the $2\Delta\Delta$Cq method, using elongation factor 1 (EFU) as the internal reference gene.

2.2.4. CYP1A activity

Microsome isolation

Microsomes from gill tissue were isolated according to Gourley and Kennedy (2009) and Siebert et al. (2017). Briefly, gill tissue was homogenized in 1:5 w/v ice-cold buffer (0.1 M TRIS-HCl at pH 7.4, 0.5 M sucrose, 0.15 M KCl, 1 mM DTT and 0.1 mM PMSF) using a glass-Teflon mortar and pestle system with ~7 strokes at 2500 rpm Homogenates were transferred to 1.5 mL microtubes and spun at 9000 *xg* for 30 min at 4°C. The supernatant was transferred to a fresh tube and was centrifuged on a Beckman Coulter Optima Max ultracentrifuge (UC) 100 000 *xg* for 70 min at 4 °C. Once again, the supernatant was removed, and the microsomal pellet was washed with 500 µL homogenization buffer without DTT and PMSF and subjected to ultracentrifugation at 100 000 *xg* for 20 min 4 °C. The supernatant was then discarded and microsomes were resuspended in assay buffer (0.1M TRIS-HCl at pH 7.4, 0.15M KCL and 20% glycerol) in a volume relating to the pellet size, ranging from 0.1-0.5 mL. Microsomes were then stored at -80°C until analysis for EROD activity.

EROD assay

Ethoxy resorufin O-deethylase (EROD) activity was determined using methods described in Gourley and Kennedy (2009) and Siebert et al.(2017). The production of the fluorescent product of the reaction, resorufin, by each microsomal sample was measured in duplicate using in black Costar™ 96-well plates (ThermoFisher Scientific). Assay reactions consisted of 300 µg of microsomal fraction, 0.1 M phosphate buffer (pH 7.4), 4 mM NADPH, and 10 µM resorufin (stock diluted in methanol at 1 mM). The amount of methanol in the standards was kept consistent with the amount of methanol in the sample wells. Samples were mixed with gentle agitation for 1 min with resorufin and incubated in the dark at 30 °C for 10 min. Then, 13.4 mM of NADPH was added to initiate the reaction. Blanks containing 150 µL of PBS were run in parallel with microsomal samples and resorufin standards (0-0.5 µM). The plates were scanned every 1 min for 60 min using a Molecular Devices SpectraMax M2e fluorimeter (Dowington, PA) at ex. λ = 530nm and em. λ = 585nm. A resorufin standard curve and protein standard curve was included on each plate. Protein content in each sample well was determined in duplicate following the BioRad™ Bradford Assay protocol, using bovine serum albumin (BSA) as the standard (0-1.4 mg/mL). To determine the resorufin production in fmol.min.mg protein in the samples, the amount of resorufin over an interval of time was divided by the amount of protein (mg) in each well.

Statistical analysis

Statistical Analyses were performed in JMP®, Version 16 (SAS Institute Inc., Cary, NC). Normalized expression values for each gene were plotted along a frequency distribution and normal quantile-quantile plots to screen for outliers and evidence of a non-normal distribution. Normality and equal variance were also confirmed using a Shaprio-Wilk goodness of fit test. Potential outliers were evaluated by plotting studentized residuals and omitted from the model if the residual value was ≥ 4. All datasets were log-transformed and re-tested for normality assumptions prior to running the model. A two-way Analysis of Variance (ANOVA) and Tukey's posthoc test was performed to determine statistical significance of least square means between each WAF concentration (0%, 25%, 50%, 100%), sampling time (7 d, 21 d, 35 d) and interaction effect. Since no EROD activity was detected, no statistical analyses were performed on this endpoint.

2.3. Results

2.3.1. PAC composition in water and oyster tissue samples

A total of 76 polycyclic aromatic compounds were quantified by LR GC-MS in both water and tissue samples, comprising of 20 parent PACs, 48 alkylated PACs and 8 dibenzothiophene (DBT) compounds. Low total PAC concentrations [TPAC] were detected in control seawater tanks (0 d = 99.12 ng/L, 3 d = 8.42 ng/L, 7 d = 13.54 ng/L) and in control oyster tissues (7 d = 94.14 ng/g, 21 d = 102.47 ng/g, 35 d = 90.88 ng/g). Marine diesel water samples showed the greatest cumulative loss in [TPAC] compared to the other petroleum products over the duration of the exposure for all WAFs, where high volatilization of lighter hydrocarbons occurred (25% WAF; D0 = 25, 000 ng/L, D7 = 20 ng/L; 50% WAF; D0 = 53, 000 ng/L, D7 = 35 ng/L; 100% WAF; D0 =150, 000 ng/L, D7 = 230 ng/L). (Figure 2.1). Predominant compounds in marine diesel water samples and tissue samples consisted of C_1-C_4 chain naphthalenes, C_1-C_2 phenanthrenes, C_1-C_3 fluorenes and C_1-C_3 biphenyls (Appendix Figures A.3-A.5). The most significant drop in water [TPAC] occurred between 0-3 d (Figure 2.1). Similarly, [TPAC] concentrations in oyster tissues exposed to marine diesel decreased in the post-exposure recovery phase (25% WAF D7 = 1020 ng/g, D35 = 138 ng/g; 50% WAF D7 = 2322 ng/g, D35 = 174 ng/g; 100% WAF D7 = 3949 ng/g, D35 = 258 ng/g), with the greatest decline occurring between d 7 and d 21 (Figure 2.2).

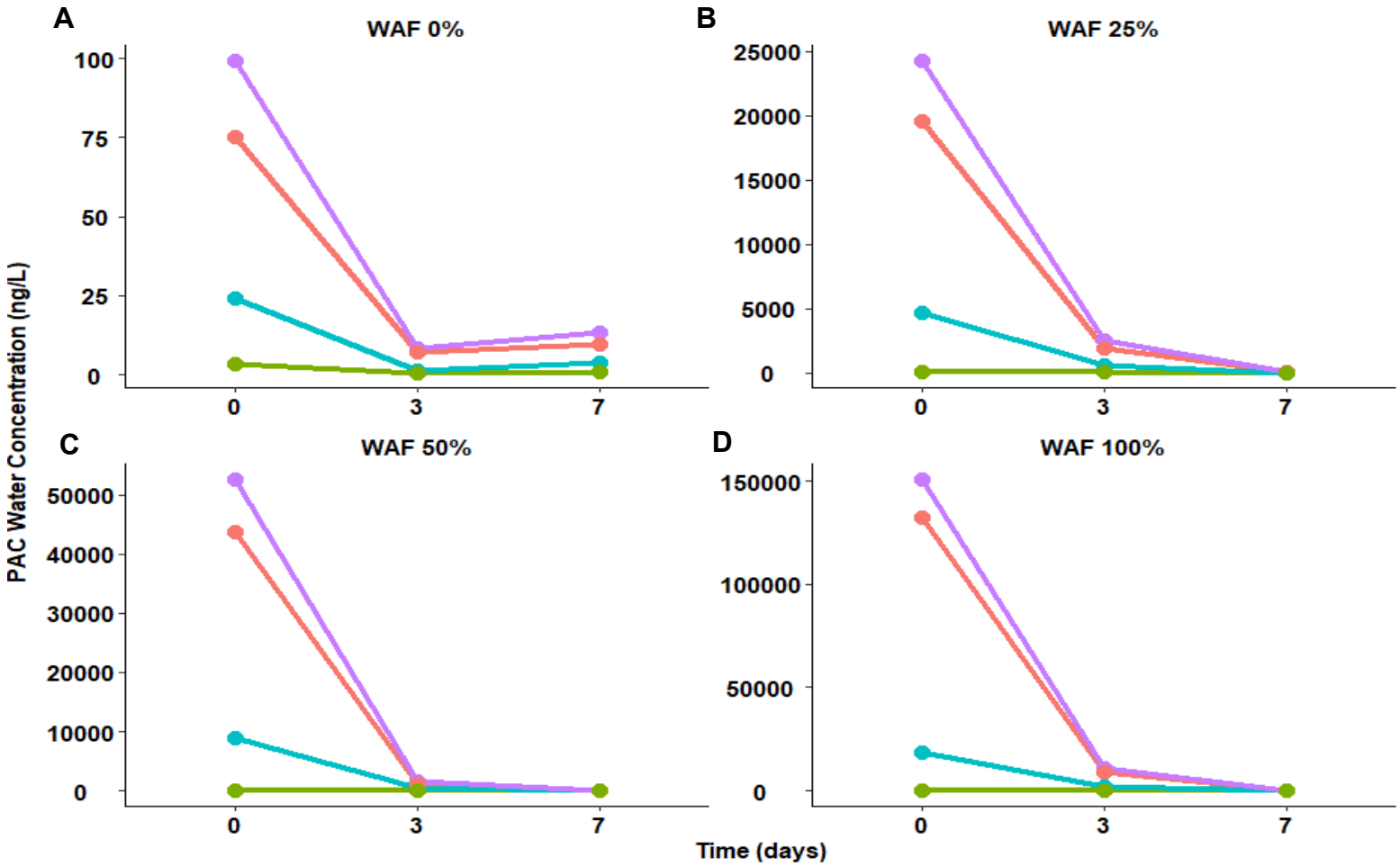

Figure 2.1. Cumulative concentrations (ng/L) of parent PACs ( ), alkylated PACs ( ) and dibenzothiophenes ( ) and all compounds combined ( ) quantified in marine diesel WAF samples on day 0, day 3 and day 7 of the exposure. 1L WAF samples were collected in amber glass bottles and treated with 0.5g of sodium azide for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).

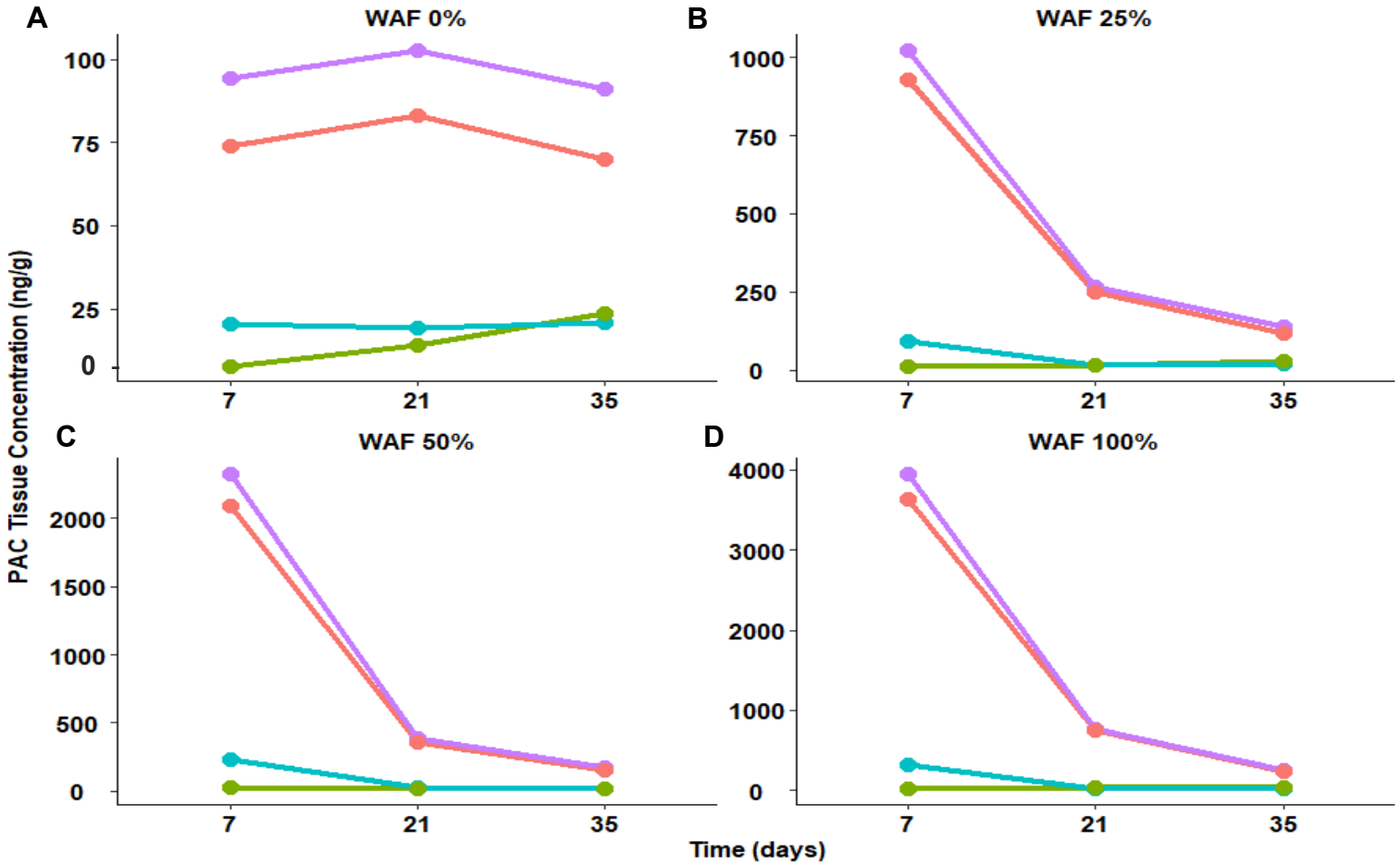

Figure 2.2. **Cumulative concentrations (ng/g) of parent PACs ( ), alkylated PACs ( ) and dibenzothiophenes ( ) and all compounds combined ( ) quantified in marine diesel tissue samples on day 7, day 21 and day 35 of the exposure. ~10g of oyster tissue samples were collected in amber glass bottles and immediately frozen on dry ice for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).**

In the crude exposure, low [TPAC] were also detected in control seawater tanks (0d = 485.46 ng/L, 3d = 89.69 ng/L, 7 d = 59.37 ng/L) and in control oyster tissues ([TPAC] at 7 d = 117.92 ng/g, 21 d = 381.75 ng/g, 35 = 307.55 ng/L). [TPAC] in crude water samples decreased during the exposure for all WAFs, (25% WAF D0 = 150,000 ng/L, D7= 280 ng/L; 50% WAF D0 = 240,000 ng/L D7 = 625 ng/L ; 100% WAF D0 = 350,000 ng/L, D7 = 24,000 ng/L Figure 2.3). The most significant decline in [TPAC] occurred between 0-3d for water samples. Predominant compounds in crude water and tissue samples were the same as marine diesel, consisting of C_1-C_4 chain naphthalenes, C_1-C_2 phenanthrenes, C_1-C_3 fluorenes and biphenyls (Appendix Figures A.6-A.8). [TPAC] concentrations in oyster tissues also decreased in the post-exposure recovery phase, with the most significant decline occurring between 7-21 d (25% WAF D7 = 4,517 ng/g, D35 = 463 ng/g; 50% WAF D7 = 14,700 ng/g, D35 = 325 ng/g ; 100% WAF D7 = 28,800 ng/g, D35 = 652 ng/g, Figure 2.4).

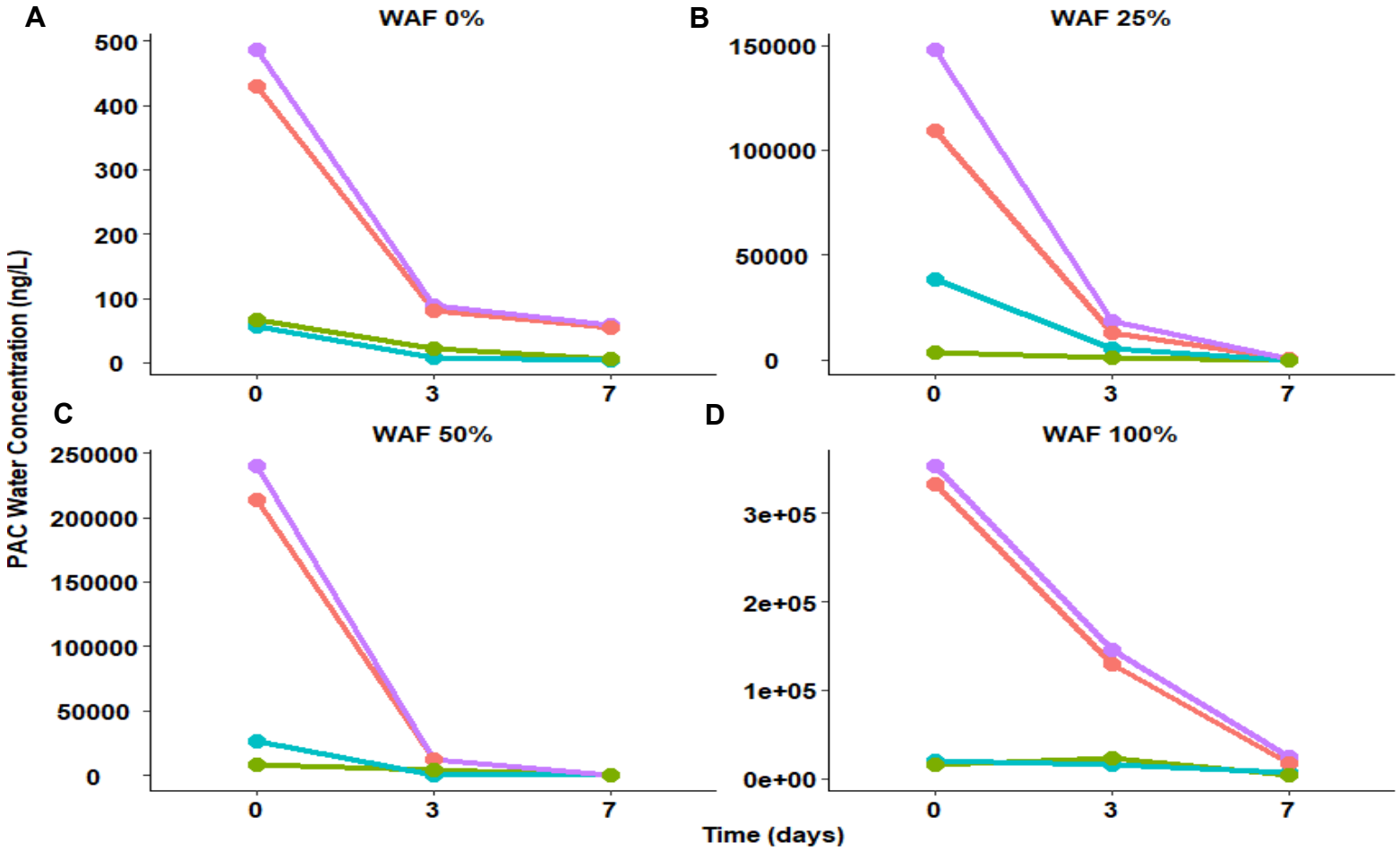

Figure 2.3. **Cumulative concentrations (ng/L) of parent PACs (■), alkylated PACs (■) and dibenzothiophenes (■) and all compounds combined (■) quantified in crude WAF samples on day 0, day 3 and day 7 of the exposure. 1L WAF samples were collected in amber glass bottles and treated with 0.5g of sodium azide for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).**

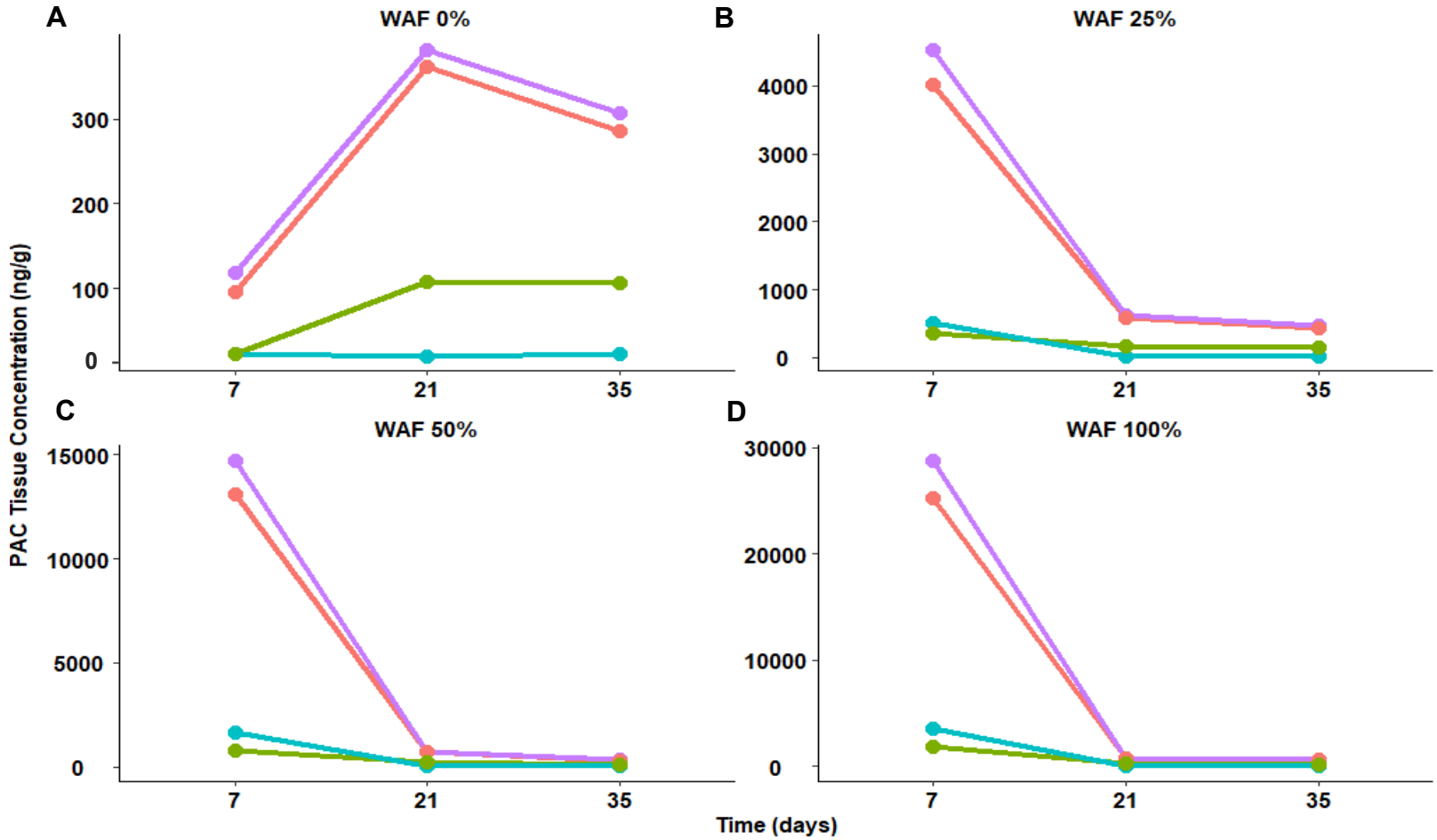

Figure 2.4. **Cumulative concentrations (ng/g) of parent PACs ( ), alkylated PACs ( ) and dibenzothiophenes ( ) and all compounds combined ( ) quantified in crude tissue samples on day 7, day 21 and day 35 of the exposure. ~10g of oyster tissue samples were collected in amber glass bottles and immediately frozen on dry ice for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).**

In the bitumen exposure, PACs were detected in control seawater tanks (0 d = 58.98 ng/L, 3 d = 390 ng/L, 7 d = 97.5 ng/L) and control oyster tissues (7 d = 68.08 ng/g, 21 d = 278.05 ng/g, 35 = 212.13 ng/g). [TPAC] in bitumen water samples decreased during the exposure for all concentrations, however to a lesser degree than crude and marine diesel WAFs (25% WAF D0 = 32,300 ng/L, D7 = 1,060 ng/L, 50% WAF D0 = 60,733 ng/L D7= 3,467 ng/L; 100% WAF D0= 128,000 ng/L, D7 = 28,132 ng/L, Figure 2.5). Predominant compounds in bitumen water and tissue samples consisted of C_1-C_4 chain naphthalenes, C_1-C_3 fluorenes and biphenyls, along with a greater variety of DBTs and chrysenes (Appendix Figures A.9-A.11). [TPAC] concentrations in oyster tissues also decreased in the post-exposure recovery phase, with the most significant decline occurring between 7-21 d (25% WAF D7 = 1,470 ng/g, D35 = 172 ng/g ; 50% WAF D7 = 1,921 ng/g, D35 =305 ng/g ; 100% WAF D7 = 2,036 ng/g , D = 229 ng/g, Figure 2.6).

