Investigating the Mutagenicity of Polycyclic Aromatic Compounds from the Athabasca Oil Sands Region in River Otters and a Mammalian Cell Line

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Abstract

Mining operations have led to an increase in polycyclic aromatic compound (PAC) concentrations in the Alberta oil sands area. However, the toxicity of most PACs and PAC mixtures is not well characterized. Some PACs and PAC mixtures are known to be mutagenic, though there is limited research on the genotoxicity of PACs from the Alberta oil sands to wildlife. This thesis tested the hypothesis that anthropogenic sources of PACs from the Alberta oil sands are mutagenic to wildlife. The objectives were: 1) to determine whether wildlife with increased exposure to PACs had increased mutations, and 2) to determine whether an anthropogenic source of PACs is mutagenic in a controlled lab setting. For the first objective, we used a single-molecule polymerase chain reaction (SM-PCR) assay to detect microsatellite mutations in river otters with differing liver tissue PAC concentrations in the Athabasca oil sand region (AOSR; Alberta, Canada). For objective two, an *in vitro* mammalian mutagenicity assay with the FE1 MutaMouse epithelial cell line (FE1) was used to determine the mutagenic potential of a bitumen extraction by-product, tailings pond bitumen. We found that PAC exposure in the AOSR was positively correlated with elevated microsatellite mutations in river otters. From the *in vitro* study, tailings pond bitumen extracts did not induce *lacZ* mutations in the FE1 cells. Differences in detection methods between the two assays and PAC profiles between the otter tissue and tailings pond bitumen are suspected reasons for contradictory results. Further investigation of the different sources and PAC profiles within the AOSR environment and wildlife food web will provide insights on what types of PACs are mutagenic. Cytotoxicity, observed following exposure to tailings pond bitumen extracts, also suggests other toxicity pathways should be considered when investigating the toxicity of bitumen from the AOSR. Overall, this thesis provided data on the potential mutagenicity of PACs in the AOSR,

which can be used to elucidate potential molecular mechanisms of toxicity in wildlife exposed to oil processing contaminants.

Résumé

Les opérations minières ont entraîné une augmentation des concentrations de composés aromatiques polycycliques (CAP) dans la région des sables bitumineux de l'Alberta. Cependant, la toxicité de la plupart des CAP et des mélanges de CAP n'est pas bien caractérisée. Certains CAP et mélanges de CAP sont connus pour être mutagènes, bien qu'il y ait peu de recherches sur la génotoxicité des CAP des sables bitumineux de l'Alberta pour la faune. Cette thèse a testé l'hypothèse que les sources anthropiques de CAP des sables bitumineux de l'Alberta sont mutagènes pour la faune. Les objectifs étaient : 1) de déterminer si la faune avec une exposition accrue aux CAP présentait une augmentation des mutations, et 2) de déterminer si une source anthropique de CAP est mutagène dans un milieu de laboratoire contrôlé. Pour le premier objectif, nous avons utilisé un test de réaction en chaîne par polymérase à molécule unique (SM-PCR) pour détecter des mutations microsatellites chez les loutres de rivière présentant des concentrations différentes des CAP dans les tissus hépatiques dans la région des sables bitumineux de l'Athabasca (RSBA ; Alberta, Canada). Pour le deuxième objectif, un essai de mutagénicité *in vitro* sur des mammifères avec la lignée cellulaire épithéliale, appelée FE1 MutaMouse (FE1), a été utilisé pour déterminer le potentiel mutagène d'un sous-produit d'extraction de bitume, le bitume des bassins de résidus. Nous avons trouvé que l'exposition aux CAP dans la RSBA était positivement corrélée avec des mutations microsatellites élevées chez les loutres de rivière. De l'étude *in vitro*, les extraits de bitume des bassins de résidus n'ont pas induit de mutations *lacZ* dans les cellules FE1. Les différences dans les méthodes de détection entre les deux analyses et les profils de CAP entre le tissu de loutre et le bitume des bassins de résidus sont des raisons présumées des résultats contradictoires. Une étude plus approfondie des différentes sources et profils de CAP au sein de l'environnement de la RSBA et du réseau

trophique de la faune permettra de mieux comprendre les types de CAP mutagènes. La cytotoxicité, observée à la suite d'une exposition à des extraits de bitume de bassin de résidus, suggère également que d'autres voies devraient être envisagées lors de l'étude de la toxicité du bitume de la RSBA. En somme, cette thèse a fourni des données sur la mutagénicité potentielle des CAP dans la RSBA, qui peuvent être utilisées pour élucider les mécanismes moléculaires potentiels de leur toxicité chez la faune exposée aux contaminants du traitement du pétrole.