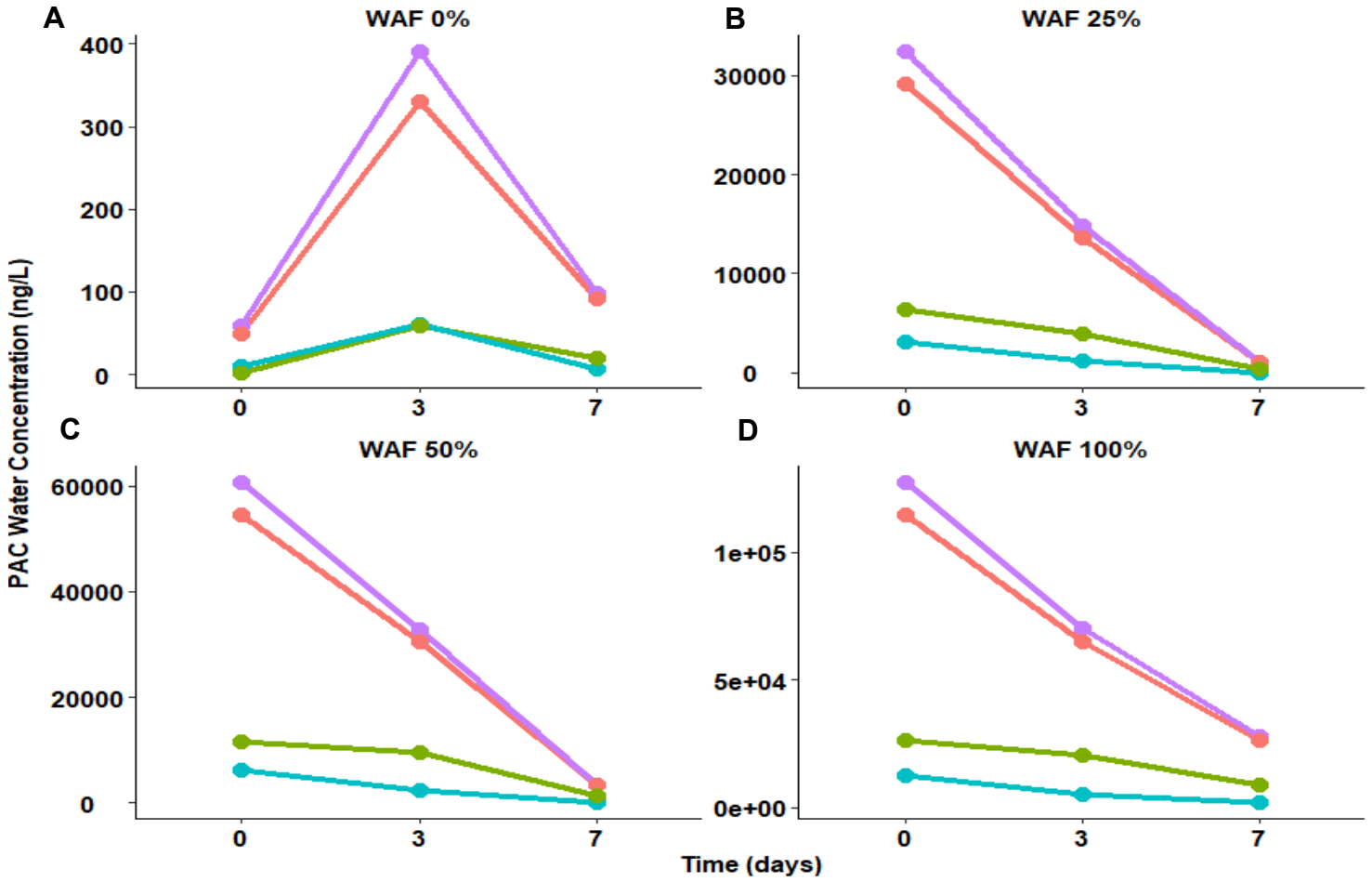

Figure 2.5. Cumulative concentrations (ng/g) of parent PACs ( ), alkylated PACs ( ) and dibenzothiophenes ( ) and all compounds combined ( ) quantified in bitumen WAF samples on day 0, day 3 and day 7 of the exposure. 1L WAF samples were collected in amber glass bottles and treated with 0.5g of sodium azide for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).

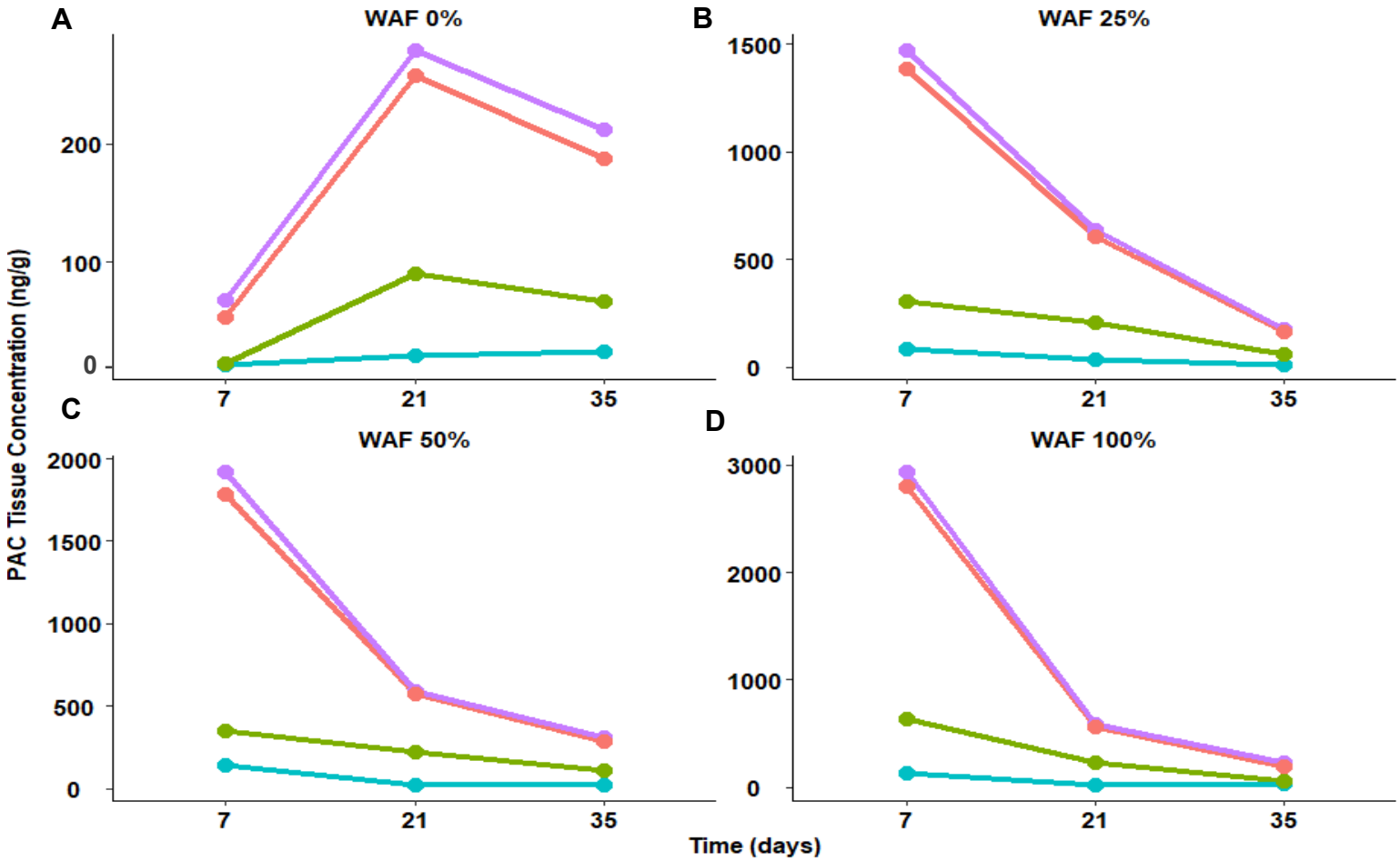

Figure 2.6. **Cumulative concentrations (ng/g) of parent PACs (■), alkylated PACs (■) and dibenzothiophenes (■) and all compounds combined (■) quantified in bitumen tissue samples on day 7, day 21 and day 35 of the exposure. ~10g of oyster tissue samples were collected in amber glass bottles and immediately frozen on dry ice for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).**

2.3.2. Gene Expression

Marine diesel exposure

For all 8 genes analyzed by qPCR, expression levels in controls were not affected by sampling time (7 d, 21 d, 35 d). Two genes in WAF exposed oysters (*m-GST-2, CYP2C50*) showed statistically significant differences in normalized expression at specific sampling times (Figure 2.7, 2.8), however, this did not translate to a consistent trend over time. Of the 8 genes analyzed by qPCR, no statistically significant differences between WAF concentrations (0%, 25%, 50%, 100%) within the same time group (Figure 2.7, 2.8, $p > 0.05$) were found.

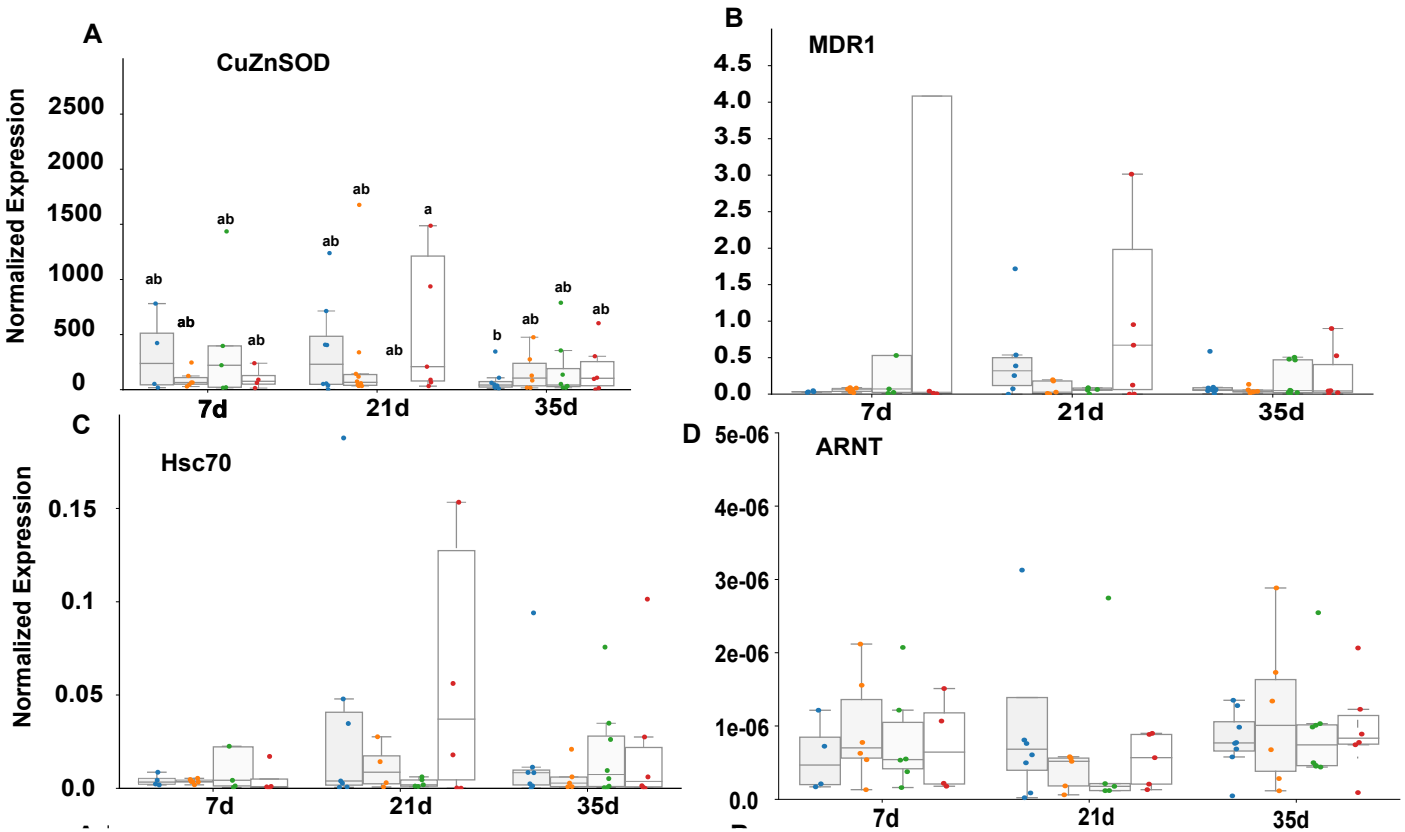

Figure 2.7. **Normalized mRNA expression levels for genes (A) CuZnSOD (B) MDR1 (C) Hsc-70 and (D) ARNT measured via qPCR in oyster gill tissues exposed to marine diesel (n=8 oysters per treatment group). Expression was calculated based on the 2ΔΔCq method using EFU as the internal reference gene. Boxplots present the the median values, lower 25th and upper 75th percentiles, minimum and maximum values (whiskers). A two-way ANOVA followed by a Tukey's post-hoc analysis was performed to determine significant differences between WAF concentration (%) , sampling time (days) and the interaction term. Significant differences between groups are represented by different superscript letters. WAF concentrations are represented by the colours inidcated : control or 0% (■), 25% (■) , 50% (■), and 100% (■).**

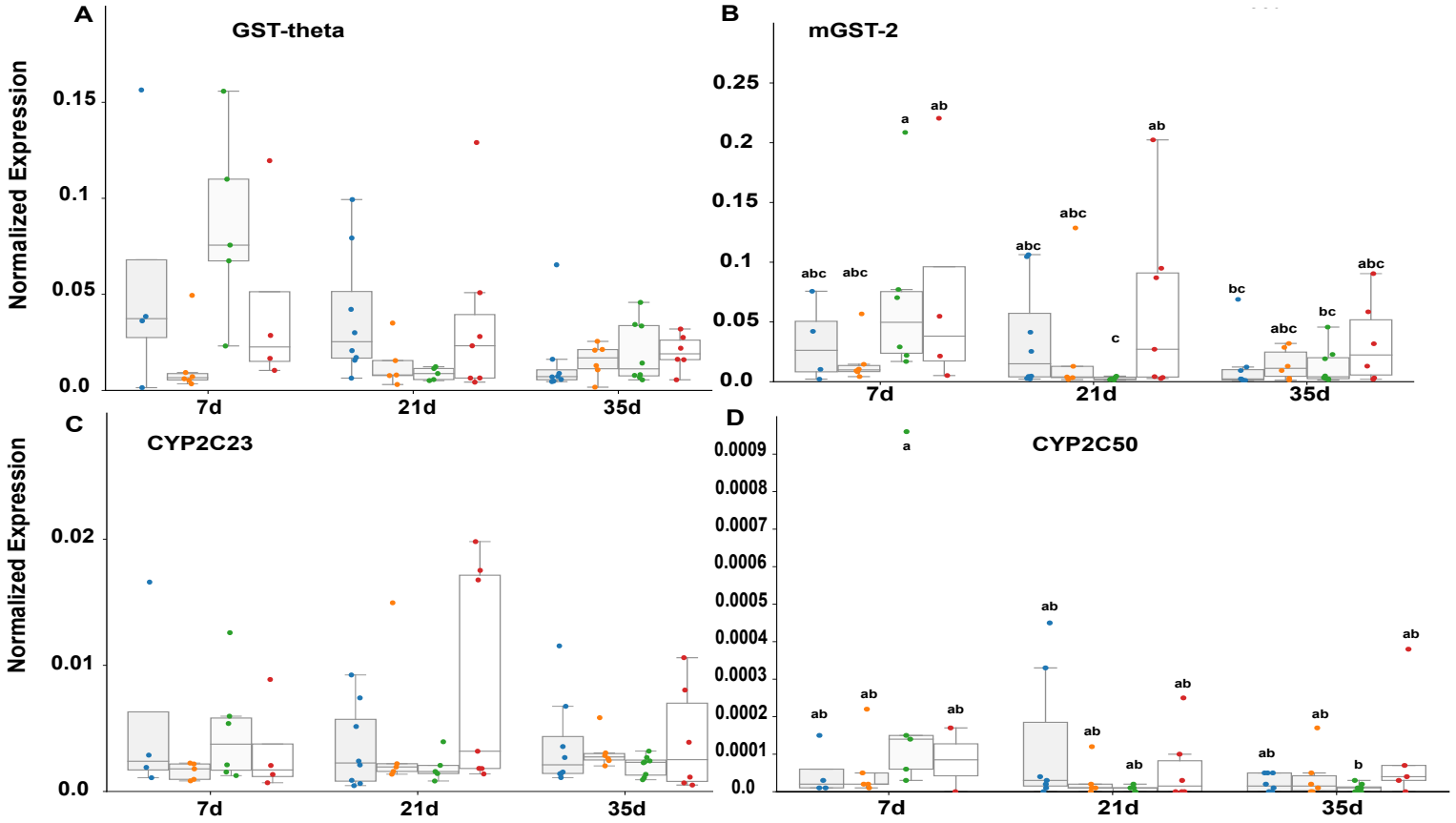

Figure 2.8 **Normalized mRNA expression levels for genes (A) GST-theta (B) mGST-2 (C) CYP2C23 and (D) CYP2C50 measured via qPCR in oyster gill tissues exposed to marine diesel (n=8 oysters per treatment group). Expression was calculated based on the 2ΔΔCq method using EFU as the internal reference gene. Boxplots present the the median values, lower 25th and upper 75th percentiles, minimum and maximum values (whiskers). A two-way ANOVA followed by a Tukey's post-hoc analysis was performed to determine significant differences between WAF concentration (%) , sampling time (days) and the interaction term. Significant differences are represented by different superscript letters. WAF concentrations are represented by the colours inidcated : control or 0% (■), 25% (■) , 50% (■), and 100% (■).**

Crude exposure

Sampling time affected controls for both glutathione genes (*GST-theta and m-GST-2*, Figure 2.10). For GST-theta, there was a statistically significant difference between 7 d and 21 d (Figure 2.10A, $p<0.001$, 95% CI = 0.41-2.82). For *m-GST-2*, there was a statistically significant difference between 21 d and 35 d (Figure 2.10B, $p = 0<0.001$, 95% CI = 0.08-2.54). In the WAF exposed oysters, 6 genes (*CYP2C23, CYP2C50, Hsc70, MDR1, m-GST-2, GST-theta*) showed statistically significant differences in normalized expression between individual treatments (7 d, 21 d, 35 d, Figure 2.3, 2.4), however this did not correspond with a consistent trend over time.

Of the 8 genes analyzed by qPCR, 3 genes (*ARNT, CuZnSOD, MDR1*) showed statistically significant differences in normalized expression between WAF concentrations within the same time group (0%, 25%, 50%, 100%, Figure 2.9). Despite individual treatment differences, no consistent concentration-dependent trends were observed.

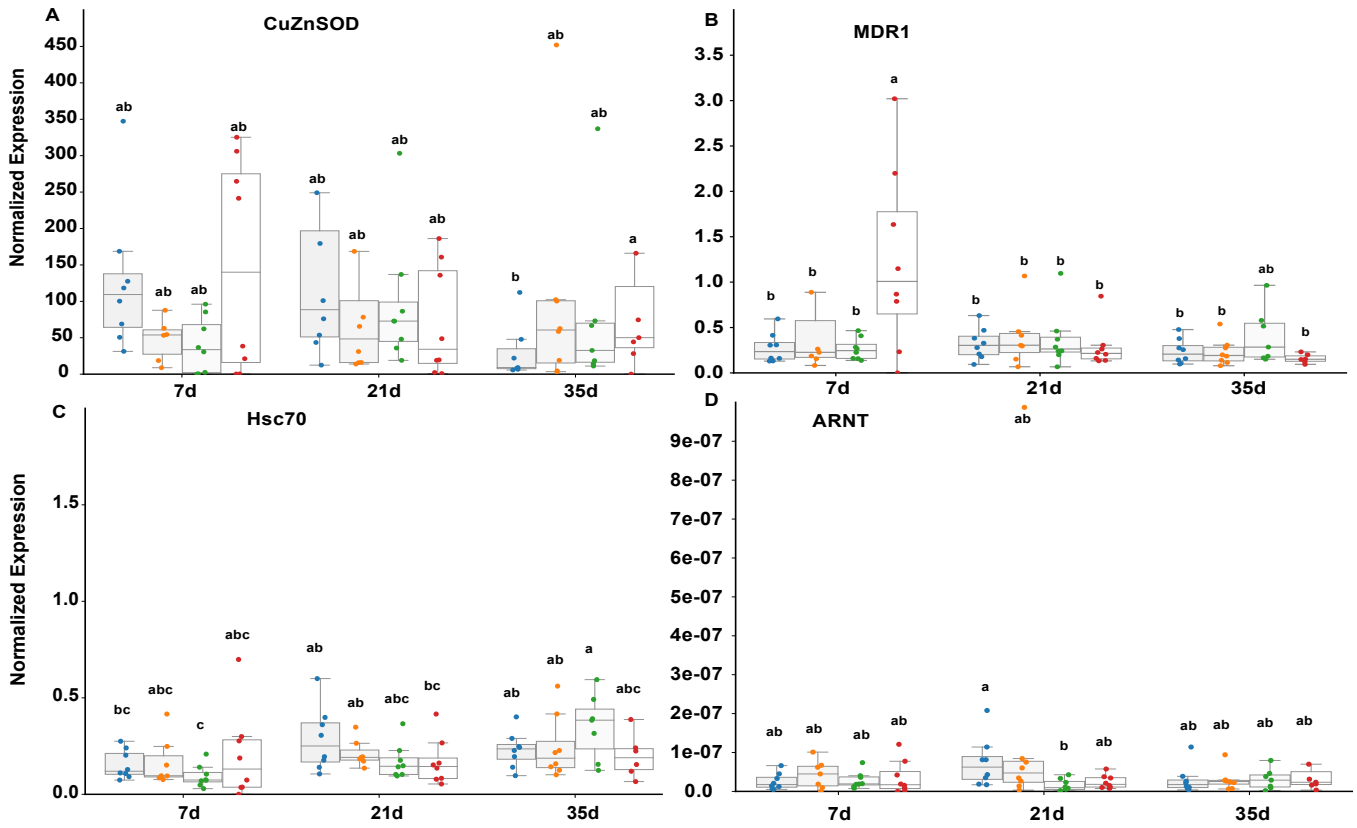

Figure 2.9. **Normalized mRNA expression levels for genes (A) CuZnSOD (B) MDR1 (C) Hsc70 and (D) ARNT measured via qPCR in oyster gill tissues exposed to crude oil (n=8 oysters per treatment group). Expression was calculated based on the 2ΔΔCq method using EFU as the internal reference gene. Boxplots present the the median values, lower 25th and upper 75th percentiles, minimum and maximum values (whiskers). A two-way ANOVA followed by a Tukey's post-hoc analysis was performed to determine significant differences between WAF concentration (%) , sampling time (days) and the interaction term. Significant differences betweem groups are represented by different superscript letters. WAF concentrations are represented by the colours inidcated : control or 0% (■), 25% (■) , 50% (■), and 100% (■).**

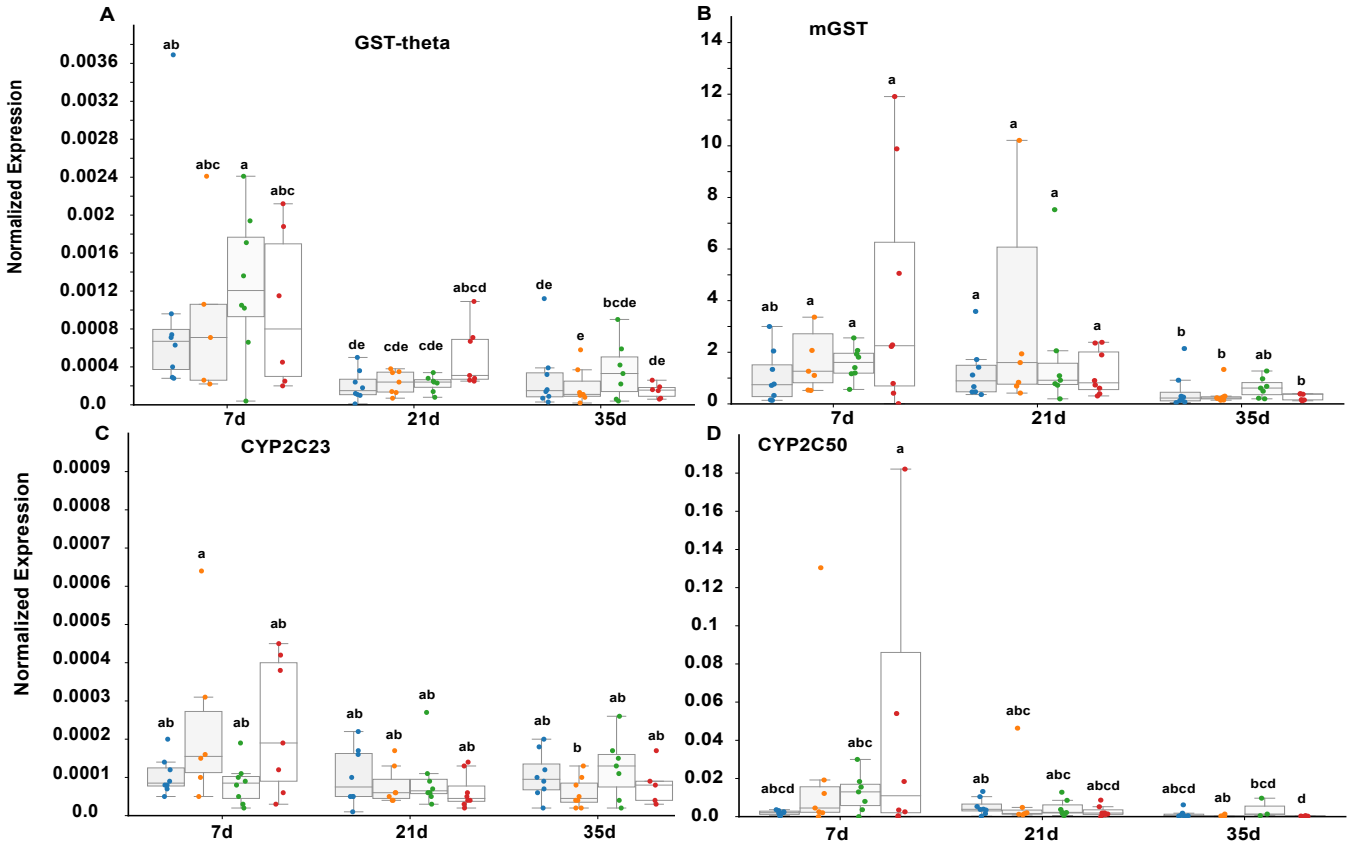

Figure 2.10. Normalized mRNA expression levels for genes (A) GST-theta (B) mGST-2 (C) CYP2C23 and (D) CYP2C50 measured via qPCR in oyster gill tissues exposed to crude oil (n=8 oysters per treatment group). Expression was calculated based on the 2ΔΔCq method using EFU as the internal reference gene. Boxplots present the the median values, lower 25th and upper 75th percentiles, minimum and maximum values (whiskers). A two-way ANOVA followed by a Tukey's post-hoc analysis was performed to determine significant differences between WAF concentration (%) , sampling time (days) and the interaction term. Significant differences between groups are represented by different superscript letters. WAF concentrations are represented by the colours inidcated : control or 0% (■), 25% (■) , 50% (■), and 100% (■).

Diluted bitumen exposure

For all 8 genes analyzed by qPCR, controls were not affected by sampling time (7 d-35 d). In WAF exposed oysters, three genes (*CuZnSOD, Hsc-70, CYP2C23*) showed statistically significant differences in normalized expression between individual treatments (7 d, 21 d, 35 d) (Figure 2.11, 2.12), however, this did not correspond to a consistent trend in expression over time.

Of the 8 genes analyzed by qPCR, two genes (*Hsc-70 and CYP2C23*) showed statistically significant differences in normalized expression between WAF concentrations within the same time group (0%, 25%, 50%, 100%). Despite individual treatment differences, no consistent concentration-dependent trends were observed.

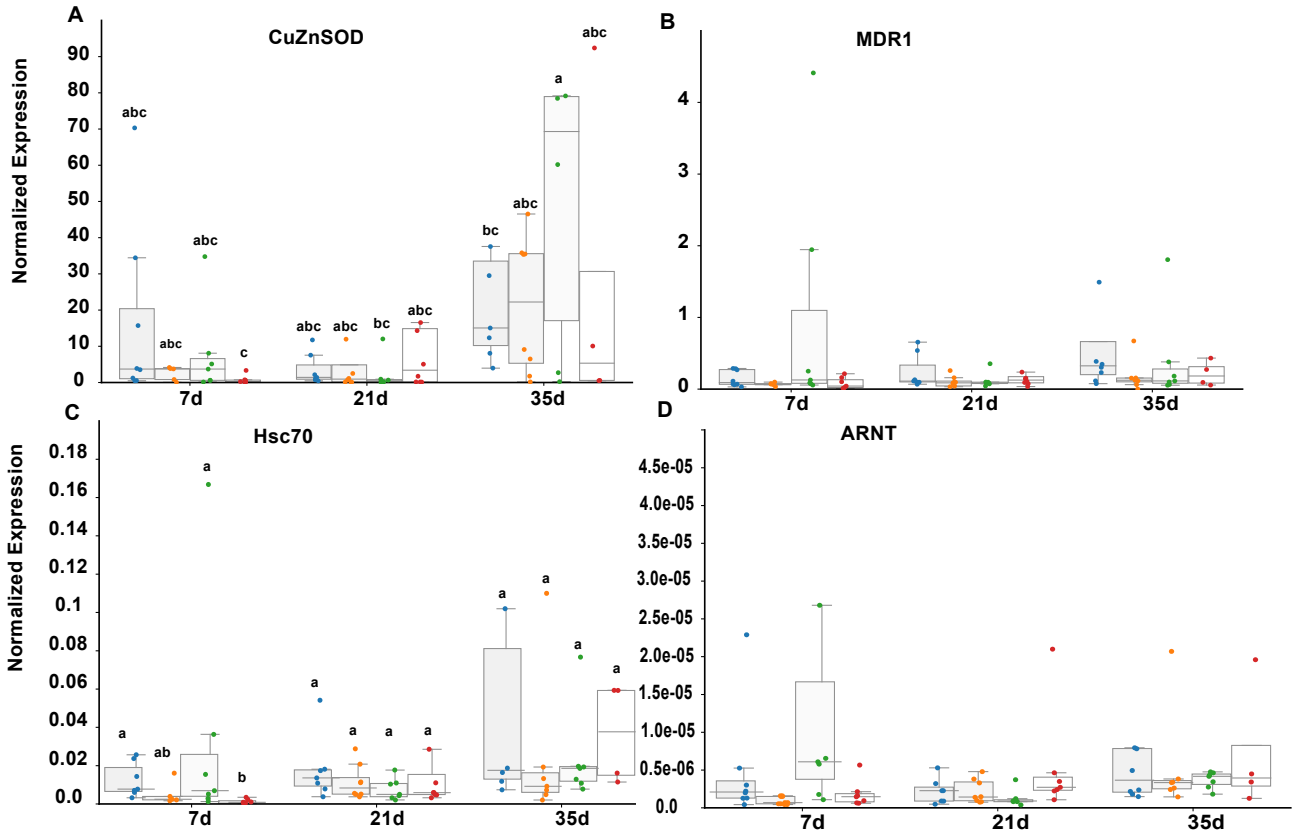

Figure 2.11. **Normalized mRNA expression levels for genes (A) CuZnSOD (B) MDR1 (C) Hsc70 and (D) ARNT measured via qPCR in oyster gill tissues exposed to diluted bitumen (n=8 oysters per treatment group). Expression was calculated based on the 2ΔΔCq method using EFU as the internal reference gene. Boxplots present the the median values, lower 25th and upper 75th percentiles, minimum and maximum values (whiskers). A two-way ANOVA followed by a Tukey's post-hoc analysis was performed to determine significant differences between WAF concentration (%) , sampling time (days) and the interaction term. Significant differences between groups are represented by different superscript letters. WAF concentrations are represented by the colours inidcated : control or 0% (■), 25% (■) , 50% (■), and 100% (■).**

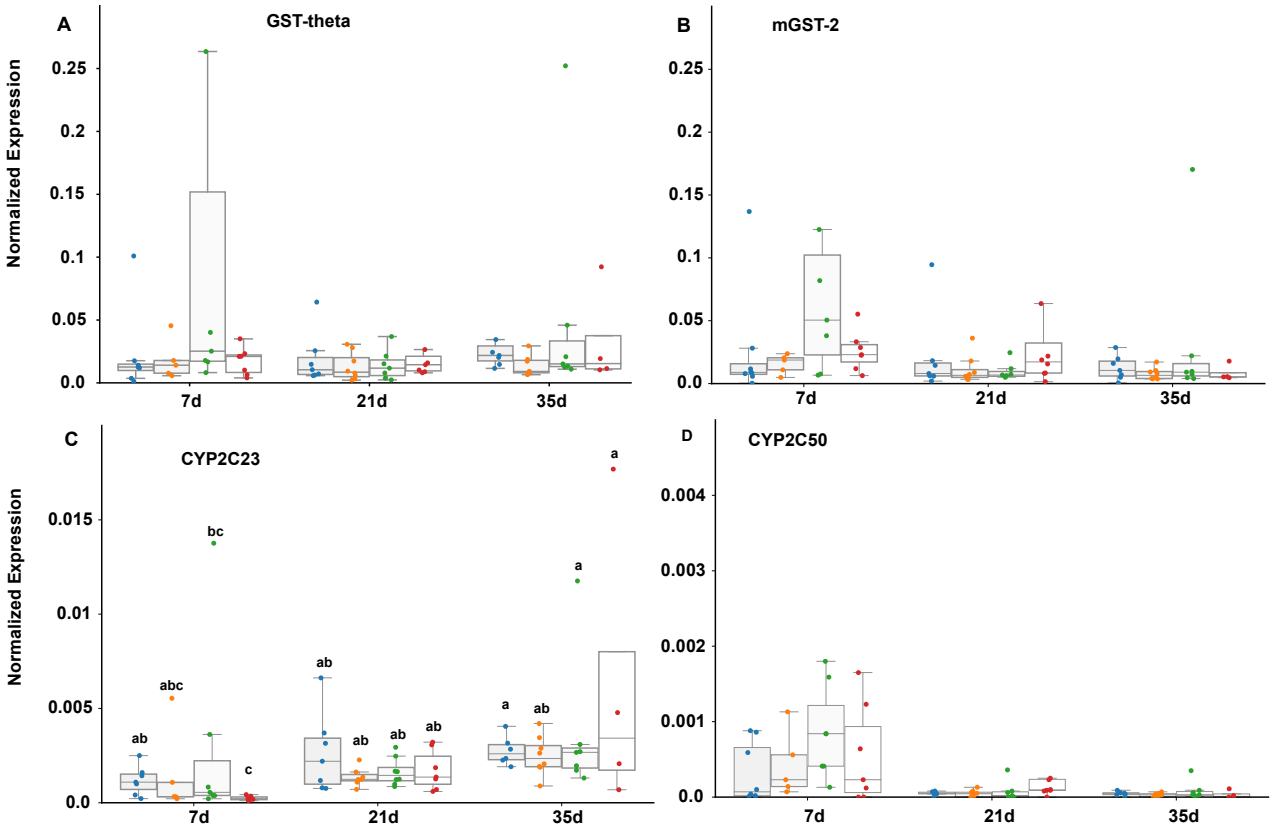

Figure 2.12. **Normalized mRNA expression levels for genes (A) GST-theta (B) mGST-2 (C) CYP2C23 and (D) CYP2C50 measured via qPCR in oyster gill tissues exposed to diluted bitumen (n=8 oysters per treatment group). Expression was calculated based on the 2ΔΔCq method using EFU as the internal reference gene. Boxplots present the the median values, lower 25th and upper 75th percentiles, minimum and maximum values (whiskers). A two-way ANOVA followed by a Tukey's post-hoc analysis was performed to determine significant differences between WAF concentration (%) , sampling time (days) and the interaction term. Significant differences between groups are represented by different superscript letters WAF concentrations are represented by the colours inidcated : control or 0% (■), 25% (■) , 50% (■), and 100% (■).**

2.3.3. CYP1A Enzyme Activity

No EROD activity above the detection limit of the assay was measured in gill tissues. To confirm this was due to lack of CYP1A baseline or induced levels in oysters, a positive control measuring EROD activity in sockeye salmon liver was performed following the same methodology. Salmon liver samples showed a typical increase in fluorescence over time upon initiation of the reaction (See results in Appendix Figure A1) and resulted in an EROD activity of 0.01 pM/min/mg.

2.4. Discussion

2.4.1. PAC content in WAFs and oyster tissues

PAC concentrations were quantified in WAF samples during the exposure phase on day 0, 3, and 7. When summarized by analyte group (total PACs, parent PACs, alkylated PACs and DBTs), alkylated PACs made up the largest fraction of the WAF mixture for all oils. The most abundant PACs detected in all WAFs were LMW hydrocarbons, specifically C_1-C_3 naphthalenes and fluorenes. Alkylated napthalenes represented the greatest proportion of the WAF for all oil types. This result is expected since naphthalene's are known to be readily soluble (log K_{ow} = 3.29) and will enter the water column easily (ATSDR, 2005). Concentrations of naphthalenes and other LMW alkylated PACs measured in marine diesel WAFs fell within the ranges of raw diesel fuel (Fuel Oil No. 2/Diesel) measured in a study conducted by the U.S EPA, which determined the complete chemical composition of several light fuel oils and conventional crudes (Wang et al., 2003). Similarly, the proportions of PACs measured in crude and bitumen WAFs aligned with fresh crude and bitumen quantified by the EPA (Wang et al., 2003). The EPA study also compared the composition of PACs in the fresh oils and 22% artificially weathered product, and found the proportions were similar, indicating that the WAFs generated in this study accurately reflect the composition of the original oil, and can be used to predict spill behaviour.

Although the specific PACs detected in the WAFs were common across oils, initial TPAC concentrations in the water were different. TPAC concentrations in crude WAFs were higher than both marine diesel and dilbit. This is likely due to the inherent physiochemical properties of each oil mixture. Marine diesel is the lightest oil because

volatile compounds such as BTEX and other monoaromatics make up the largest component of oil by mass, so fewer PACs would be present in the dissolved phase compared to crude (Dupuis and Ucan-Marin, 2015). Conventional crudes contain a greater proportion of 2-3 ring PACs, while dilbit source oil contains a greater proportion of 3-5 ring PACs, which are less soluble and would therefore be less likely to end up in the dissolved phase initially (Dupuis and Ucan-Marin, 2015).

TPAC concentrations measured in WAFs throughout the exposure phase showed a significant decline over time. This decline reflects a combination of natural weathering and uptake of PACs into oyster tissues. Marine diesel exhibited the greatest [TPAC] loss, particularly between 0-3 d, likely due to volatilization of lighter hydrocarbons. Volatilization can account for up to 75% of mass lost in condensates and ultra-light oils, and 20-30% of mass lost in light-medium oils when released into marine environments (NRC 2003). These rates are consistent with the cumulative [TPAC] reduction observed in this study. Typical mass loss due to evaporation is only around 10% for heavy oils (NRC 2003). Cumulative [TPAC] loss was observed in bitumen WAFs from 0-7 d, but at a slower rate than crude or marine diesel. This aligns with the bimodal weathering pattern often displayed by dilbit blends, where lighter diluent compounds initially evaporate quickly, followed by a slower rate as spilled oil becomes more viscous (Lee et al., 2015).

PAC concentrations were quantified in oyster tissues (whole-soft tissue) at the end of the exposure at 7 d, and in the recovery phase at 21 d and 35 d. Predominant PACs detected in tissues were the same PACs that were most abundant in the water samples (C_1-C_3 naphthalenes, fluorenes, and phenanthrenes) , showing that these compounds were readily taken up by the oysters. Additionally, the PAC composition profile of 7 d tissue samples closely resembled the profile of 0 d WAFs (Appendix A), indicating rapid uptake and storage of PACs. Alkylated naphthalene, fluorenes and phenanthrenes have also been the primary constituents quantified in tissues from other recent studies that measured PAC uptake following exposure to oil (Sundt et al., 2011; Breitwieser et al., 2018; Lourenco et al., 2015; Uno et al., 2010; Soriano et al., 2006; Thompson et al., 2017; Seguin et al. 2022; de Lapuente et al., 2015). For all oils in this study, tissue TPAC concentrations were significantly higher at 7 d compared to water concentrations for all treatment groups except for the 100% bitumen WAF, where water and tissue concentrations were similar; showing that bioconcentration of PACs can occur. Overall uptake of PACs also reflected what was in the WAF samples, with crude having the

highest [TPAC] accumulation in tissues, followed by marine diesel and then bitumen. The overlap in both composition and overall concentration of PACs between WAF and tissue samples reaffirms that bioavailability of PACs is directly related to the proportion of water-soluble PACs in the oil.

Tissue concentrations at 7 d fell within the medium-high contamination range for PACs (828-2512 ng/g) according to NOAA Mussel Watch Program body burden thresholds (Kasiotis and Emmanouil, 2015). Concentrations in tissues of the control oysters at 7 d also fell within the typical baseline range for PACs (300-500 ng/g) reported from urban sites around coastal U.S by the NOAA Mussel Watch Program (Kasiotis and Emmanouil, 2015). A sharp decline in tissue concentrations occurred between 7- 21 d once oysters were transferred to clean seawater in the recovery phase, followed by a steadier decline between 21 – 35 d for all oils. Since the same PACs accumulated in the oysters for all oils, similar trends in depuration rates between oil types would be expected in the recovery phase. Rapid bioaccumulation of PACs in bivalves has been well documented in the literature (Reviewed by Wallace et al., 2020). Consistency in accumulation of PACs between conventional oil and bitumen (Cold Lake Blend and Access Western Blend) was also observed in the blue mussel, where the highest PAC concentrations were recorded after 3 d of exposure to both products (Schmutz, 2018). Mussels from this study also exhibited a similar depuration process, where 50% of the hydrocarbons accumulated were eliminated during the first two weeks following exposure to both crude and bitumen products (Schmutz, 2018). Similarly, a recent study using water-soluble fractions of oil found that 90% of the accumulated hydrocarbons in clams were eliminated by the end of a 15 d depuration period (Li et al., 2020). Earlier studies in mussels, clams and eastern oysters where elimination rate constants of multiple PACs were calculated typically observed a biphasic elimination process; rapid depuration occurred in the first 8-20 d after exposure, followed by a slower rate (Gewrutz et al., 2002; Gobas and Morrison, 2000; Akcha et al., 2000; Bender et al., 1988; Sericano et al., 1996; Pruell et al., 1986; Tanacredi and Cardenas, 1991), which aligns with the depuration pattern observed in this study.

Elimination rate constants of the mentioned studies generally displayed an inverse relationship with PAC hydrophobicity, that is, more water-soluble compounds were eliminated quickly (Hwang et al., 2008). Gewrutz and colleagues concluded that 9 priority EPA PACs tested were being lost passively via diffusion over the gill surface in mussels

because elimination kinetics displayed trends typical of unmetabolized compounds (Gewrutz et al., 2002). Other early depuration studies with PACs and petroleum mixtures revealed that the length of exposure greatly influenced the rate of hydrocarbon loss during the initial depuration phase, where short term exposures (2-7 d) resulted in half-lives of approximately 5-6 d in bivalve tissues when subjected to a post-exposure depuration period (Mason, 1988; Farington et al., 1983; Lee, 1977). In contrast, long-term exposures (45-90 d) resulted in half-lives of 15-30 d in tissues (Mason, 1988; Boehm et al., 1996). Therefore, it is likely that the duration of exposure in the current study was not long enough for HMW PACs to become bioavailable in the water column via weathering and subsequently accumulate in oyster tissues at higher proportions. If a higher proportion of HMW PACs were accumulated during the exposure, depuration rates would have likely levelled off earlier in the recovery period.

Bivalves are also known to have a poor ability to biotransform PACs compared to fish and other marine vertebrates due to having lower quantities of detoxification enzymes; including those involved in the CYP540 monooxygenase system (Livingstone, 1996). One study compared the accumulation of parent and alkylated PACs following a heavy fuel oil spill (*Solar I spill)* in fish and bivalves (Uno et al., 2010). The authors found that concentrations of alkylated PACs were much higher in shellfish compared to their parent/unsubstituted counterparts, which were more prevalent in fish (Uno et al., 2010). Similarly, alkylated PACs detected in tissues of floater mussels were an order of magnitude greater than parent compounds following exposure to dilbit-contaminated water (Seguin et al., 2022). The retention of alkylated PACs aligns with low phase I enzyme capacity, which would be responsible for dealkylation via monooxygenase reactions (Livingstone, 1997; Moore et al., 1987). Other bivalve toxicokinetic studies with individual LMW PACs detected very low levels of their associated radiolabelled metabolites, suggesting that biotransformation is not the driving mechanism for elimination of these compounds in a short-term exposure scenario (James, 2000; Michel et al., 1995; Bustamente et al., 2012).

2.4.2. CYP1A enzyme activity

In this study, CYP1A-mediated enzyme activity (EROD) was below detection limits in oyster gill tissues. Levels detected in the sockeye liver displayed comparable activity (0.01-0.03 pM/min/mg) observed in other EROD studies using salmon (Goksoyr and

Larsen, 1991; Seubert and Kennedy, 1996; Blanc et al., 2010) confirming that that the lack of activity in oysters was not attributed to errors in the experimental protocol. In this study, various methods were used to obtain quantifiable EROD activity. First, gill tissue from 2 oysters were pooled to concentrate the microsomal pellet sample during the homogenization and resuspension steps. The molarity of the 7-ER stock solution was assessed with a spectrophotometer to ensure that it was fully dissolved, as the premature addition of 7-ER to wells can result in negligible readings. To prevent deterioration of the enzyme during the assay, a protease inhibitor (phenylmethylsulphonyl fluoride) and dithiothreitol (DTT) were added to homogenization buffers. Fresh microsomes were also tested immediately after dissection during the method development phase to assess the effect of flash-freezing gill tissue, and this did not make a difference in detecting activity levels.

The absence or low level of EROD activity has been reported in previous invertebrate studies in this regard. Early literature reviews on CYP450 systems in bivalves concluded that CYP1A enzymatic responses are completely absent or extremely low and variable, rendering them inconclusive for biomonitoring or environmental assessment (Livingstone, 1998; Lopez-Barea et al., 1998). An early phylogenetic study determined that a major split in the evolution and distribution of CYP450 enzymes occurred between deuterostomes (vertebrates) and proteosomes (molluscs and arthropods), where CYP2 families are represented in both evolutionary groups, but specific CYP1A was only found in vertebrates (Nelson, 1988). This distinction seems to hold true in more recent years, where upon the whole-genome sequencing of *C. gigas* in 2012, CYP2 sequences were the most abundant CYP family represented (Zhang et al., 2012).

Other studies have found what have been characterized as CYP1-*like* proteins in mussels and oysters, based on their similarities to vertebrate CYP proteins, however no evolutionary relationship has been established, suggesting that their metabolic functions may not be the same (Dejong and Wilson, 2014; Luchmann et al., 2015; Zanette et al., 2013). AhR homologues have also been identified in molluscs, but AhR target genes and agonist molecules were not present in these organisms (Butler et al., 2001; Hahn, 2004; Zanette et al., 2013). Another study assessed baseline EROD activity in conjunction with transcript levels of CYP1-*like* and CYP2-*like* genes in pacific oysters and found a positive correlation between CYP expression and enzyme activity in gill tissue (Siebret et al., 2017). However, EROD activity in this study was detected in the femtomolar range (137-

292 fmol/min/mg), which is still quite low in comparison to fish and other vertebrates (Siebret et al., 2017). Metabolite studies in fish and bivalves have revealed marked differences in the primary products formed following exposure to PACs; quinones were the most prevalent in shellfish (Uno et al., 2010; Livingstone, 1998; James, 2000; Michel et al., 1994) while other hydroxylated derivatives (phenols, diols, epoxides) were most prevalent in fish (Uno et al., 2010). Formation of quinones can be a combination of enzymatic and non-enzymatic processes such as interactions with glutathione and other endogenous reducing agents (Tierney et al., 2013), the latter of which could account for a greater contribution in bivalves.

The low or absent enzyme activity is also explained by the chemistry results; water-soluble PACs that accumulated in tissues at the highest proportions are not considered strong inducers of CYP1A and failed to produce a detectable signal. Alkylated naphthalenes and fluorenes did not induce CYP1A in early life-stage fish exposed to PAC mixtures (Hodson et al., 2017; Basu et al., 2001; Gagnon and Holdway, 2020). This has also been demonstrated with *in vitro* studies using teleost cell lines, where the magnitude of EROD induction was strongly dependent on PAC structure; LMW PACs (naphthalene, fluorene, anthracene) failed to induce CYP1A activity while 3-5 ring PACs (BaP, BeP, chrysene) demonstrated a dose-response relationship (Fent and Batscher, 2000). Fewer studies have compared PAC induction potencies in bivalves. EROD activity was also below the assay detection limit in oysters exposed to pyrene and fluorene (dos Reis et al., 2020), suggesting that the structure-activity relationship applies to bivalves.

Another reason for negligible activity could be because gill tissue is not the major site of CYP1A enzymes. Other studies have measured CYP1A activity in other tissues in bivalves such as the gonad, mantle and digestive gland. (Baussant et al., 2009 ; Luchman et al., 2015 ; Siebert et al., 2017; Lopes et al., 2012; dos Reis et al., 2010; Liu et al., 2010; Li et al., 2021). The digestive gland is considered the most closely related organ to the vertebrate hepatopancreas (Rebelo and Moreira, 2003), but it is also subjected to CYP inhibition by endogenous ligands (Snyder, 1999). Although these studies produced some variation in activity between tissues, all tissues consistently exhibited low metabolic capacity. Therefore, using gill is still a suitable tissue to assess the effects of PACs and oil, especially uptake rates since it has the greatest surface area exposed to water-borne contaminants (Gan et al., 2021). Overall, the combination of weak CYP inducers

representing the largest proportion of PACs accumulated in tissues and inherently low enzyme capacity in oysters resulted in negligible EROD activity.