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List of Abbreviations

AHMW	Alkylated higher molecular weight
AIHTS	Agreement on International Humane Trapping Standards
ALMW	Alkylated lower molecular weight
ANT	Anthracene
AOSR	Athabasca oils sands region
ASE	Accelerated solvent extraction
B[*a*]P	Benzo[*a*]pyrene
BghiP	Benzo[*ghi*] perylene
bp	Base pair
CNRL	Canadian Natural Resources Ltd.
COGRAD	Centre of Oil and Gas Research and Development
CYP1A4	Cytochrome P450 1A4
CYP450	Cytochrome P 450 enzymes
DCM	Dichloromethane
DE	Diatomaceous earth
DMBA	7,12-dimethylbenz[*a*]anthracene
DMEM/F-12	Dulbecco's modified eagle medium/Nutrient mixture F-12
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco’s phosphate buffered saline
ECCC	Environment and Climate Change Canada
EEM	Environmental Effects Monitoring
EI	Electron ionization
EROD	Ethoxyresorufin-*O*-deethylase
ESTR	expanded simple tandem repeats
FBS	Fetal bovine serum
FE1	FE1 MutaMouse lung epithelial cell line

GC/MS/MS	Gas chromatograph coupled with a triple quadrupole mass spectrometer
GHG	Greenhouse gases
GPC	Gel permeation chromatography
HMW	High molecular weight
HPACs	Halogenated polycyclic aromatic hydrocarbons
HTT	Heritable translocation test
IcdP	Indeno[*1,2,3-c,d*]pyrene
JOSM	Joint Canada-Alberta Implementation Plan for Oil Sands Monitoring
LMW	Low molecular weight
MRM	Multiple-reaction monitoring
NIST	National Institutes of Standards and Technology
NSO-heterocycles	Heterocyclic polycyclic aromatic hydrocarbons
NWRC	National Wildlife Research Center
PAC	Polycyclic aromatic compound
PAD	Peace Athabasca Delta
PAH	Polycyclic aromatic hydrocarbon
PCR	Polymerase chain reaction
PEF	Potency equivalent factor
Pfu	Plaques forming units
P-Gal	Phenyl-β-D-galactopyranoside
PHE	Phenanthrene
PHMW	Parent higher molecular weight
PLWM	Parent lower molecular weight
RICC	Relative increase in cell counts
SCO	Synthetic crude oil
SLT	Specific locus test
SMA	Surface minable area
SM-PCR	Single molecule-polymerase chain reaction

SRM	Standard reference material
TGR	Transgenic rodent
UHP	Ultra-high-purity
US EPA	United States Environmental Protection Agency

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Study design	Helina Gyasi Jory Curry Kelsey Ha Philippe J. Thomas Jason M. O'Brien
Sample Collection	Northern Alberta commercial trappers
Chemical Analysis	Centre of Oil and Gas Research and Development
Source Determination	Helina Gyasi
River otter Physiological Analysis & Dissection	National Wildlife Research Center
DNA Extraction	Jory Curry
Single Molecule PCR Microsatellite Mutation Assays	Helina Gyasi Jory Curry Jared Browning Kelsey Ha
Data Analysis	Helina Gyasi Jason M. O'Brien
Manuscript Preparation	Helina Gyasi Philippe J. Thomas Jason M. O'Brien

Chapter 3. Polycyclic Aromatic Compound Extracts from Athabasca Oil Sands Tailings Pond Bitumen Do Not Induce *LacZ* Mutations in FE1 MutaMouse Lung Epithelial Cells

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Cytotoxicity Assays	Helina Gyasi
LacZ Mutation Assays	Helina Gyasi
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Chapter 1. General Introduction