2.4.3. Gene Expression

Phase I Biotransformation Genes

mRNA transcript levels for three Phase I biotransformation-related genes (*ARNT, CYP2C23, and CYP2C50*) were analyzed by qPCR in oyster gill tissues. A CYP1A-like gene was also initially included in the primer validation phase but was eventually eliminated from qPCR analysis due to low/negligible expression. This may partially explain the lack of EROD activity observed at the protein level in the gills, providing further evidence that oysters may not metabolize PACs *via* the AhR pathway, or that CYP-mediated induction did not occur. The ARNT homolog in this study was chosen as a potential candidate gene for the presence of an AhR-like pathway. In vertebrates, xenobiotics that bind to the AhR form a ligand-receptor complex with aryl hydrocarbon nuclear translocator (*ARNT*) once into the nucleus (Tierney et al., 2013). *ARNT* binding initiates the downstream transcription of several detoxification enzymes (Hahn, 2002). Overall, *ARNT* mRNA exhibited relatively low levels of normalized expression in all samples compared to the other genes examined. Despite PAC accumulation in tissues, this did not appear to affect the expression of this gene, as no clear temporal or WAF concentration-dependent effects were seen. Mussels injected with other potent vertebrate AhR agonists (e.g., ß-naphthoflavone and polychlorinated biphenyls) also did not have an influence on *ARNT* gene expression in the blue mussel (Zanette et al., 2013). Other studies that have tested affinity of various AhR homologs with typical vertebrate substrates found that they failed to bind and elicit a response in bivalves (Rebelo and Moreira, 2003; Hahn, 2002; Butler et al., 2001). The combination of negligible EROD activity and *CYP1A* gene expression in the gill also corroborates lack of AhR-mediated biotransformation.

CYP2 genes measured (*CYP2C50 and CYP2C23*) remained stable between 7-35 d in the controls for all oils. CYP2 expression was not influenced by increasing WAF concentration for any of the oils. Concentration-dependent affects may have been masked by the timeframe of induction at the transcriptional level. Time-course of induction is highly dependent on basal levels of expression in the target tissue, and crosstalk with multiple metabolic systems (Cajaraville et al., 2000). Few studies have examined natural

background expression levels of these genes. A controlled study compared expression levels of a CYP-2 isoform from field collected oysters with laboratory acclimated control oysters prior to an acute thermal stress exposure (Farcy et al., 2008). The authors found agreement in expression ranges between the two groups. Several studies have quantified expression levels of CYP-like transcripts in invertebrates following exposure to PACs and have produced a wide range of results, with upregulation observed as early as 24 h to up to 7 d (Luchman et al., 2015; Boutet et al., 2020; Lei et al., 2021; Zacchi et al., 2020; Tian et al., 2017; dos Reis et al., 2020), and the magnitude of induction was also highly variable. Despite the wide range of exposure conditions in these studies, mRNA expression was generally found to be sensitive to PACs, however expression often returned to background or control levels by the end of an experiment. (Bustamante et al., 2012 ; Boutet et al., 2020; Jenny et al., 2016; Tian et al., 2017; dos Reis et al., 2015). A study exposing scallops to phenanthrene for 96 h followed by a 96 h depuration period found that CYP2 transcript expression in the gill tissue was nearly identical to non-exposed controls in the depuration period (Piazza et al., 2016), aligning with the results observed in this study. There is a need for more studies that assess the effect of depuration on gene expression for PAC mixtures and whole-oil products. Transcriptomic analysis has revealed that the *C. gigas* genome contains 136 CYP genes, compared to mammals which have ~57 CYP genes (Zhang et al., 2012). Efforts to further characterize expression patterns and chemical inducers for these additional CYPs should be a focus of future biomarker research, as bivalves may have more diverse biotransformation pathways than mammals (and fish) that contribute to detoxification.

Phase II Biotransformation Genes

mRNA transcript levels for two Phase II biotransformation genes (*GST-theta-1, and mGST-2*) were analyzed by qPCR in oyster gill tissues. Glutathione-S-transferases (*GST*s) are a multifunctional family of enzymes that play a significant role in biotransformation of hydrophobic and polar compounds by catalyzing the conjugation of these with reduced glutathione. In general, Phase II genes were detected at higher normalized expression levels than the Phase I genes measured. This may be because GST enzymes have multiple roles in addition to Phase II metabolism, including mitigation of lipid peroxidation and catalysis of endogenous ligands. (Coughlan et al., 2002). In this study, *GST-theta-1* and *mGST-2* expression remained stable in control oysters during the entire sampling period. Like CYP2 genes, there was no clear evidence of induction in a

concentration-dependent manner for any of the oils. This is likely a reflection of the rapid depuration observed between 7-21 d in gill tissues and low uptake of potent inducers. Maximum induction may have occurred between the 0-7 d exposure period and transcript levels did not remain elevated once oysters were transferred to clean seawater in the recovery phase.

Early and relatively short-lived mRNA induction (24 h – 3 d) has been observed in other bivalve studies examining GST activity following PAC exposure (Zacchi et al., 2020; dos Reis et al., 2020; Liu et al., 2010; Boutet et al., 2004; Yao et al. 2017). Field-collected oysters from the *Deep Water Horizon* and *Don Pedro* oil spills found GSTs belonging to the theta class were highest in early deployments (i.e. closer to the time of the initial spill) and decreased in later deployments over a 2-month monitoring period (Jenny et al., 2016; Sureda et al., 2012). GSTs belonging to the omega and sigma classes have also been upregulated by 6-9 fold compared to controls following exposure to phenanthrene (1000 µg/L) in both mangrove oysters and pacific oysters, reaching maximum induction levels after 24 h (Lüchmann et al., 2015). The lack of upregulation at 7 d in our study is likely also attributed to the specific PACs that accumulated in the oysters, which differ from the field studies mentioned where the oils were significantly weathered for up to 6 months in the natural environment (Jenny et al., 2016; Sureda et al., 2012). Microsomal GSTs have received less focus in the literature because there is a larger abundance of cytosolic GSTs. There are 15 classes of GSTs that have been characterized across taxa with varying substrate/inhibitor specificity (Park et al., 2009). A laboratory study exposed scallops to benzo[a]pyrene, chrysene and a combination mixture and measured mRNA expression of 9 different GST classes (Yao et al., 2017). The authors found that sensitivity to B[a]P alone, chrysene alone or the combination mixture varied between GSTs. In all cases, maximum induction levels were reached between 1-3 d (Yao et al., 2017). Further efforts should be directed towards analyzing multiple classes of GSTs to determine their degree of sensitivity to PACs.

Multidrug Resistance Gene

mRNA transcript levels for the *MDR1* gene were analyzed by qPCR in oyster gill tissues. Multidrug resistance proteins belong to a superfamily of ATP-Binding Cassette (ABC) transporters responsible for efflux of xenobiotics and endogenous compounds (Luchman et al., 2015). Nine different MDRs exist within the ABC transporter family. In

mammals, P-glycoprotein (P-gp) is associated with the *MDR1* phenotype due to its large substrate base for even moderately hydrophobic compounds. There have been several studies using functional assays with ABC transporter substrates and inhibitors that indicate expression and activity of MDR efflux pumps in bivalve gills (Minie et al. 1995, Keppler and Ringwood, 2001; Smital et al., 1998). In this study, *MDR1* expression remained stable in controls over time. MDR1 was upregulated immediately after the exposure in the highest WAF concentration and decreased over time in crude oil, but no changes in expression were observed for marine diesel or diluted bitumen. Based on the relatively broad efflux function of this protein, it is not clear why the significant change expression only occurred in crude-exposed oysters, since all oil types contain moderately hydrophobic compounds that would be typical substrates for *MDR1*. There was also large variation in this treatment group at 7 d, so it is unlikely that components specifically crude affected expression of this gene in a consistent temporal and concentration-dependent manner.

Oxidative Stress Response Genes

mRNA transcript levels for two oxidative stress response genes (*CuZnSOD* and *Hsc-70*) were analyzed by qPCR in oyster gill tissues. These genes were stable throughout the sampling period in the control group for all oils. Any significant differences in expression that were observed in WAF exposed oysters occurred between the 21 d – 35 d. Heat shock proteins (HSPs) provide cellular protection through their chaperone activity. (Miller and Fort, 2018). Molecular chaperones facilitate protein stabilization, translocation, re-folding and degradation. SODs scavenge for free oxygen radicals by catalyzing the conversion of superoxide (O_2^-) into molecular oxygen and hydrogen peroxide (H_2O_2) (Gonzalez et al., 2005). Copper-zinc binding SODs (*CuZnSOD*) make up two of three evolutionarily conserved SOD families. Despite their generality, SODs and HSPs are useful indicators of cellular stress and are often co-expressed with phase I and phase II genes (Giuliani et al., 2013). Whole-genome characterization of *C. gigas* revealed large expansions in the HSP 70 family compared to other taxa (Zhang et al., 2012). In the current study, *CuZnSOD* and *Hsc70* were detected at the highest basal levels compared to all other genes measured. Biotransformation of PACs are a known source of ROS (Gan et al., 2021), that could induce upregulation of these genes. Another study using pacific oysters observed a two-fold increase in *CuZnSOD* expression after 7 d of exposure to water-soluble fractions of oil and a continued increase after 21 d (Boutet et al., 2004).

Similarly, Hsc-70 expression remained high during for the entire exposure duration (Boutet et al., 2004). On the contrary, a study that measured both enzymatic and transcriptional activity of selected detoxification genes in mussels following exposure to PAC mixtures observed no change in *CnZnSOD* at the mRNA level (Giuliani et al., 2013). Since there was not strong evidence of active metabolism occurring in the gill during the recovery phase in the current study, upregulation towards the end of the experiment may be attributable to mitigating cellular stress following depuration (Li et al., 2020; Giuliani et al., 2013). The expansion of antioxidant and oxidative stress response sequences in the bivalve genome likely contributes to their robustness and adaptability to oil-contaminated environments. Although both *CuZnSOD* and *Hsc-70* have been characterized as sensitive to hydrocarbon exposure and are routinely monitored in aquatic toxicity studies with bivalves, it is important to recognize that they are not petroleum or hydrocarbon-specific biomarkers. Rather, they are a consequence of cellular stress and an antioxidant response that can be broadly induced by other classes of contaminants including PACs (Snyder et al., 2001). For this reason, it is recommended that for studies attempting to elucidate mechanisms of action for detoxification in bivalves, these genes should be consistently analyzed along others that have greater specificity to the target group of contaminants.

2.5. Conclusions

This study is the first to examine CYP1A enzyme activity and gene expression in adult Pacific Oysters exposed to the WAFs of three different oil types. The results provide insight into the degree of PAC uptake, bioconcentration, and depuration of these contaminants post-exposure in tissues. LMW PACs (C_1-C_3) and their alkylated homologs were detected at the greatest proportions in the WAF samples, and this corresponded directly with PACs that were most abundant in oyster tissues. The composition profile was consistent across all three oils, indicating that bioavailability of PACs in the dissolved phase is similar across oil types in a short-term exposure scenario. The concentrations, however, were greatest in crude oil for both water and tissue samples. This reflects the composition of other components in the oil that influence the weathering and partitioning of PACs into the aqueous phase. Marine diesel is made up of a larger proportion of light components that are susceptible to rapid volatilization, resulting in a lower proportion of bioavailable PACs. Conversely, heavier components in bitumen are less water-soluble and reduce bioavailability to tissues in the short-term.

Tissue chemistry data also show that oysters rapidly bioaccumulated water-soluble PACs, as tissue concentrations were higher than water samples at the end of the exposure period on d 7. During the depuration phase, PACs were rapidly eliminated, with an exponential decrease in TPAC between 7 d – 21 d. These results align with other studies that have monitored depuration following exposure to oil. This suggests that unless exposure is continuous at concentrations that exceed the threshold rate of depuration, oysters will effectively eliminate most LMW compounds.

An overall lack of AhR and CYP-mediated activity at both the enzyme level (negligible detection of EROD) and mRNA level for all oil exposures was observed. Extremely low or absent EROD activity has been a consistent challenge in previous bivalve studies. Likewise, the expression of CYP2 genes did not appear to be correlated with PAC concentrations in tissues. The lack of induction observed at both the enzyme and mRNA level suggest the AhR pathway may not the primary biotransformation system in the gills and that the PACs accumulated were not inducers of CYP-mediated metabolism. Alternative metabolic pathways should continue to be investigated to validate transcript biomarkers, ideally using high-throughput methods (RNA-seq, microarray, proteomics) that allow identification of clusters of genes that could be co-expressed in multiple metabolic pathways.

Phase II genes were present at higher background levels than Phase I genes, therefore may be more likely to produce a consistent detectable signal. Antioxidant and heat shock genes are good indicators of cellular stress that may be a downstream product of PAC biotransformation but are not PAC specific on their own. Future studies with the goal of validating biomarkers should continue to corroborate transcript expression with protein or enzyme activity to further elucidate the magnitude and time-frame of induction.

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Chapter 3.

Effects of Petroleum WAF exposure on DNA Integrity

3.1. Introduction

The hallmarks of genotoxicity were outlined in Chapter 2 (section 2.1.2). In addition to modifying gene expression, genotoxic chemicals can alter chromosome structure and cause DNA damage in the form adducts and strand breaks (Hayes et al., 2014). As mentioned previously, some PAC metabolites can lead to DNA adduct formation by either direct covalent binding to DNA, or through the production of ROS species that intercalate with DNA (Baird et al., 2005; Santana et al., 2018). Oxidized metabolites, particularly epoxidated PACs, cause DNA lesions that can also result in chromosomal strand breaks (Hwa et al., 2018; Tarantini et al., 2009). Certain PAC intermediates can also completely evade recognition by DNA repair enzymes once bound to DNA (Hwa et al., 2018). Generally, genotoxicity from strand breakage occurs when the capacity of DNA repair mechanisms in the organism is exceeded and mutations occur (Dupuis and Ucan-Marin, 2015).

Common methods used to detect DNA damage in environmental and health science applications include the micronucleus assay, anaphase aberration test and the comet assay (also known as the single-cell electrophoresis assay) (Bolognesi and Cirillo, 2014). The micronucleus and anaphase aberration assays target defects in mitosis that can be used as an index for chromosomal damage during cell division (Barsiene et al., 2000; Hose and Brown, 1998), while the comet assay detects single-strand breaks in individual cells (Kohn, 1991). Of these methods, the micronucleus and comet assays are currently standardized by OECD guidelines (OECD TG guideline 489) for assessing genotoxicity in organisms (Zhang et al., 2018). The comet assay detects strand breaks using gel electrophoresis; DNA fragments from strand breakage migrate faster along the gel away from the nucleoid body of the cell, forming a “comet” tail (Bandi et al., 2014). The amount of accumulated DNA in the tail can be quantified to measure the degree of DNA damage in a particular cell (Bandi et al., 2014). The comet assay has unique advantages compared to the other assays mentioned because of its high sensitivity for measuring DNA integrity in single cells and its versatility in terms of being able to perform the assay

on many different cell types (de Lapuente et al., 2015). Additionally, the comet assay can be performed in either neutral or alkaline conditions depending on the desired sensitivity. The alkaline version of the comet assay can detect lower levels of DNA damage than the neutral version since it detects both double and single-strand breaks, while the neutral comet assay only detects double-strand breaks (Sing et al., 1988). Damage induced by oxidized PAC metabolites tend to produce single-strand breaks and can therefore be quantified by the alkaline-comet assay (Tarantini et al., 2009). An advantage of using the comet assay over other standardized methods is that the cells do not have to be actively dividing. This is particularly useful for testing in bivalves, as they have low rates of cell division (Pérez-Cadahía et al., 2004).

Assessment of DNA damage in bivalves using the comet assay has been performed in laboratory-based exposures to single PAC constituents (Large et al., 2002; Aksha et al., 2000) and petroleum mixtures (Brooks et al., 2009; Sarker et al., 2017), as well as field-based monitoring programs following oil spills (Perez-Cadahia et al., 2004; Hamoutene et al., 2002). A significant portion of these studies have focused specifically on DNA damage following exposure to benzo[a]pyrene (B[a]P), which is arguably the most well-researched PAC regarding DNA damage because of its classification as a human carcinogen (CEPA, 1997). B[a]P-induced DNA damage has been reported in oysters, clams, and mussels (reviewed by Lee et al., 2003). Oil exposure studies that assessed DNA damage using bivalves have primarily been performed with conventional crudes (Hamoutene et al., 2002; Taban et al., 2004; Laffon et al., 2006; Large et al., 2002). Considering the complexity and variability in the composition of petroleum formulations, additional studies with other types of oil would further contribute quantifying constituents that are more likely to induce DNA damage in susceptible marine organisms. The objective of this study was to measure DNA damage in the Pacific oyster using the comet assay following exposure to three different petroleum products (marine diesel, crude, and diluted bitumen) and to determine if DNA damage is repaired following cessation of exposure.

3.2. Methods

3.2.1. Oyster Maintenance

Pacific oysters (approximately 2 y old) were obtained from Deep Bay Marine Field Station (Vancouver Island University, Centre for Shellfish Research, BC) and transported

dry in a cooler to Simon Fraser University (<24h). Oysters were acclimated for at least 7 d in 60 L aerated tanks with filtered seawater at 10°C. Oysters were fed algae: *Isocrysis galbana* (University of British Columbia) daily up to 24 h before the exposure (1 L of algae per 60 L tank at a concentration of 100 000 cells/mL). Algae stock cultures were prepared and maintained in a Guillard's f/2 enrichment (GE) solution at 16°C under a 12/12 h light-dark cycle according to the natural seawater medium (HESNW) protocol described by the Canadian Centre for the Culture of Microorganisms (University of British Columbia). Algae stock cultures were diluted every 2-3 days to maintain the desired cell density and health. The density of both the stock culture and diluted aliquots for oyster feeding were determined with a fluorescence spectrophotometer (Varian Cary Eclipse, Agilent Technologies) using a 12-point standard curve. Both a low-density curve ($15x10^3$–$350x10^3$ cells/mL) and high-density curve ($1.2x10^6$ – $7.0x10^6$ cells/mL) were generated based on haemocytometer cell counts to encompass the range of algae concentrations in stocks and diluted aliquots. The spectrophotometer was set to ex. λ = 341 nm and em. λ = 682 nm for both standard curves. Slit lengths and reading times differed between low-density (20 nm, 3 sec) and high-density (10 nm, 0.3 sec) curves to adjust for limits of fluorescence detection. Fluorescence readings were obtained using PS 4-sided polystyrene BRANDO macro-cuvettes (Sigma, Cat No. BR759053).

3.2.2. WAF preparation, exposure, and sampling

Oysters were exposed to the water accommodated fractions (WAFs) of each of the petroleum products in 6 L glass aquaria (15 oysters per tank) submerged in 10°C water baths for 7 d. There was a total of sixteen 6 L tanks for all exposures, resulting in 4 replicate tanks per WAF concentration. The 3 different unweathered oil products were marine diesel, crude oil, and diluted bitumen (obtained from Centre of Offshore Oil and Gas, Nova Scotia). WAFs were prepared in a 500 L distribution tank (Singer, 2001). Eight liters of an individual oil was added to 240 L of filtered sweater to generate a 1:30 oil/water ratio mixture. The prepared WAF was then sealed and mixed at 92 rpm with a stainless-steel stir rod for 24 h to encourage oil components to partition to the water phase. After mixing, the oil was allowed to settle for 1 h with the lid remaining sealed. Once settled, the dissolved fraction of the WAF was siphoned into the 6 L oyster exposure tanks at either 25, 50 or 100 % dilutions (plus a control group containing regular sea water). Oysters and

water parameters (temperature and salinity) were monitored for 7 d until termination of the exposure. Oysters were not fed during the exposure.

Water samples were taken from each treatment group (control, 25 %, 50 %, 100 %) from the 6 L exposure tanks on d 0, 3 and 7 of the exposure. Water (1 L) from each treatment group was collected in amber bottles and treated with sodium azide (50 mg) as a preservative and stored at 4 °C for a maximum of 2 d before being sent to SGS Axys Analytical (Sydney, BC) for quantification of PACs by low resolution GC/MS. Approximately 10 g of whole-oyster tissue samples were also collected for each treatment group (approximately 2 oysters per tank) immediately after exposure termination on d 7, as well as d 21 and d 35 recovery timepoints, put on dry ice and stored at -80°C in 60 mL amber glass vials. Tissue samples were shipped to SGS Axys Analytical on dry ice for chemical analysis quantification of PACs by low resolution GC/MS. A complete list of analytes for WAF and whole tissue samples are displayed in Appendix Figures A9-A26.

A subset of 2 oysters were sampled from each replicate tank at each WAF concentration (resulting in biological replicates of 8 oysters per treatment group) and hemocytes (100 µL) were collected immediately for the COMET; the remaining live oysters in each tank were sorted into mesh bags corresponding to the WAF concentration and transferred back to the 60 L acclimation tanks for subsequent monitoring. Sampling was repeated at 21 and 35 d post-exposure to measure endpoints after a 14 and 28 d recovery in uncontaminated seawater.

3.2.3. Comet Assay

Hemolymph collection and microscope slides were prepared using the Trevigen® Alkaline Comet Assay kit according to the manufacturer's instructions (BioTechne, Minneapolis, USA) .100 µL Hemolymph was collected from the oyster heart cavity using a 1 mL syringe, and then suspended in ~100 µL of Hanks Balanced Salt Solution (HBSS) and adjusted to a cell concentration of ~$1x10^5$ cells/mL on the day of sampling and stored at 4 °C until processing (maximum 2 days). Cell concentration was determined based on haemocytometer counts. Within 1-2 d of haemolymph collection, 30 µL of the cell suspension was combined with 300 µL of LMAgarose (Trevigen ® , Cat No. 4250-050-02) (1:10 v/v ratio) and mixed by inversion with a wide-mouth pipette tip. 30 µL of the mixture was then dispensed onto a pre-coated NMAgarose microscope slide (Trevigen®, Cat No.

4250-050-K). The microscope slide was placed in a 4°C refrigerator for 30 min to allow the gel to solidify. Slides were then submerged in a lysis solution (Trevigen® Cat No. 4250-050-01) overnight at 4°C. Excess lysis solution was drained, and slides were then placed in an alkaline unwinding solution (200 mM NaOH, 1 mM EDTA) for 20 min at room temperature in the dark. Slides were then placed in a gel electrophoresis tank (Bio-Rad Technologies) equidistant from electrodes. Alkaline electrophoresis solution (300 mM NaOH, 1mM EDTA) was added to the gel tank and run for 35 min at ~20 V and 300 mA at 4°C. Following electrophoresis, slides were then washed in deionized water and 70% ethanol for 5 min each. Slides were then incubated at 37°C for 15 min to bring cells into a single plane for microscope observation. Slides were then stained with 50 µL of SYBR® Green nucleic acid stain for 30 min in the dark at room temperature. After staining, slides were rinsed with deionized water and dried completely at 37°C prior to viewing under microscope. Slides were imaged using the Ziess Axio Observer WaveFX spinning disc confocal system (Quorum Technologies) with the dry 10x objective lens. Comet images for all exposures were saved as bitmap files and manually scored using Comet Assay IV Software Version™ 4.3.2. (Perspective Images Ltd.) calibrated to 100 microns per pixel. The following parameters measured by the software were reported for 50 cells per treatment group: tail % intensity, tail length, tail migration and olive tail moment (See Appendix Figure A.2 for details on each specified parameter).

3.2.4. Statistical Analysis

Statistical Analysis was performed in JMP®, Version 16 (SAS Institute Inc., Cary, NC). For the analysis, 50 scored cells were pooled from 8 oysters per treatment group (n= 2 from each tank). To account for pseudo replication, mean values for each comet parameter (tail intensity, tail length, tail migration, and olive tail moment) were calculated according to tank number. Mean values were then plotted along a frequency distribution and normal quantile-quantile plots to screen for possible outliers and evidence of a non-normal distribution. Normality and equal variance were also confirmed using a Shaprio-Wilk goodness of fit test. Potential outliers identified in the initial screening were further evaluated by plotting studentized residuals and omitted from the model if the residual value was ≥ 4. All datasets passed normality assumptions and therefore did not have to be transformed prior to running the model. A two-way Analysis of Variance (ANOVA) and Tukey's posthoc test was performed to determine statistical significance of least square

means between each WAF concentration (0%, 25%, 50%, 100%), sampling time (7 d, 21 d, 35 d) and interactions between these two variables.

3.3. Results

3.3.1. PAC Composition in WAFs and Oyster Tissue Samples

A total of 76 polycyclic aromatic compounds were quantified by LR GC-MS in both WAF and tissue samples, comprising of 20 parent PACs, 48 alkylated PACs and 8 dibenzothiophene (DBT) compounds. These results are described in Chapter 2, section 2.2.2.

3.3.2. Marine Diesel

Controls were not affected by sampling time for any of the comet parameters measured (% tail intensity; olive tail moment; and tail migration and tail length, Figure 3.1 A-D, $p > 0.05$). As well, no significant differences in DNA damage in hemocytes were observed over sampling times (7 d, 21 d, 35 d) in exposed oysters for any of the comet parameters (Figure 3.1 A-D, $p > 0.05$). No statistically significant differences in DNA damage in hemocytes were observed between WAF concentrations (0% , 25% , 50%, 100%) for any of the comet parameters (Figure 3.1 A-D, $p > 0.05$).

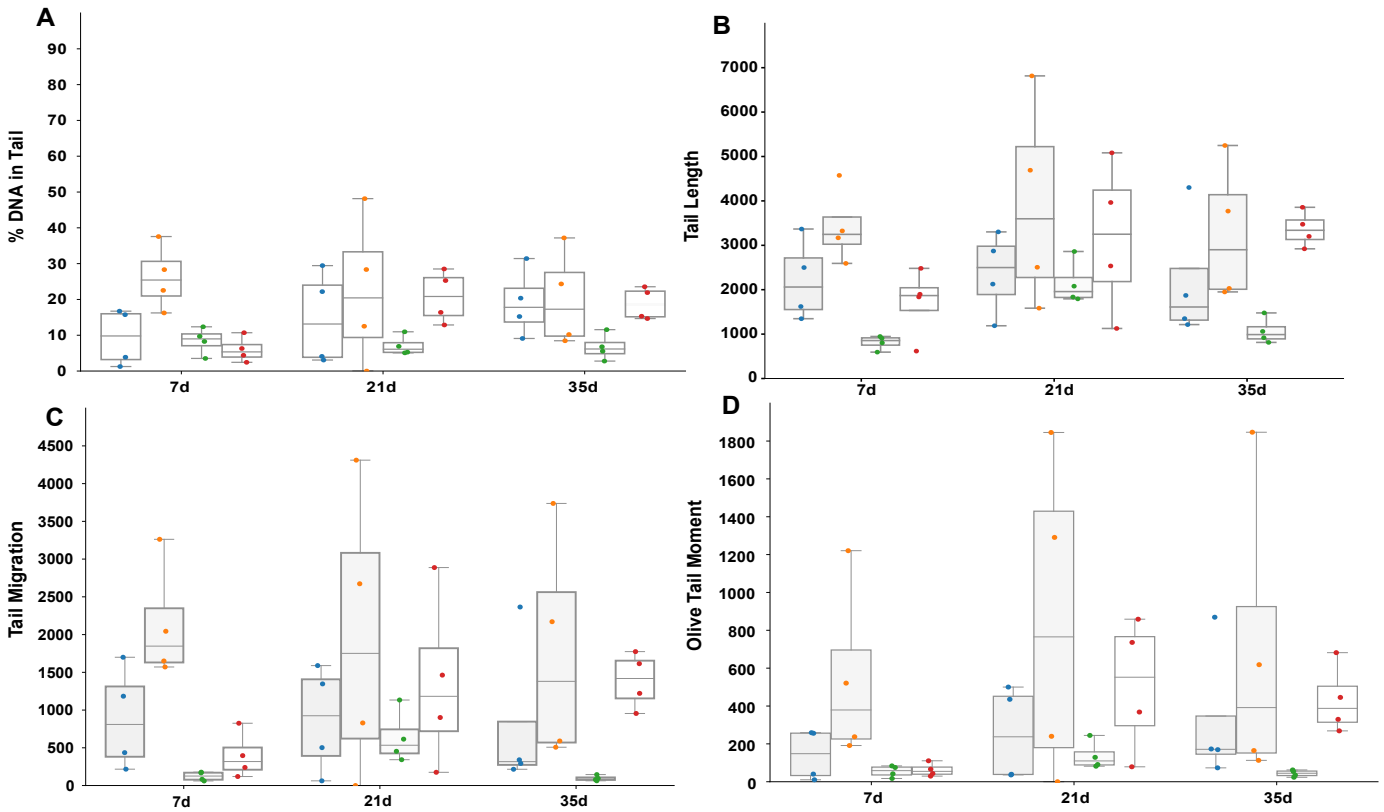

Figure 3.1. **DNA damage levels measured with the comet assay for oyster hemocytes exposed to marine diesel (n= 8 oysters per treatment group). Boxplots present the the median values, lower 25th and upper 75th percentiles, minimum and maximum values (whiskers). A) % DNA in tail, B) tail length, C) tail migration and D) olive tail moment . A two-way ANOVA followed by a Tukey's post-hoc analysis was performed to determine significant differences between WAF concentration (%) , sampling time (d) and the interaction term. Significant differences between groups are represented by different superscript letters. WAF concentrations are represented by the colours indicated : control or 0% (■), 25% (■) , 50% (■), and 100% (■).**

3.3.3. Crude oil

Measured DNA damage in control oysters did not change over time (7 d, 21 d, 35 d) for any of the comet parameters (Figure 3.2B, $p > 0.05$). WAF exposed oysters exhibited some individual treatment differences, but these did not correspond to any trends overtime or with increasing concentration for any of the comet parameters (Figure 3.2 A-D).

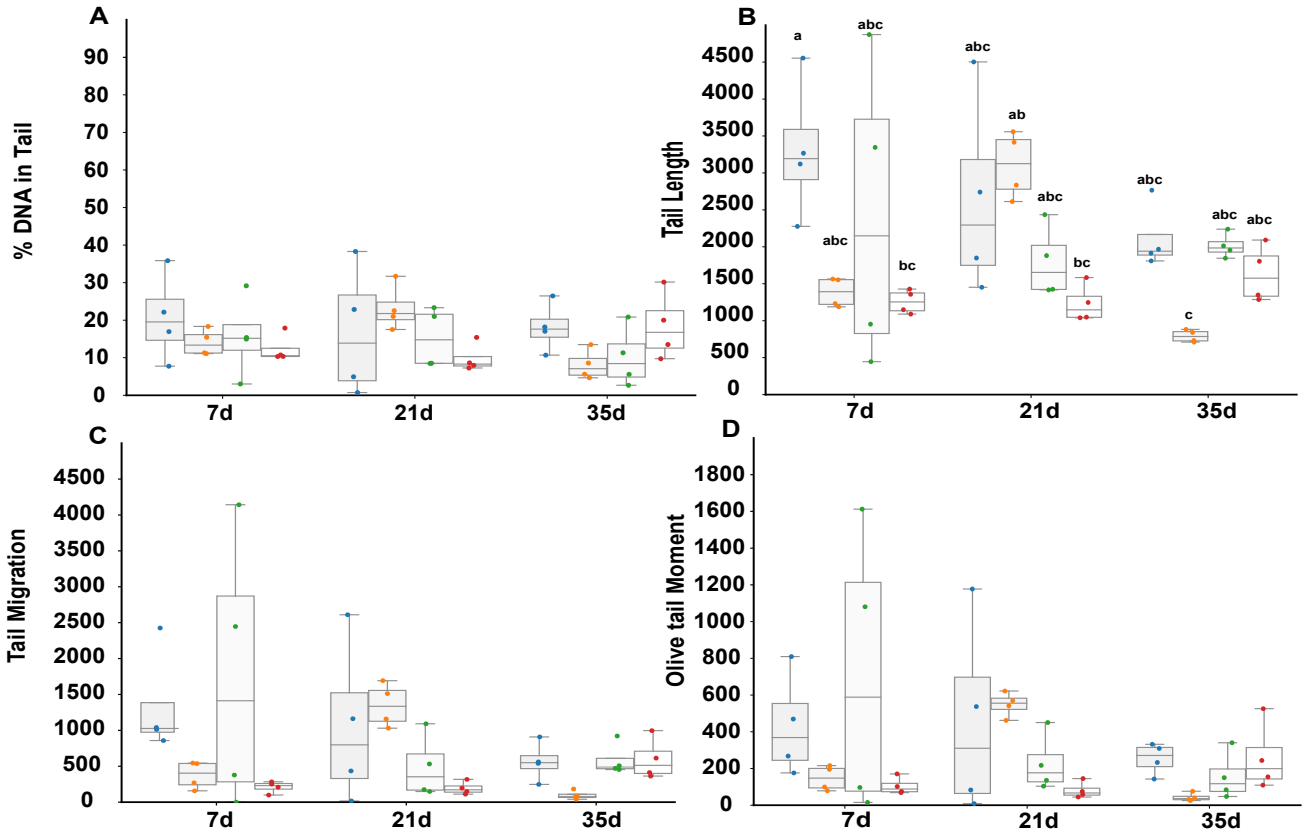

Figure 3.2 **DNA damage levels measured with the comet assay for oyster hemocytes exposed to crude oil (n= 8 oysters per treatment group). Boxplots present the the median values, lower 25th and upper 75th percentiles, minimum and maximum values (whiskers). (A) % DNA in tail (B) tail length (C) tail migration and (D) olive tail moment . A two-way ANOVA followed by a Tukey's post-hoc analysis was performed to determine significant differences between WAF concentration (%) , sampling time (days) and the interaction term. Significant differences between groups are represented by different superscript letters. WAF concentrations are represented by the colours indicated : control or 0% (■), 25% (■) , 50% (■), and 100% (■).**

3.3.4. Diluted Bitumen

Controls were not affected by sampling time for any of the comet parameters measured (Figure 3.2C, $p > 0.05$). WAF exposed oysters exhibited some individual treatment differences, but these did not correspond to any trends overtime or with increasing concentration for any of the comet parameters (Figure 3.3 A-D).

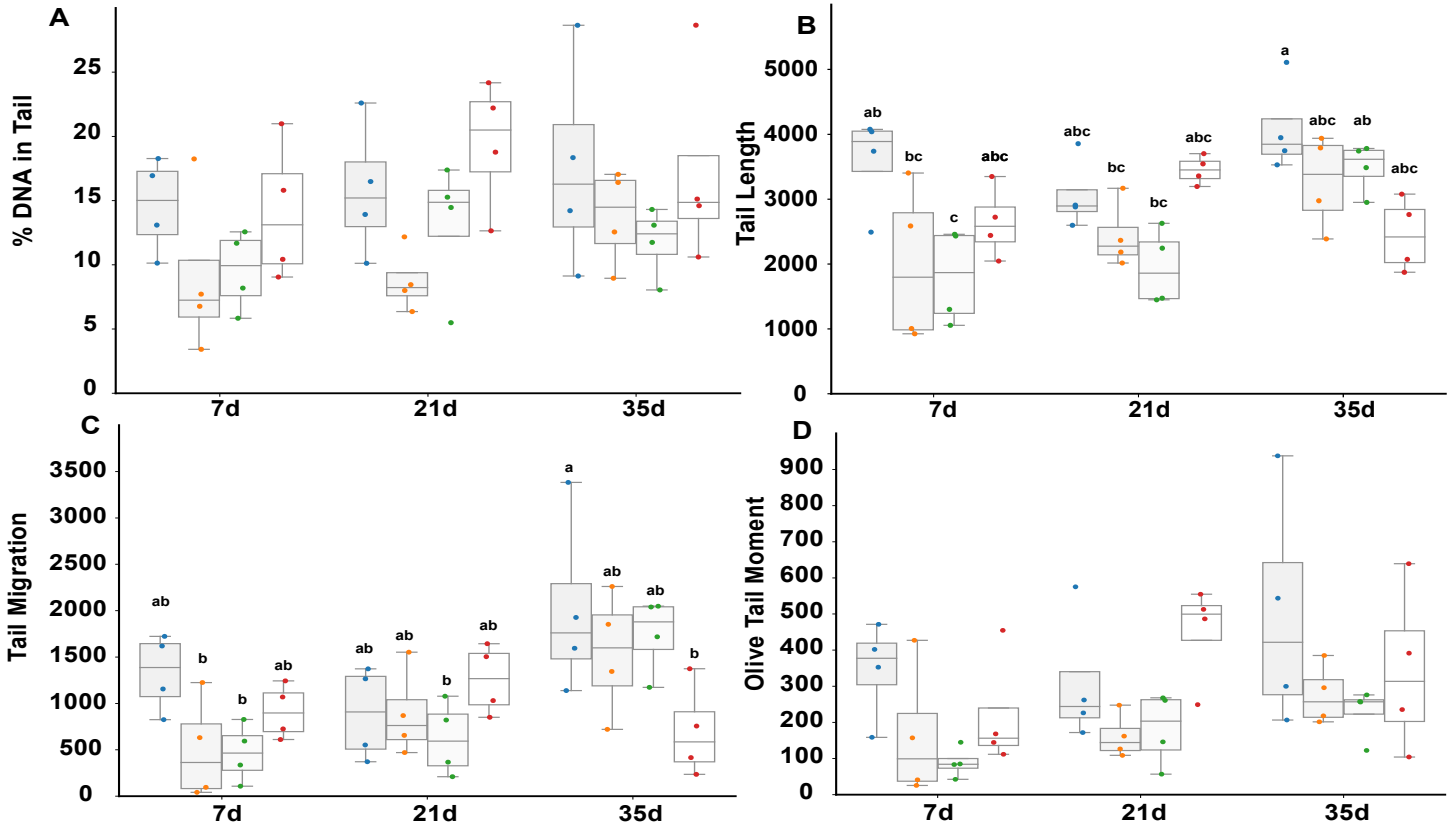

Figure 3.3 **DNA damage levels measured with the comet assay for oyster hemocytes exposed to diluted bitumen (n= 8 oysters per treatment group). Boxplots present the the median values, lower 25th and upper 75th percentiles, minimum and maximum values (whiskers). (A) % DNA in tail (B) tail length (C) tail migration and (D) olive tail moment . A two-way ANOVA followed by a Tukey's post-hoc analysis was performed to determine significant differences between WAF concentration (%) , sampling time (days) and the interaction term. Significant differences between groups are represented by different superscript letters. WAF concentrations are represented by the colours indicated : control or 0% (■), 25% (■) , 50% (■), and 100% (■).**

3.4. Discussion

3.4.1. Comet Assay Parameters

In this study, an assessment of DNA damage in oyster hemocytes following exposure to three petroleum products and a recovery period was conducted. This is the first study to report measurements for four different comet parameters. Scores can be divided into two types of measurement across all available image analysis software programs: primary and derived. Primary parameters include tail length, % DNA in tail, tail intensity, and tail distribution, while all other measurements are derived by converting one or more of these 3 primary measurements into derived parameters (Kumaravel and Jha, 2006). Primary measurements are generally recommended for biomonitoring as they are more representative of DNA damage accumulation across the entire distribution of the cell, while derived parameters only encompass a horizontal cross-section from the centre of the nucleoid to the end of the tail (Collins et al., 2014). In this study, there were no biologically significant trends for any of the comet parameters, indicating that results between each parameter are comparable.

3.4.2. Petroleum PAC Exposure and DNA Damage Levels

The interplay between uptake of PACs from each petroleum product, and the efficiency of DNA repair mechanisms influence the extent of DNA damage in oyster hemocytes. It is important to note that variability in background levels of DNA damage was observed in controls, however this variability remained stable over time for all exposures (between 10-20% tail DNA). Elevated background levels of DNA damage in bivalves have been observed in other studies (Perez-Cadahia et al., 2004; Barranger et al., 2014). The Inter-individual coefficient of variation for baseline DNA damage in mussel and clam hemocytes was between 17-30%, which aligns with the range observed for controls in this study (Hamoutene et al., 2002). Similarly, another baseline study conducted found that % tail DNA ranged from 17-34% across four different invertebrate species (Sahlmann et al., 2017).

As described in detail in Chapter 2 (section 2.4.1), predominant PACs (C_1-C_2 naphthalenes, fluorenes and phenanthrene) in water samples were also the most abundant PACs accumulated in oyster tissue samples, which consisted of C_1-C_2

naphthalenes for all oils. Napthalenes are considered to have low genotoxic potential based on standardized in-vivo and in-vitro genotoxicity assays (U.S EPA, 1993). Considering naphthalene and alkylated homologs were most abundant PAC detected in tissue samples at all concentrations measured, the lack of significant differences in DNA damage between WAF treatments is not surprising. It is also worth noting that benzo[a]pyrene was not analytically detected in any of the WAF or tissue samples for any oil. Benzo[a]pyrene is the most well-researched PAC in terms of its mutagenic properties and ability to form DNA adducts that lead to strand breakage (Ewa et al., 2016), therefore the absence of this compound in our samples may also be a reason for the lack of significance in DNA damage observed between WAF concentrations. A study exposed mussels to water soluble fractions of *Prestige* crude oil measured concentrations of aqueous [TPAC] and [benzo[a]pyrene] separately (Perez-Cadahia et al., 2004). The authors only found a significant increase in % tail DNA compared to controls for the oil mixture with the higher relative benzo[a]pyrene content. Additionally, other PACs classified as carcinogenic (Benzo[a]anthracene, Benzo[b]fluoranthene, Dibenzo[a,h]anthracene, Indeno[1,2,3,cd]pyrene], U.S EPA, 1993) were also completely absent or detected at extremely low levels (<10 ng/g) in tissues. These compounds are less water-soluble (K_{ow} values between 6.11 and 6.58, respectively; ATSDR, 2005), therefore were not as bioavailable to tissues and did not accumulate to significant levels during the exposure timeframe compared to the LMW PACs. A study exposing mussels and clams to water soluble fractions of crude oil exhibited a very similar PAC composition profile and exposure duration to the current study, and likewise no significant change in haemocyte DNA damage levels were detected (Hamoutene et al., 2002).

DNA damage results were also a reflection of the time-course depuration of PACs. Once oysters were placed in clean sea water in the recovery phase, TPAC levels in oyster tissues decreased between 7-35 d by 80-90% in all oils, with the greatest reduction occurring between 7-21 d. For all 3 oils in our study, % tail DNA did not exceed 40% and no significant temporal differences were observed, potentially suggesting any DNA damage incurred during the exposure phase was not great enough to exceed DNA repair rates in the depuration phase. Studies exposing mussels to benzo[a]pyrene found that the upper limit of DNA damage detected was between 25-30% tail DNA (Rank et al., 2003; Mitchelmore et al., 1988). In the latter study, the most significant effects were seen between 2-4 days of in vivo exposure, whereafter the effects decreased (Rank et al., 2003)

This threshold effect has also been observed in bivalve studies using PAC mixtures and water-soluble fractions of crude oil (Taban et al., 2004, Coughlan et al., 2002). Previous studies have also shown that complete recovery of DNA damage occurs within a few hours or days in sea urchins and other bivalves (Akcha et al., 2000; El- Bibany et al., 2014; Dixon et al., 2002). However, studies on baseline DNA repair rates in bivalves are limited (Sahlmann et al., 2014). Additionally, a significant source of DNA damage is ROS production and generation of reactive metabolites that can intercalate with DNA; both which are by-products of CYP-mediated metabolism (Baird et al., 2005; Santana et al., 2018). The lack of CYP-related enzyme activity and gene expression observed in tissues in the previous chapter suggest low evidence of biotransformation, therefore, sources of ROS would be minimal. Overall, the absence of PACs that have affinity for DNA adduct formation in tissues, partnered with the rapid elimination and lack of biotransformation observed led to negligible differences in DNA damage quantified by the comet assay.

3.5. Conclusions

To our knowledge, this was the first study that examined DNA damage in adult Pacific Oysters exposed to three different oil types. As stated in Chapter 2, the PAC composition profile in both WAF samples and oyster tissues was consistent across all oils, with LMW PACs (C_1-C_3 napthalene, fluorene and phenanthrene) and their alkylated homologs were detected in the greatest proportions. This consistency indicates that bioavailability of PACs in aqueous solution is similar across oils in a short-term exposure scenario, and directly reflects PAC uptake into tissues. Concentrations of these PACs however, differed between oil types, where crude TPAC concentrations were greatest in both water and tissue samples. This reflects the physio-chemical properties of the oil and initial weathering processes. Marine diesel is made up of a larger fraction of ultra-light components that are susceptible to rapid volatilization, resulting in a lower proportion of bioavailable PACs. Conversely, heavier components in bitumen are less water-soluble and this reduces bioavailability to tissues in the short-term.

Rapid bioaccumulation of water-soluble PACs was also apparent from the tissue chemistry data. Tissue concentrations were orders of magnitude higher than water samples at the end of the exposure period on day 7. Although uptake of PACs into tissues was rapid, chemistry data from the recovery phase showed that PACs were also rapidly eliminated, with an exponential decrease in TPAC between 7 d – 21 d. These results align

with other studies that have monitored depuration following exposure to oil. This suggests that unless exposure is continuous at concentrations that exceed the threshold rate of depuration, oysters will effectively eliminate most water-soluble compounds passively rather than through active metabolism. Future studies that measure primary metabolite products in multiple tissues of bivalves that are exposed to whole-oils rather than single PACs would help elucidate mechanisms of elimination and biotransformation in these organisms.

The absent to minimal presence of HMW PACs in water and tissues that are classified as carcinogenic by the U.S EPA likely explains the lack of biological significance in the DNA damage results. Previous studies that have quantified DNA damage in bivalves exposed to PAC mixtures have been somewhat correlated with the concentrations of benzo[a]pyrene and other compounds from this list. Low evidence of CYP-mediated metabolism may also reduce accumulation of ROS species, which are an indirect source of strand breaks that would be that would be detected by the comet assay. Additionally, studies have shown rapid recovery of DNA damage in short-term exposures, suggesting that any DNA damage potentially induced by the PACs accumulated did not exceed DNA repair rates during the depuration phase of the experiment.

Pacific Oysters and other bivalves are often chosen as bioindicators for monitoring studies because of their tendency to accumulate contaminants. The results of this study show that they are indeed effective indicators of exposure due to rapid uptake of contaminants that enter the water column, and the magnitude of TPAC concentration can provide incident responders information about the source oil spilled. On the other hand, Pacific oysters may not be as ideal for measuring toxic effects if an oil spill is short-lived because of their ability to depurate the initially bioavailable compounds coupled with low metabolic capacity. On the contrary, if spill remediation efforts are delayed and significant oil weathering occurs, the rate of depuration does level off and remaining compounds are likely to persist in the organism. Future studies should continue to compare bivalve depuration rates for fresh and significantly weathered products in longer-term exposure scenarios.

3.6. References

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Appendix.

Supplementary Information

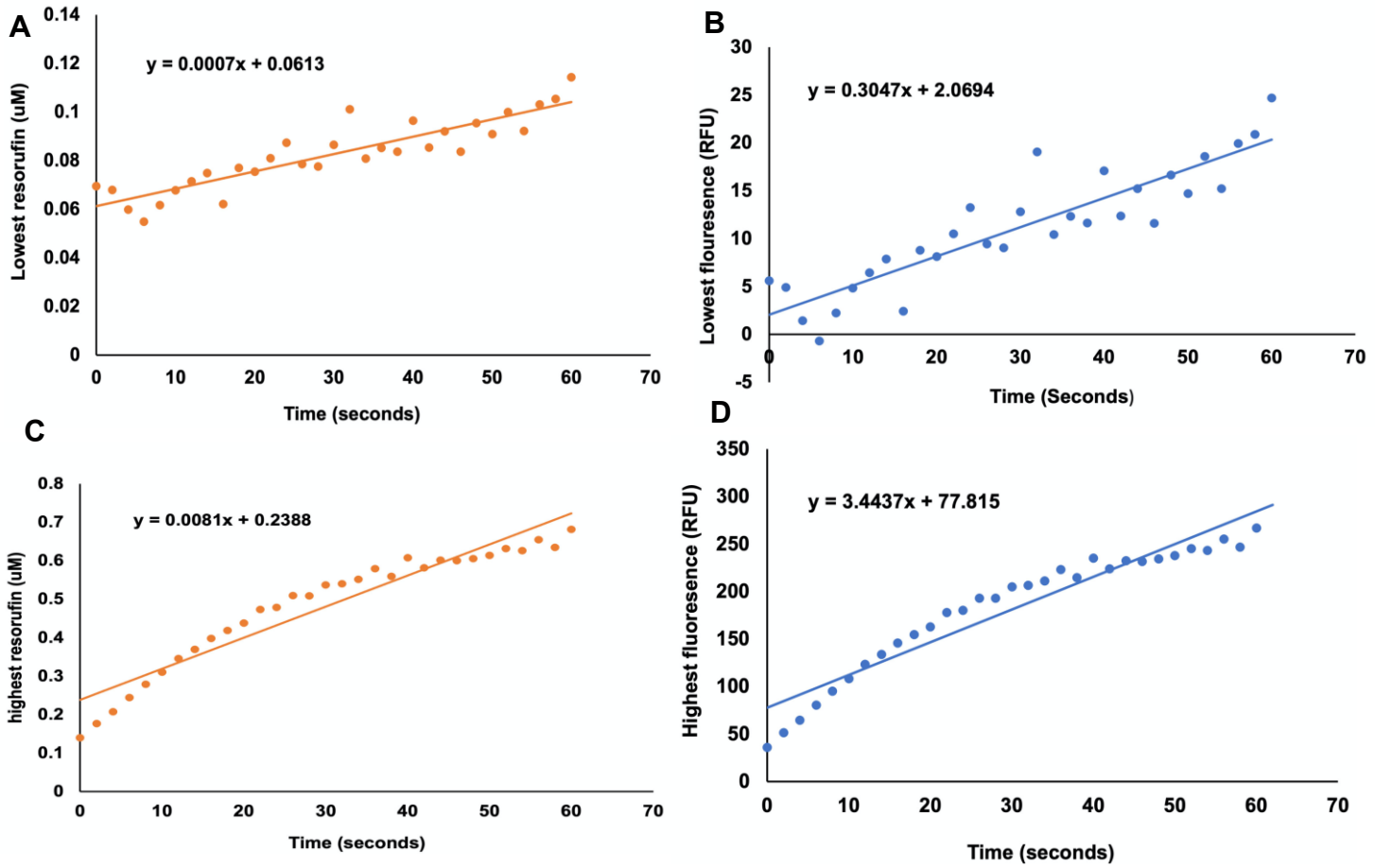
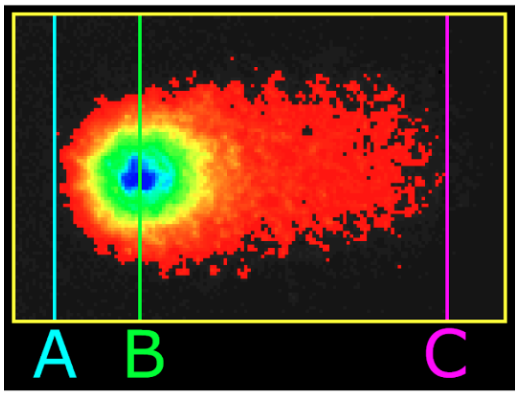


Figure A.1. EROD fluoresence (RFU) and resorufin (uM) curves over time (seconds) for positive control test with sockeye salmon liver tissue samples (N=5). Slopes were calculated using (A) lowest resorufin (B) lowest fluoresence (C) highest resorufin and (D) highest fluoresence.