1.1 The Alberta Oil Sands Area

1.1.1 Alberta Oil Sands Area

Oil sands are a vital source of energy for transportation, residential, and industrial purposes. Worldwide, Canada has the third-largest oil reserve where 98% of the reserves are in Northern Alberta oil sands found in three deposits: 1) Athabasca (~93,000 Km^2) , 2) Peace River (~29,000 Km^2), and 3) Cold Lake (~18,000 Km^2) (1–3). The oil sand deposits comprise a mixture of water (5%), sand (85%), and bitumen (10%) (4). Bitumen is a viscous crude oil made up of complex hydrocarbons that can be mined from the surface or extracted by drilling (in-situ extraction using steam-assisted gravity drainage) into deposits found deeper than 70 meters (200 ft.). In the Alberta oil sands area, the surface minable area (SMA) accounts for 20% of the oil sands reserve, whereas the bitumen found deeper underground makes up about 80% of the oil sands resource (1,5). Currently there are four surface mining upgrading facilities in Alberta; Shell Scotford, Suncor Base and Millennium, Syncrude Mildred Lake, and Canadian Natural Resources Ltd. (CNRL) Horizon (6). In 2019, Canadian oil sands produced 2.9 million barrels of raw bitumen per day and 80.5% of Canada's total production came from Alberta (7). Overall, in Canada, oil sands production is anticipated to increase by 1.27 barrels per day by 2035 (8). The global energy demand is expected to rise by 24% by 2040 and oil will supply about 28% of this demand. As a result of the demand for oil, oil sands mining and extraction operations will continue to rise (9). Bitumen mining operations have been shown to have negative impacts to the Alberta oil sands environment. Therefore, there are concerns that increase activity will further alter and pollute the surrounding aquatic and terrestrial ecosystems with petroleum extraction and processing by-products (10,11).

To address pollution concerns from the Alberta oil sands operations the Minister of the Environment created the Federal Oil Sands Advisory Panel in 2010 (12). The panels' report revealed the lack of a monitoring system in the oil sands. In response, Environment and Climate Change Canada (ECCC) released the Integrated oil sands Environment Monitoring Plan to monitor the air and water quality, and biodiversity in the Alberta oil sands area. Following this plan, in 2012, the Joint Canada-Alberta Implementation Plan for Oil Sands Monitoring (JOSM) by ECCC and Alberta Environment and Sustainable Resource Development was formed and released a collaborative plan to implement and improve the monitoring of the oil sands environment, which included wildlife toxicology and habitat disturbance surveillance (12). Since then, many initiatives and projects have revealed contaminant concentrations/deposition, emission levels, and biodiversity disturbances in the oil sands. However, further work is required to understand and address the cumulative environmental effects of Alberta oil sands development (12–14).

1.1.2 Bitumen Processing By-Products & Polycyclic Aromatic Compounds

By-products from bitumen mining, extraction and upgrading processes are major sources of pollution to the Alberta oil sands surrounding environment. Surface mining of bitumen involves exposing the petroleum source by shoveling and removing the bitumen ore from the surface, which results in the release of sulfur and nitrogen oxides, hydrocarbons, and fine particulate matter into the atmosphere (15,16). The ore is then processed by crushers and hot water is added to allow the bitumen to be transported and extracted. At the extraction plant, bitumen is separated from the mixture of sand, clay, debris, and water by a gravity separation vessel. In the separation vessel, the diluted bitumen is separated by attaching to air bubbles and floating to the top of the vessel in a froth leaving behind the water, sand, clay, and residual

bitumen to be pumped into tailings ponds for storage and water recovery (17–19). These tailings ponds are susceptible to evaporation, seepage, or leakage into ground water (20,21). Another source of pollutants in the Alberta oils sands area is through bitumen upgrading. After extracting the bitumen, the recovered raw and thick bitumen can be processed into synthetic crude oil (SCO; a lighter oil) using upgraders. During the upgrading process, the C:H ratio is increased through carbon injecting (coking) or hydrogen addition (hydroconversion). Coking involves thermally cracking (breaking down) the bitumen residue (complex long-chain hydrocarbon molecules) into the light hydrocarbons (simpler short-chain hydrocarbon molecules) leaving behind petroleum coke or "petcoke" (18,22). Due to the high sulfur content, petcoke cannot be used as fuel and is stored in large piles on mine sites making it susceptible to wind erosion and therefore, a major source of contaminants in the Alberta oils sands environment (22,23).