A: The blue line marks the **start of the head**
B: The green line marks the **centre of the head**
C: The magenta line marks the **end of the tail**

Tail % intensity

Tail intensity expressed as a percentage of the comet's total intensity.

Tail length

The horizontal distance from the centre of the head (start of tail) to the end of the tail.

Tail migration

The horizontal distance from the end of the head to the end of the tail.

Olive tail moment

Product of the proportion of tail intensity and the displacement of tail centre of mass relative to the centre of the head.

Figure A.2. Parameter measurements in oyster hemocytes using Comet Assay IV Software Version™ 4.3.2. (Perspective Images Ltd.). Comet images for all exposures were saved as bitmap files and manually scored for: tail % intensity, tail length, tail migration and olive tail moment. 50 cells were scored per treatment group for the mentioned parameters (calibration factor = 100µm per pixel).

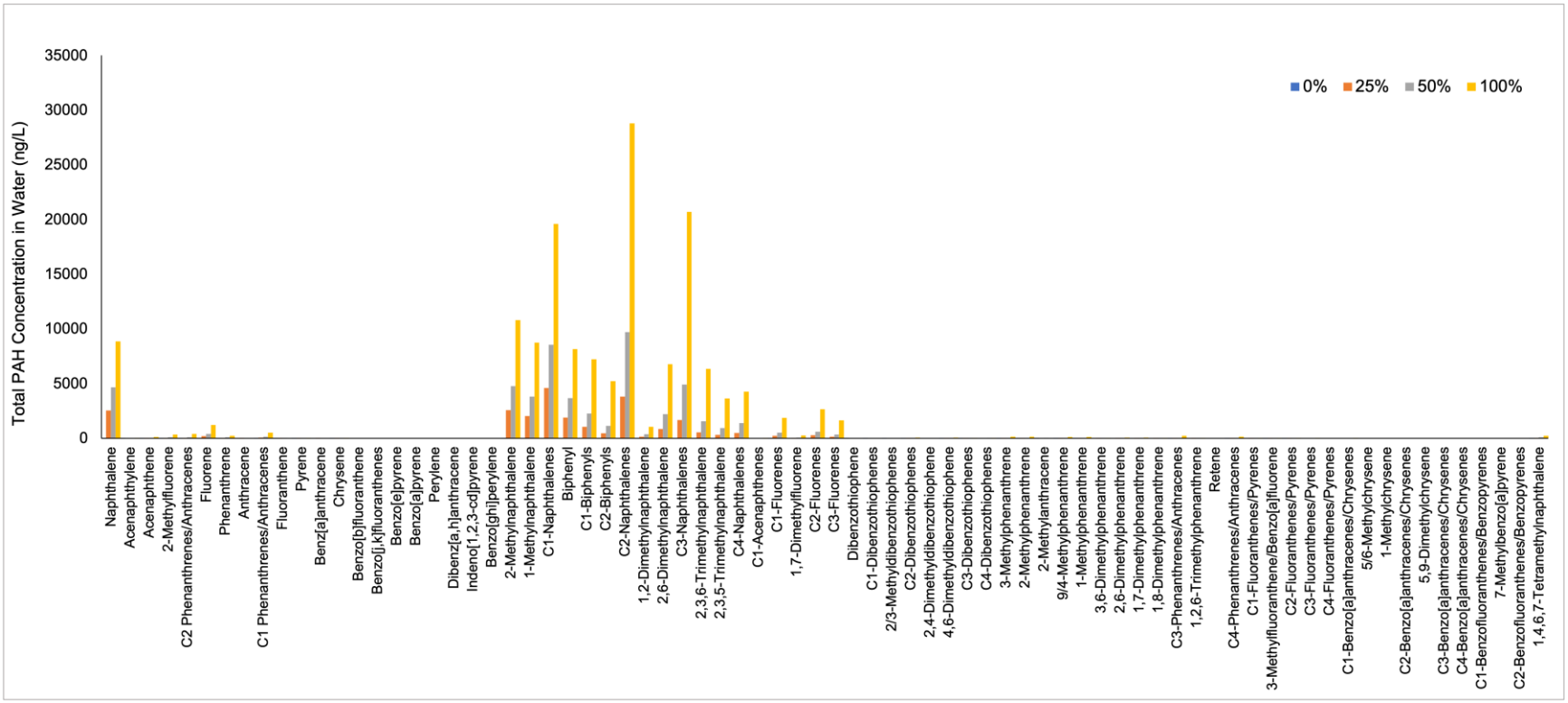


Figure A.3. Analytical chemistry profile for TPAC concentrations (ng/L) in marine diesel WAF samples measured on day 0 of exposure. 1L WAF samples were collected in amber glass bottles and treated with 0.5g of sodium azide for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).

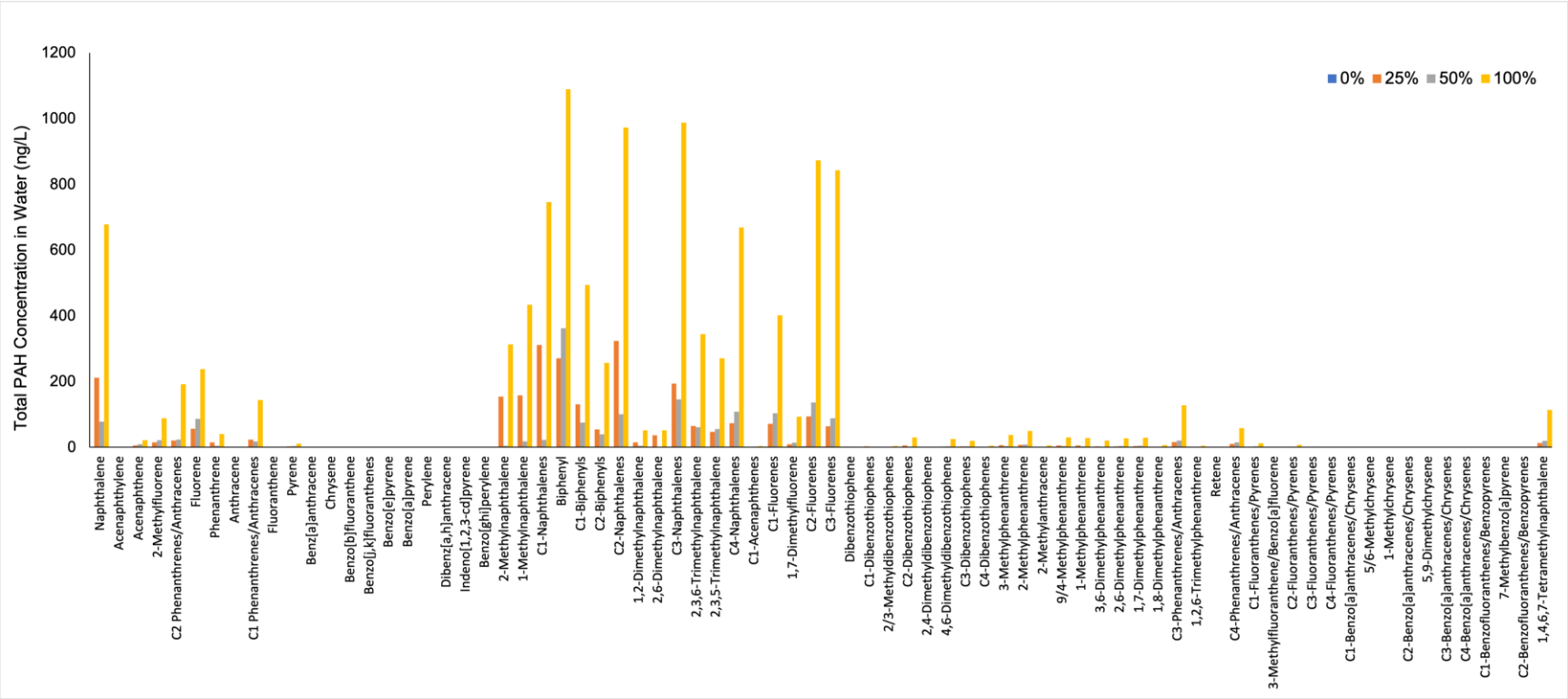


Figure A.4. Analytical chemistry profile for TPAC concentrations (ng/L) in marine diesel WAF samples measured on day 3 of exposure. 1L WAF samples were collected in amber glass bottles and treated with 0.5g of sodium azide for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).

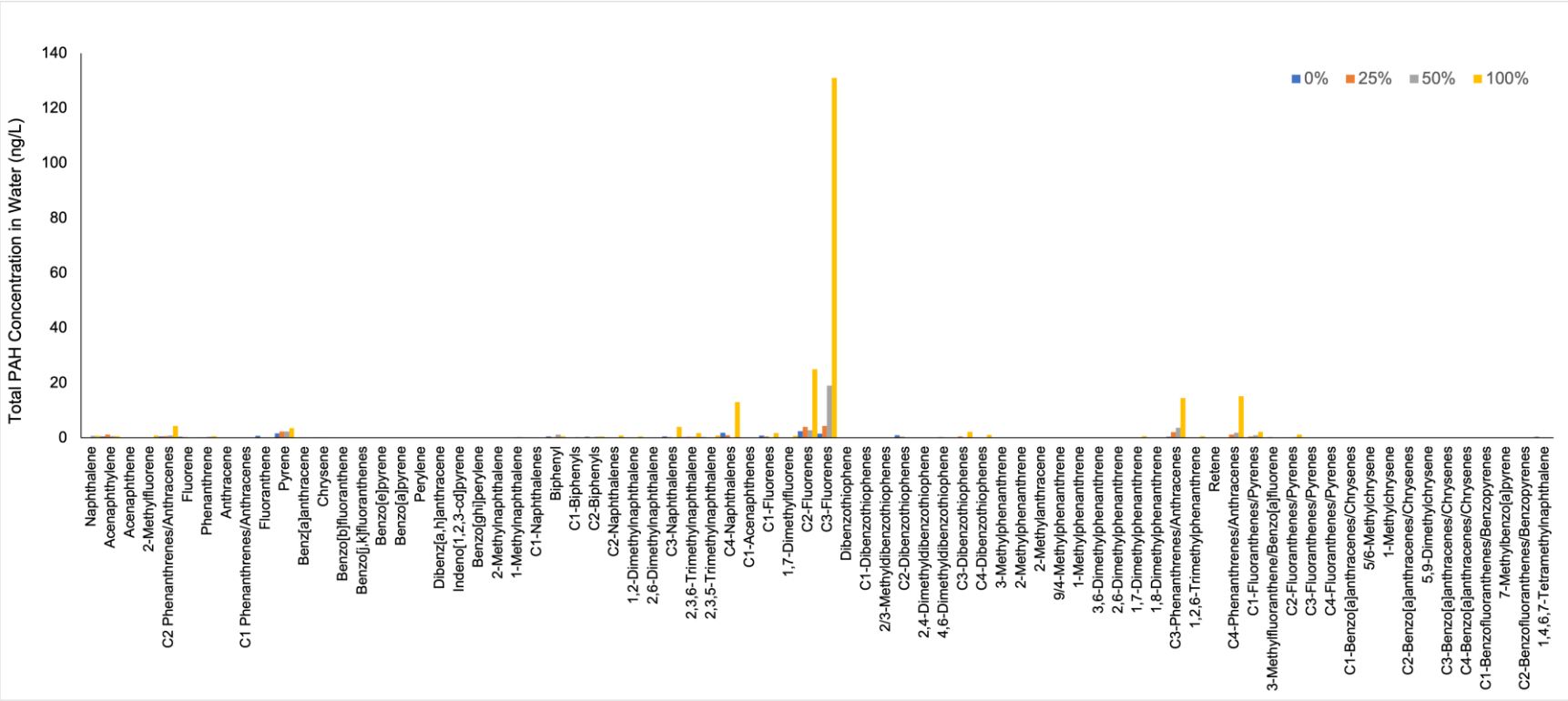


Figure A.5. Analytical chemistry profile for TPAC concentrations (ng/L) in marine diesel WAF samples measured on day 7 of exposure. 1L WAF samples were collected in amber glass bottles and treated with 0.5g of sodium azide for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).

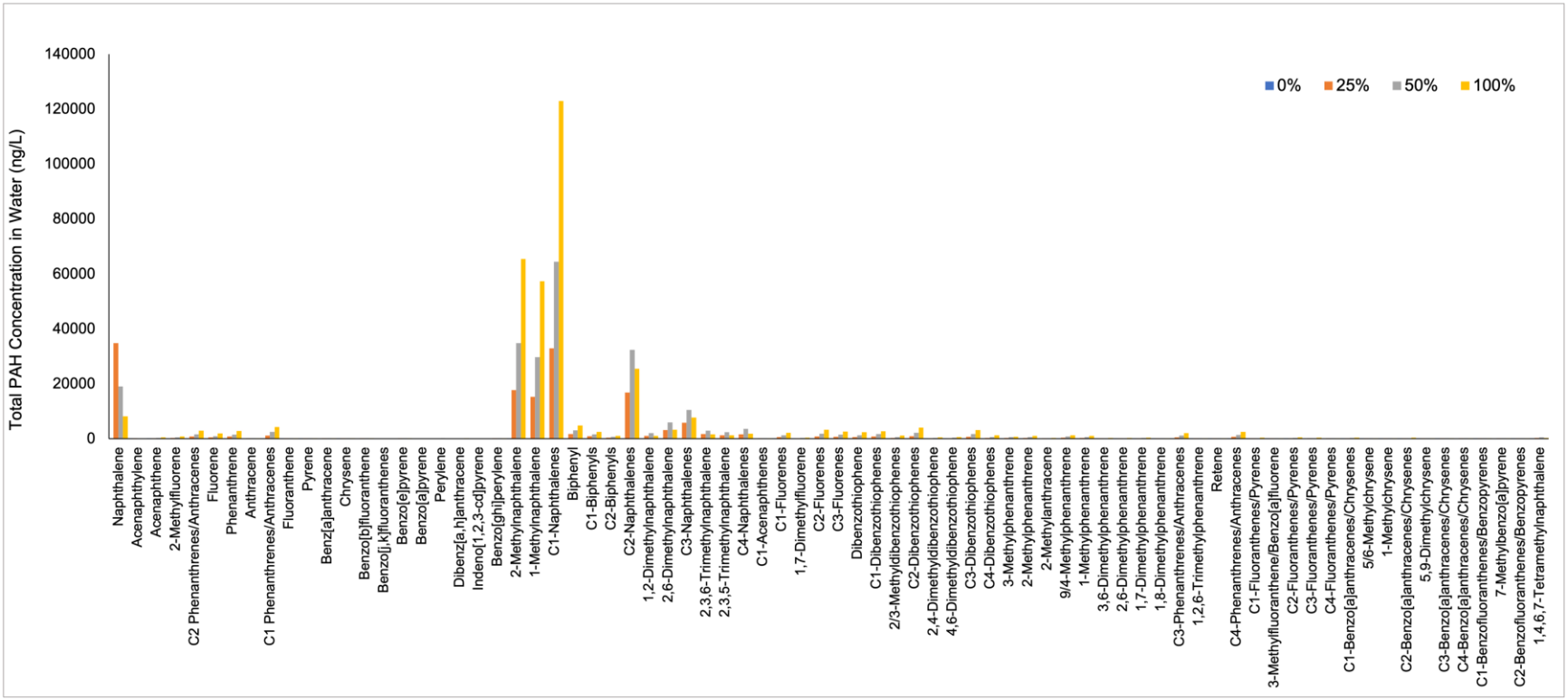


Figure A.6. Analytical chemistry profile for TPAC concentrations (ng/L) in crude WAF samples measured on day 0 of exposure. 1L WAF samples were collected in amber glass bottles and treated with 0.5g of sodium azide for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).

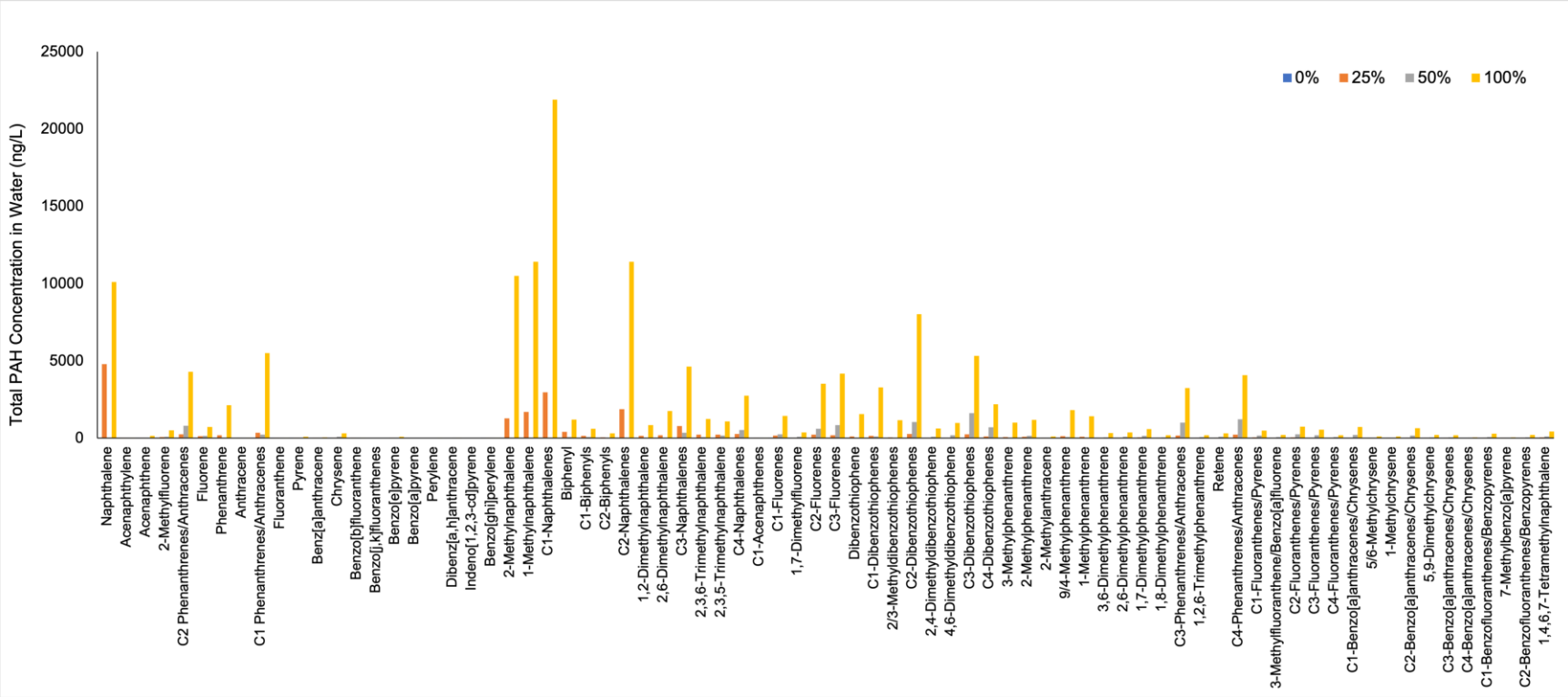


Figure A.7. Analytical chemistry profile for TPAC concentrations (ng/L) in crude WAF samples measured on day 3 of exposure. 1L WAF samples were collected in amber glass bottles and treated with 0.5g of sodium azide for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).

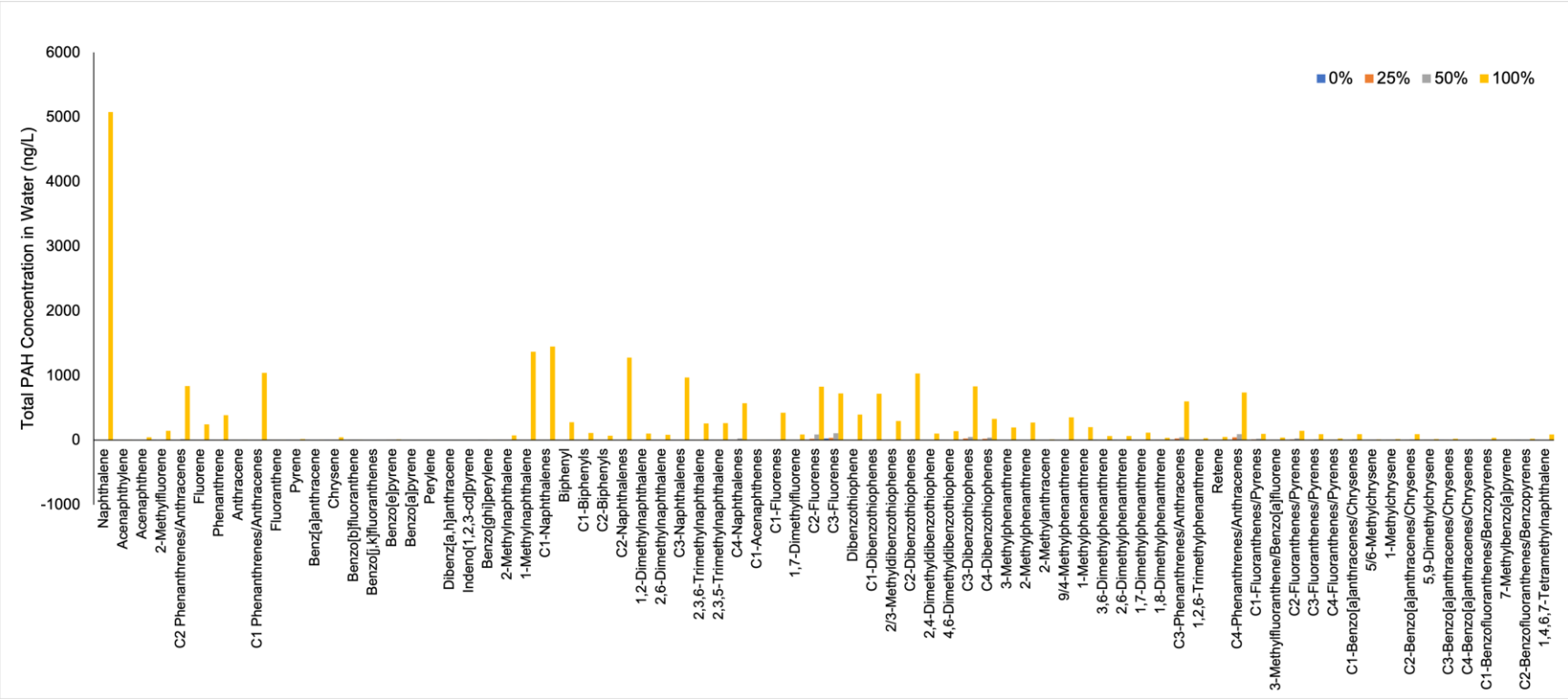


Figure A.8. Analytical chemistry profile for TPAC concentrations (ng/L) in crude WAF samples measured on day 7 of exposure. 1L WAF samples were collected in amber glass bottles and treated with 0.5g of sodium azide for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).

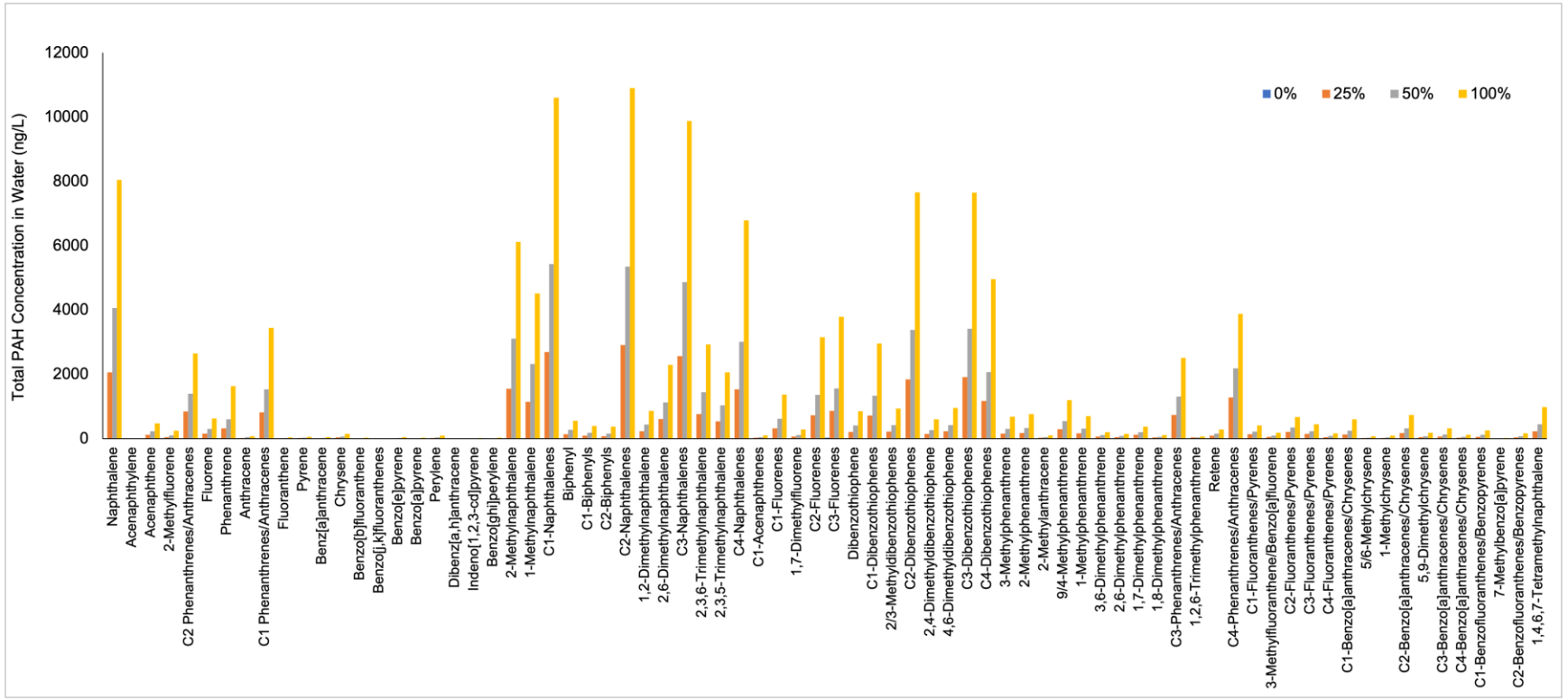


Figure A.9. Analytical chemistry profile for TPAC concentrations (ng/L) in diluted bitumen WAF samples measured on day 0 of exposure. 1L WAF samples were collected in amber glass bottles and treated with 0.5g of sodium azide for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).

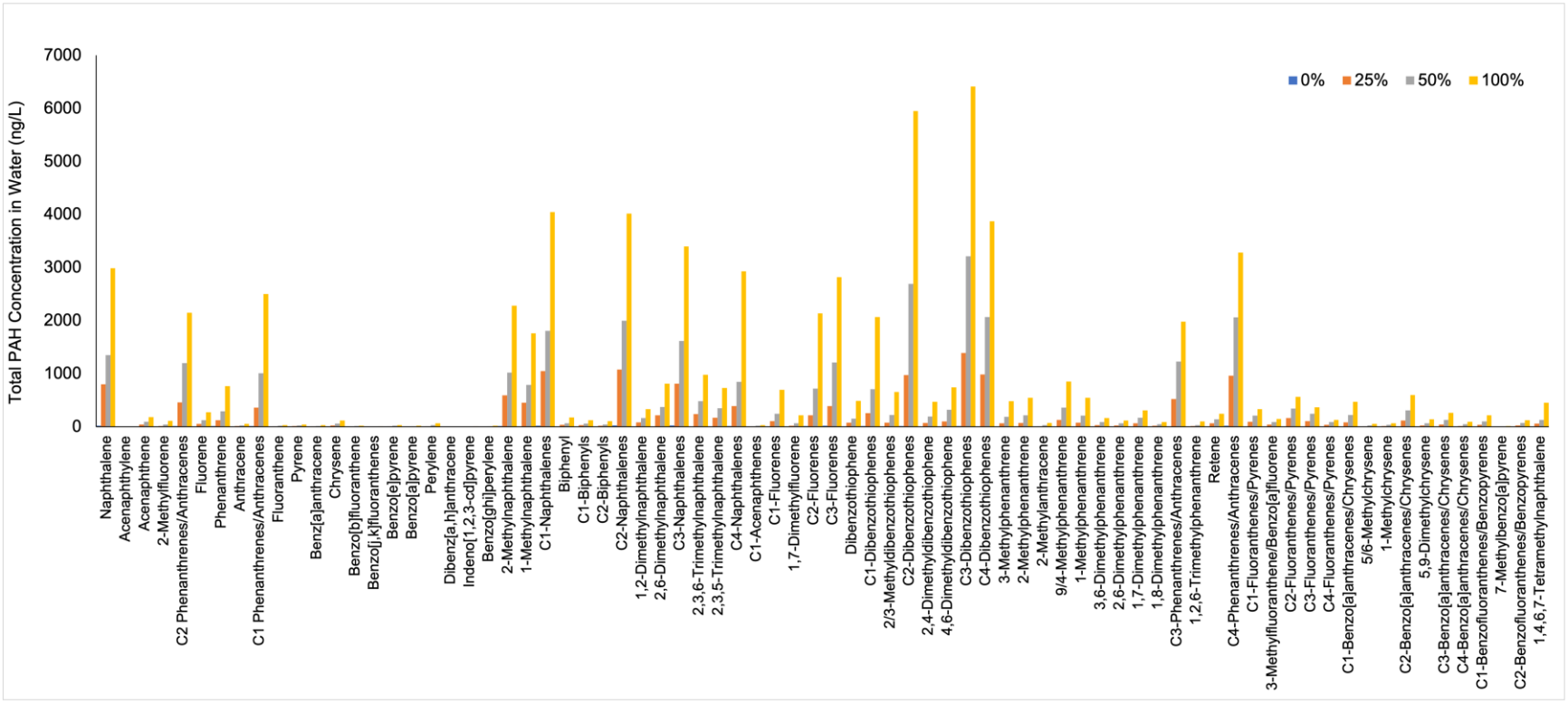


Figure A.10. Analytical chemistry profile for TPAC concentrations (ng/L) in diluted bitumen WAF samples measured on day 3 of exposure. 1L WAF samples were collected in amber glass bottles and treated with 0.5g of sodium azide for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).

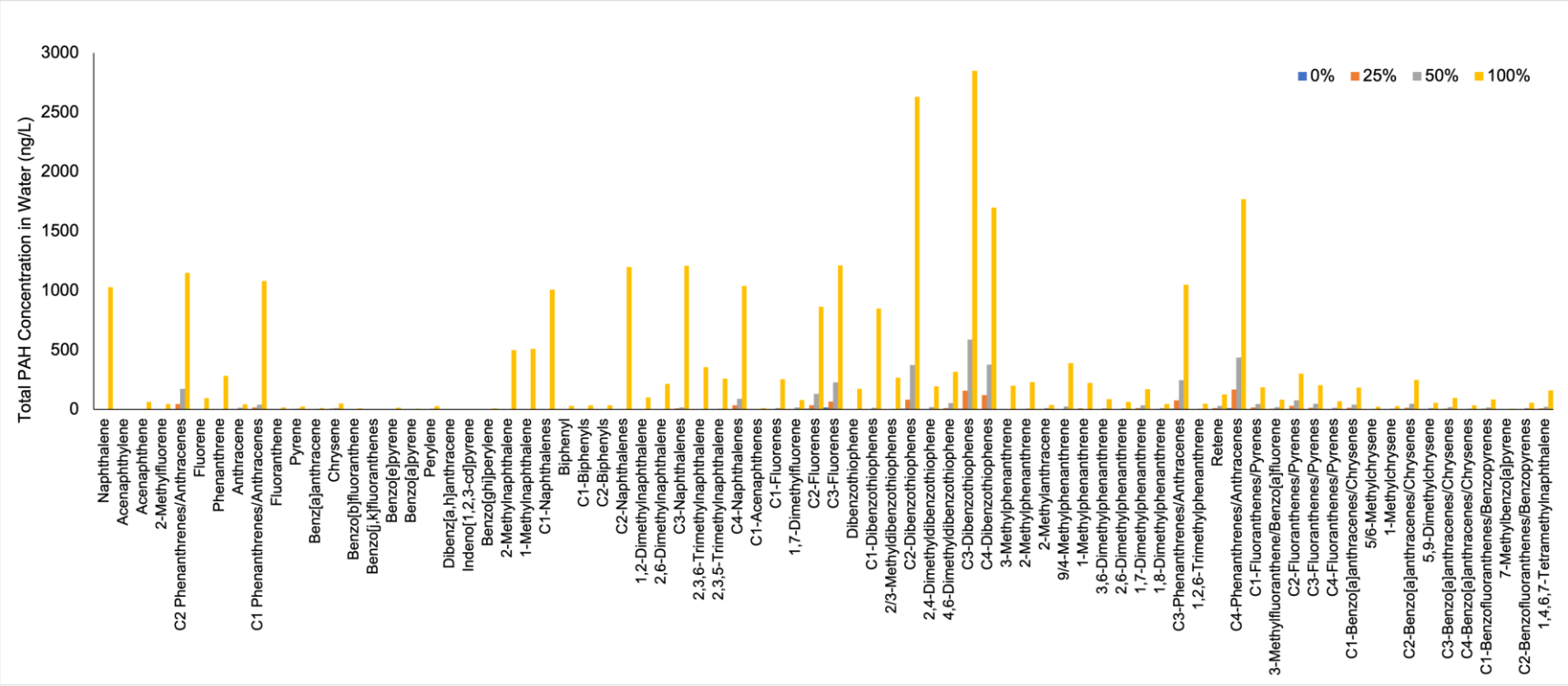


Figure A.11. Analytical chemistry profile for TPAC concentrations (ng/L) in diluted bitumen WAF samples measured on day 7 of exposure. 1L WAF samples were collected in amber glass bottles and treated with 0.5g of sodium azide for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).

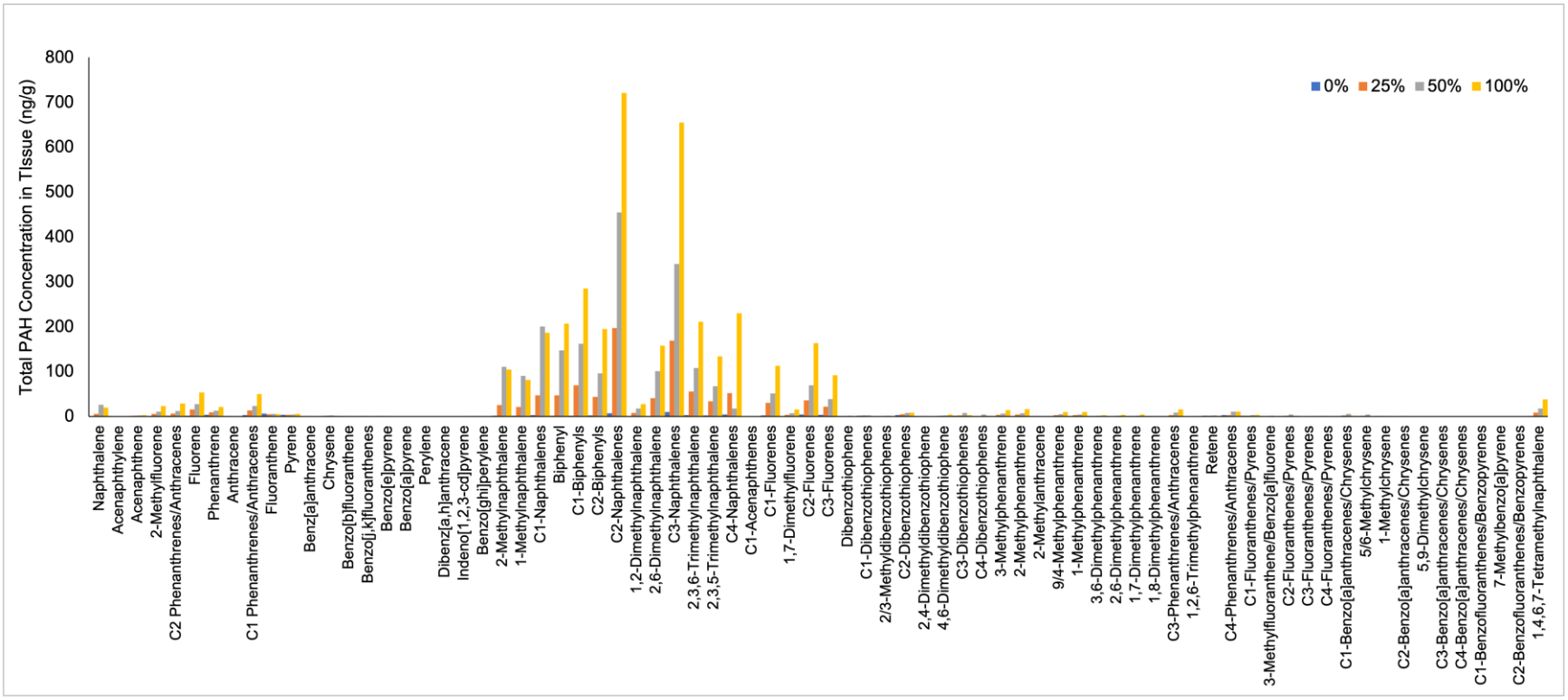


Figure A.12. Analytical chemistry profile for TPAC concentrations (ng/g) wet weight in marine diesel whole tissue samples measured on day 7 of exposure. ~10g of oyster tissue samples were collected in amber glass bottles andimmediately frozen on dry ice for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).

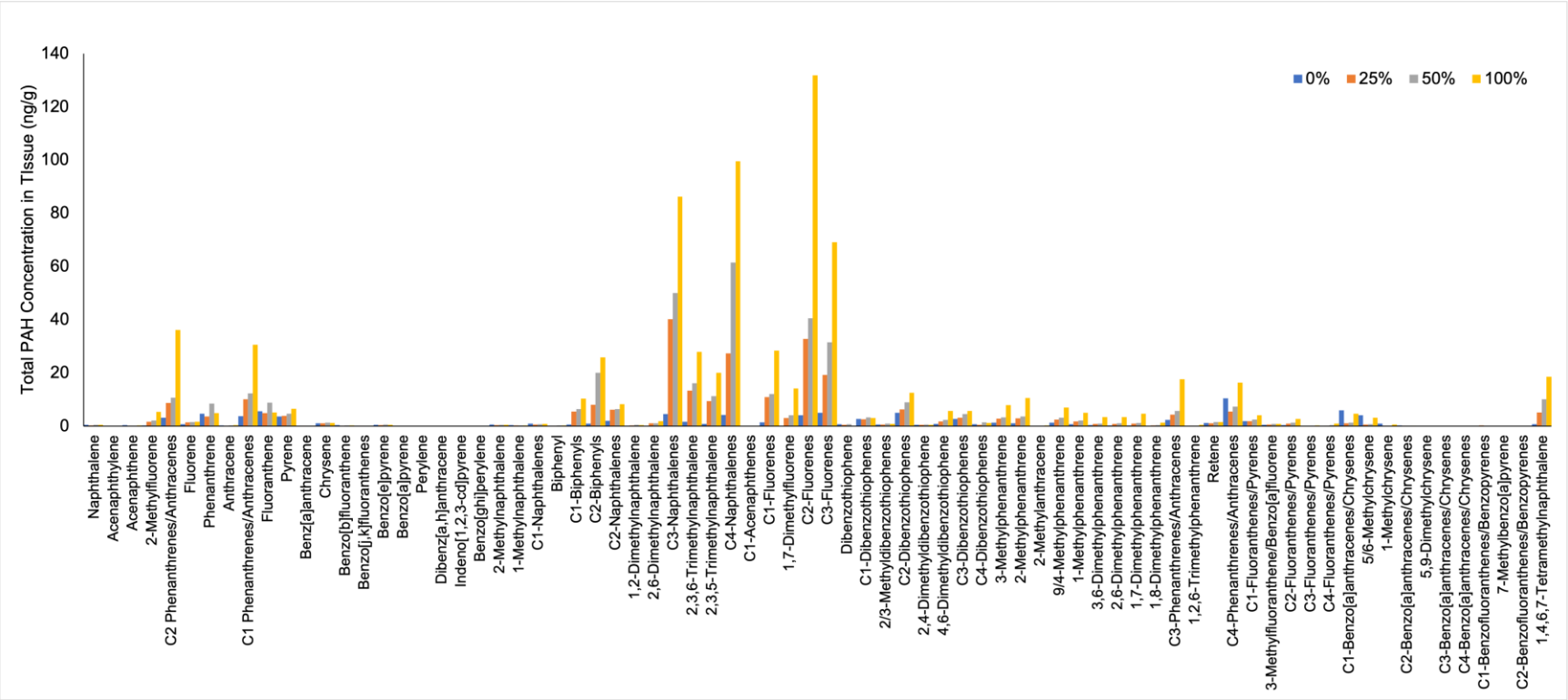


Figure A.13. Analytical chemistry profile for TPAC concentrations (ng/g) wet weight in marine diesel whole tissue samples measured on day 21 post exposure. ~10g of oyster tissue samples were collected in amber glass bottles andimmediately frozen on dry ice for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).

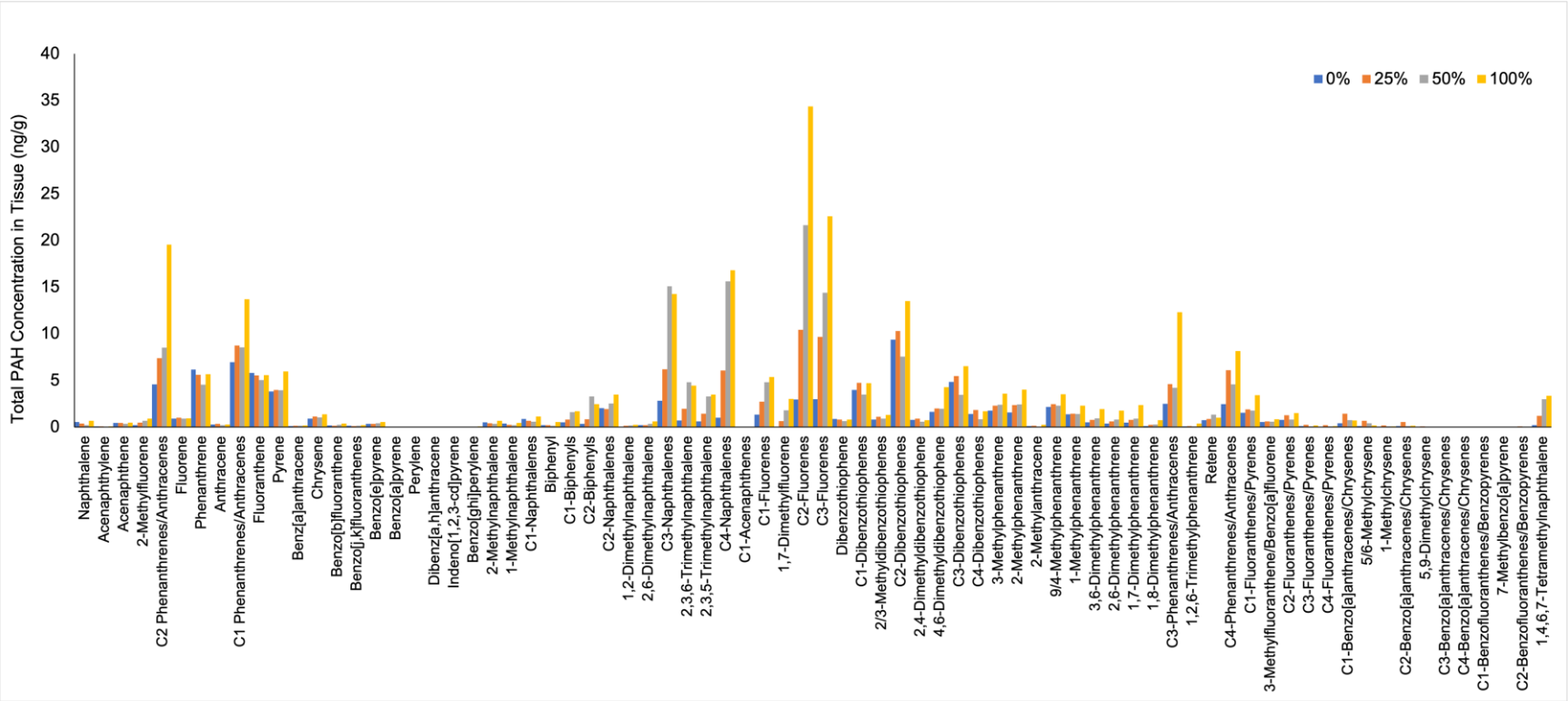


Figure A.14. Analytical chemistry profile for TPAC concentrations (ng/g) wet weight in marine diesel whole tissue samples measured on day 35 post exposure. ~10g of oyster tissue samples were collected in amber glass bottles andimmediately frozen on dry ice for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).

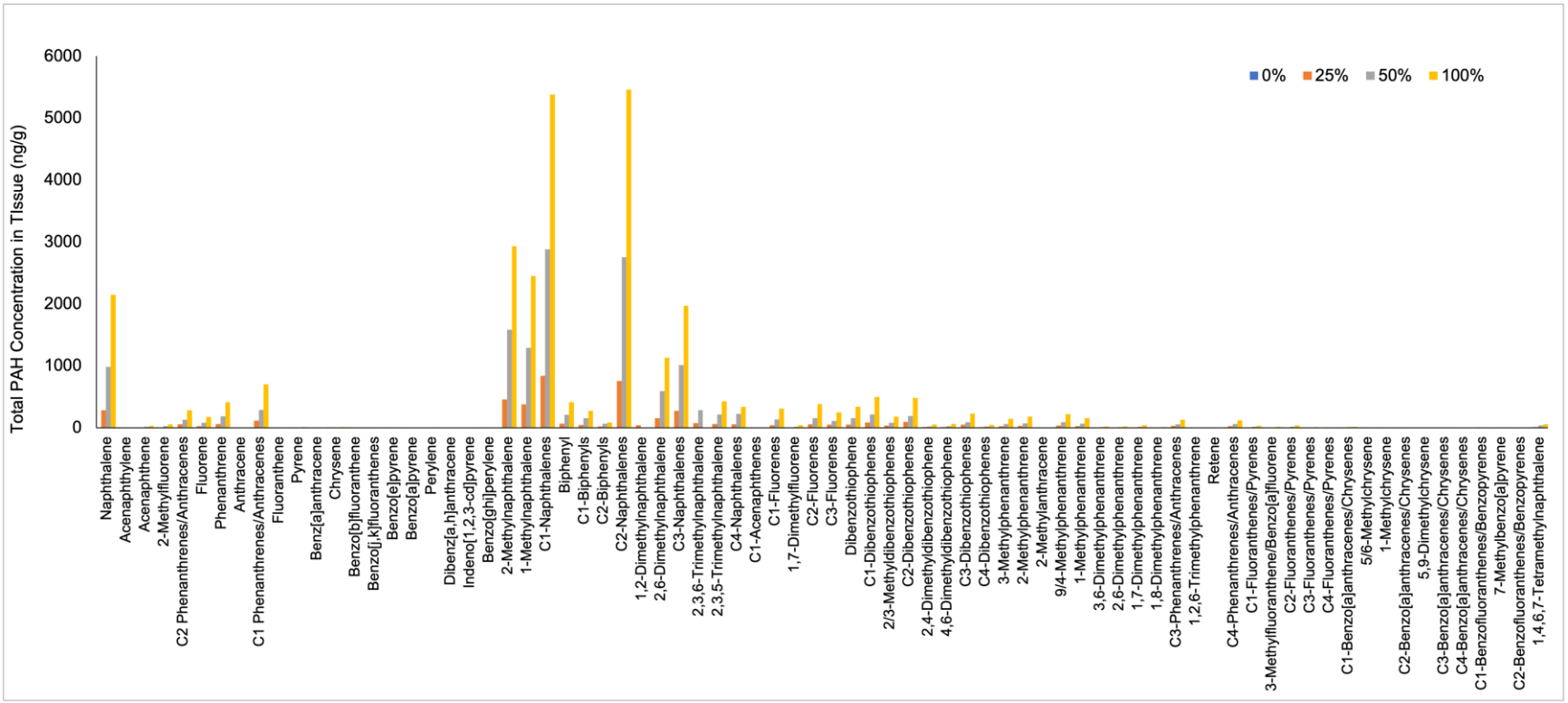


Figure A.15. Analytical chemistry profile for TPAC concentrations (ng/g) wet weight in crude whole tissue samples measured on day 7 of exposure. ~10g of oyster tissue samples were collected in amber glass bottles andimmediately frozen on dry ice for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).

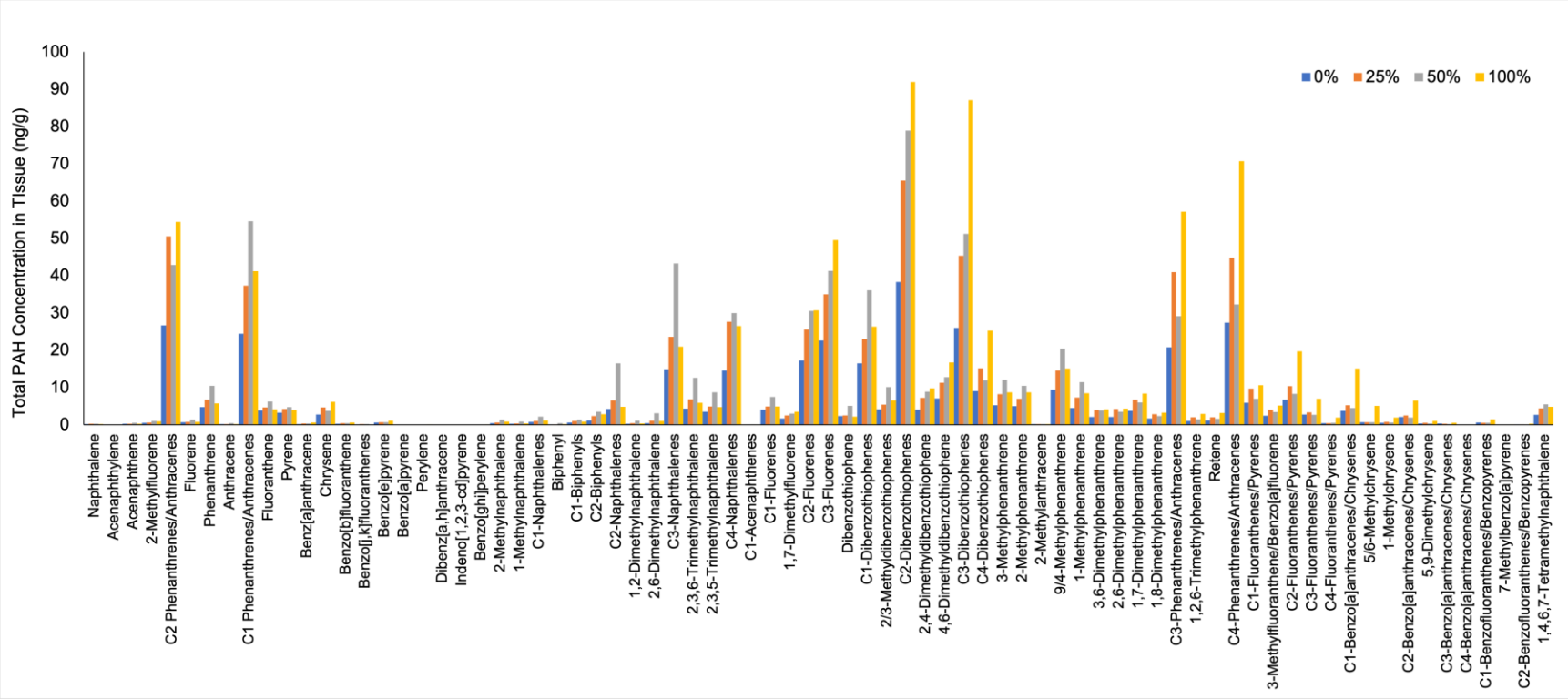


Figure A.16. Analytical chemistry profile for TPAC concentrations (ng/g) wet weight in crude whole tissue samples measured on day 21 post exposure. ~10g of oyster tissue samples were collected in amber glass bottles andimmediately frozen on dry ice for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).

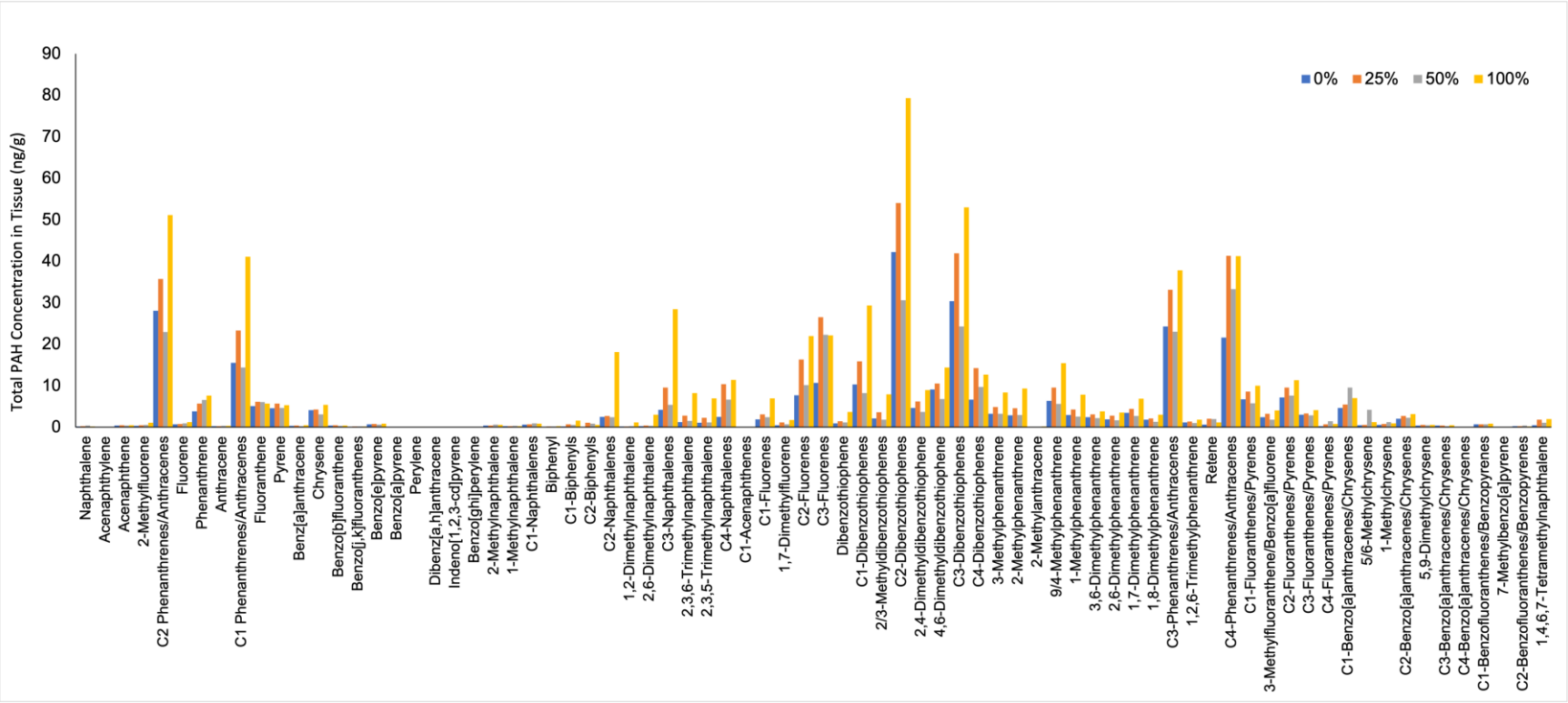


Figure A.17. Analytical chemistry profile for TPAC concentrations (ng/g) wet weight in crude whole tissue samples measured on day 35 post exposure. ~10g of oyster tissue samples were collected in amber glass bottles andimmediately frozen on dry ice for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).

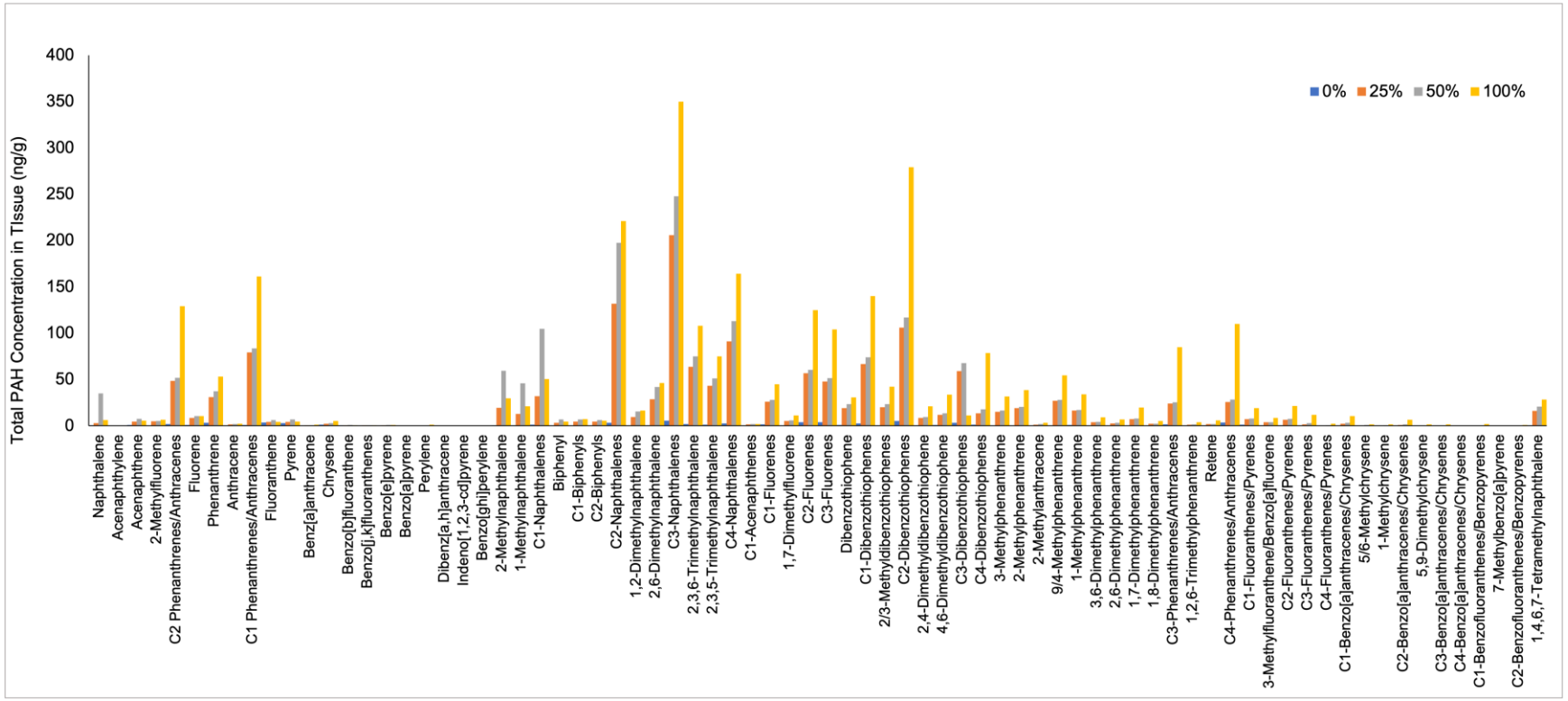


Figure A.18. Analytical chemistry profile for TPAC concentrations (ng/g) wet weight in diluted bitumen whole tissue samples measured on day 7 of exposure. ~10g of oyster tissue samples were collected in amber glass bottles andimmediately frozen on dry ice for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).

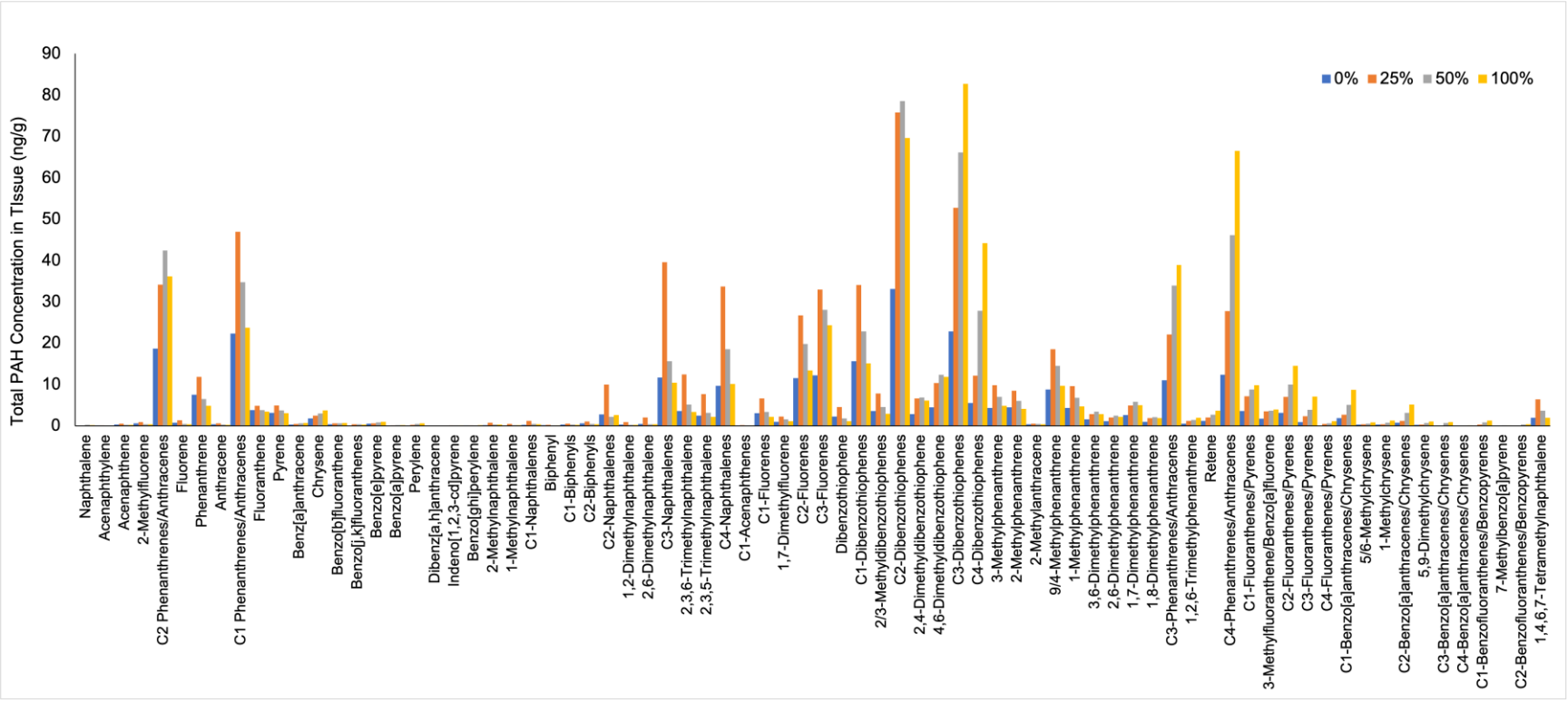


Figure A.19. Analytical chemistry profile for TPAC concentrations (ng/g) wet weight in diluted bitumen whole tissue samples measured on day 21 post exposure. ~10g of oyster tissue samples were collected in amber glass bottles andimmediately frozen on dry ice for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).

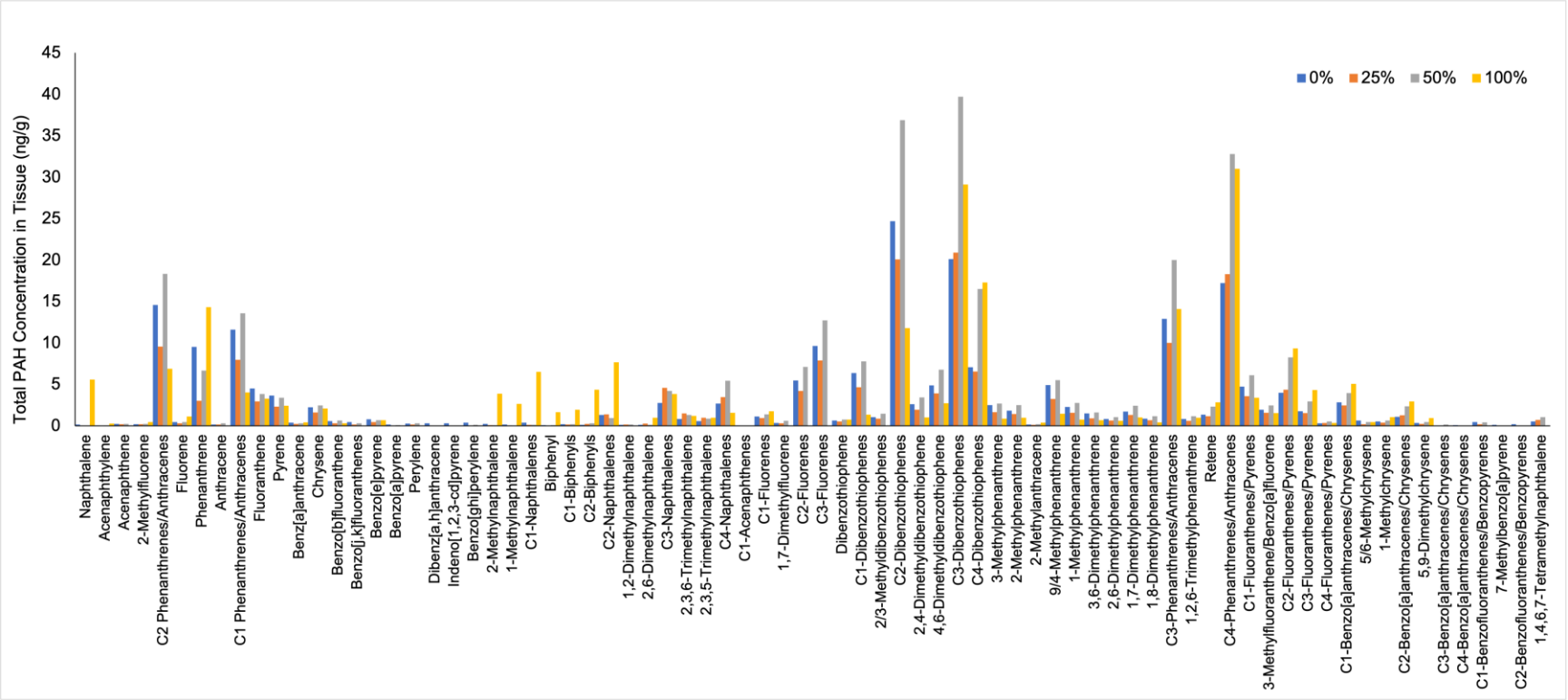


Figure A.20. Analytical chemistry profile for TPAC concentrations (ng/g) wet weight in diluted bitumen whole tissue samples measured on day 35 post exposure. ~10g of oyster tissue samples were collected in amber glass bottles andimmediately frozen on dry ice for preservation and transportation to SGS Axys Analytical Services (Nanaimo, BC.). Concentrations of PACs were quantified using LR GC/MS for each WAF dilution (0%, 25%, 50%, 100%).

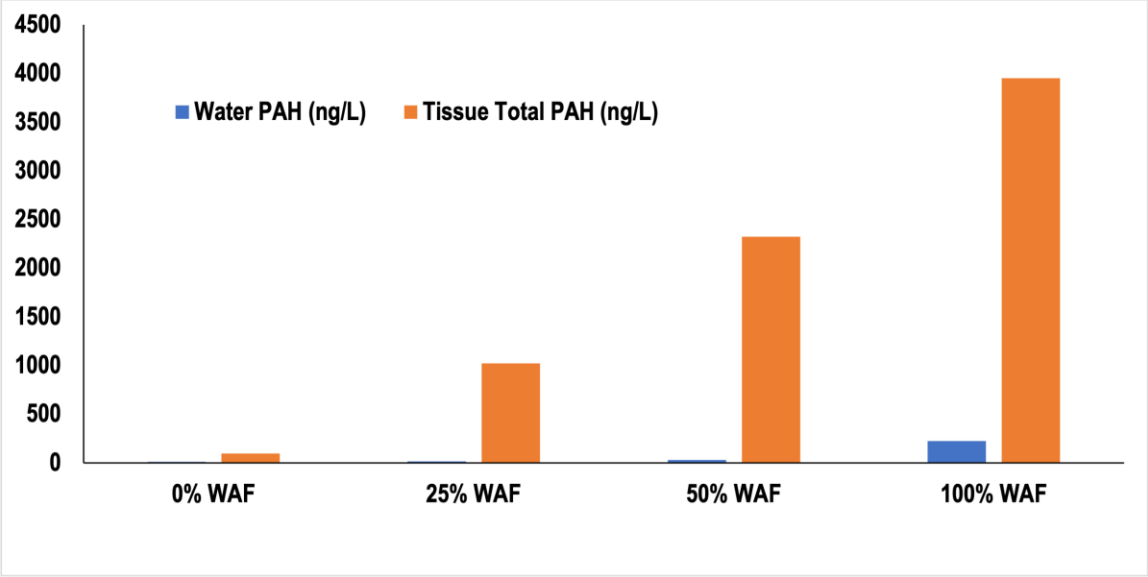


Figure A.21. Bioconcentration of TPAC in WAF samples (ng/L) and whole oyster tissue (ng/L) wet weight from marine diesel day 7 exposure measurements.

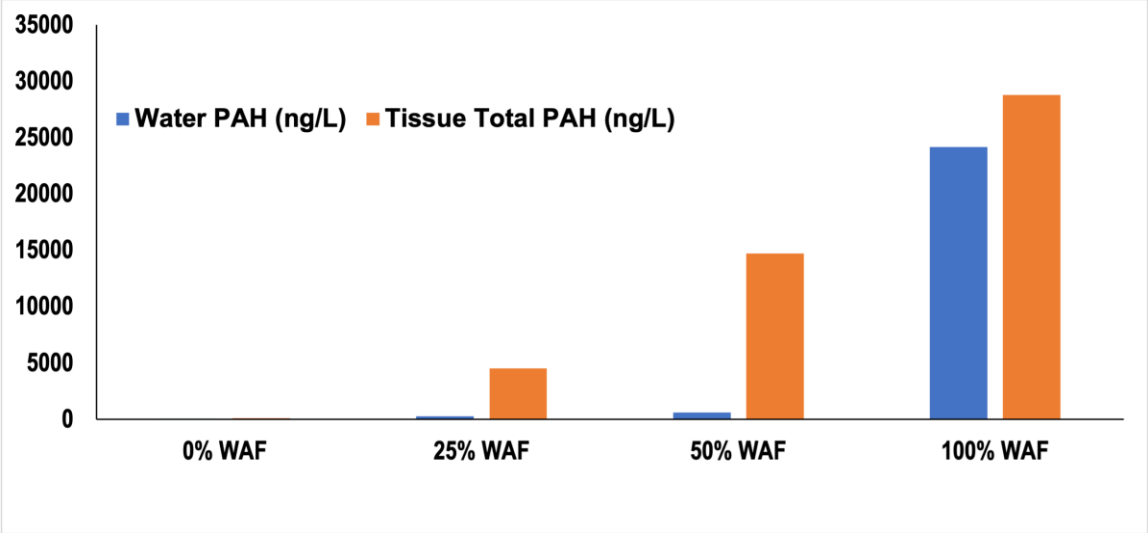


Figure A.22. Bioconentration of TPAC in WAF samples (ng/L) and whole oyster tissue (ng/L) wet weight from crude day 7 exposure measurements.

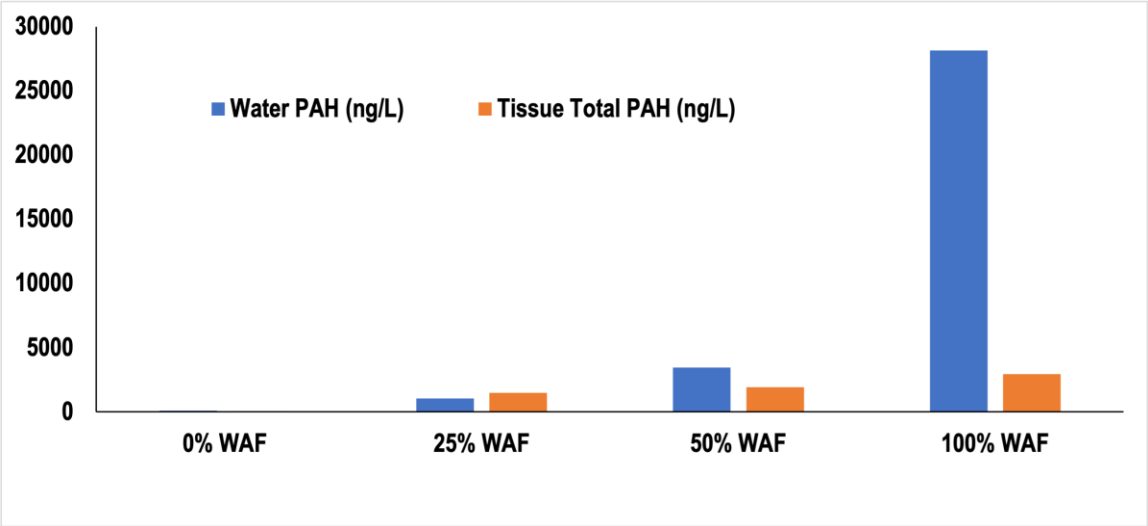


Figure A.23. Bioconcentration of TPAC in WAF samples (ng/L) and whole oyster tissue (ng/L) wet weight from bitumen day 7 exposure measurements.