Polycyclic aromatic compounds (PACs) are one of the main contaminant classes of concern found in bitumen, SCO, and their processing by-products. PAC is a general term describing several thousand highly lipophilic organic compounds made up of two or more aromatic rings. Unsubstituted PACs are known as polycyclic aromatic hydrocarbons (PAHs) or parent PACs. PACs can also be alkylated, heterocyclic, where one carbon group in the aromatic ring is replaced with a sulfur, nitrogen, or oxygen (NSO-heterocycles), or halogenated, where a halogen is attached to the aromatic ring (HPACs). PACs with two to three fused rings are considered low molecular weight (LMW) and those with 4 or more fused rings are high molecular weight (HMW) (20,24,25). PACs can be produced in four ways: 1) diagenesis of organic matter after deposition in sediments; 2) biosynthesis of certain individual PACs by plants and animals (i.e., biogenic); 3) formation of fossil fuels such as petroleum and coal (i.e., petrogenic); 4) incomplete combustion of organic matter (i.e., pyrogenic), which can occur

naturally (e.g., forest fires) or anthropogenically (e.g., burning of fossil fuels). Each source will produce mixtures with varying PAC profiles. For example, petrogenic sources generally have a higher proportion of alkylated and lower molecular weight PACs, whereas pyrogenic sources tend to have a greater proportion of parent and high molecular PACs (26). Sources of PACs in the Alberta oil sands environment are both natural and anthropogenic. Natural sources include petrogenic PACs from oil seepages and erosion and pyrogenic PACs from forest or grass fires. Anthropogenic environmental inputs, which can produce different PACs and mixtures to those of natural origin, can come from release of petrogenic PACs into the environment through accidental petroleum spills or erosion, seepage, and emissions from industrial operations and by-products; or pyrogenic PACs from thermal cracking and burning fossil fuels (20,24,25).

Sixteen parent PACs were identified as priorities by the United States Environmental Protection Agency (US EPA) in 1976 based on their prevalence in nature, availability of analytical standards, detection capability with existing analytical methods, and whether they were known to be toxic. Most monitoring and laboratory studies to date have focused on these US EPA priority PACs. However, one consequence of this is that our understanding of non-priority PACs, including alkylated, heterocyclic, and halogenated PACs, is currently very limited. Some of these less-studied compounds also have high toxicity; in fact, some alkylated PACs have been shown to be more toxic and mutagenic than their parent counterparts. For example, three to five ring alkylated PACs, such as retene and other related alkylated phenanthrenes, have been shown to be more toxic to developing fish than their unsubstituted congeners by inducing blue sac disease. Blue sac disease results in hemorrhages, yolk sac edemas, and skeletal deformities in these young fish (27–30). In addition, the binding affinity of 7, 12- dimethylbenzo[*a*]anthracene (DMBA) to mouse skin DNA was shown to be 20 times

greater than its parent compound and twice that of benzo[*a*]pyrene (B[*a*]P; a known carcinogen) (31,32). Thus, there is concern that due to our currently incomplete understanding of the total makeup of PAC mixtures found in the environment, ecological risk assessments may be overlooking toxicologically important compounds (26,33,34).

1.1.3 Wildlife Exposure to PACs from Bitumen Mining

Multiple environmental studies have revealed the distribution and deposition of contaminants, such as PACs, from oil sand operations in the Alberta oil sands area. Contaminant profiles in sediment cores show how PAC levels have changed over time. These studies have revealed that since the onset of mining, total PAC concentrations within river sediments near oil sand operations in the Athabasca region have increased 2.5-23 times greater (35), and now contain more petrogenic-sourced PACs as opposed to pyrogenic PACs (mainly from wood burning) compared to pre-mining times (pre-1960), suggesting industrial origin (36). In the Athabasca oils sands region (AOSR), an exponential decline of PACs within the air was observed with increasing distance from mining sites (37). Similarly, it was reported that 391 kg of PACs accumulated in snowpack within 50 Km of upgrading facilities (10). These studies indicate that by-products from oil sands bitumen extraction and upgrading are major sources of PAC contamination in the local environment.

There are also multiple lines of evidence that bitumen mining and processing operations are also major source of PAC contamination to wildlife and their food webs. For example, high PAC concentrations (ranging from 11 to 120 ng/g wet weight [ww]) were found in muscle tissue from Athabasca River fish collected close to oil sand extraction and upgrading activities (38). PACs have also been detected in apical predators suggesting bioaccumulation through the food chain. Tree swallow nestlings situated at mining sites had greater total PAC concentrations (31-

106 ng/g ww) than at reference sites (13-27 ng/g ww) (39). It was determined that the elevated PAC exposure was due to contaminated diet (via consumption of aquatic-emerging insects that accumulate PACs) (39). Wildlife (e.g., birds) can also be exposed to PACs from tailings ponds through direct contact by entering the water (40,41). Long distance migratory birds can use tailings ponds during inclement weather as a pre-migratory fuelling site or resting spot along their migratory pathway, especially in colder climates as these ponds don't completely ice over like natural freshwater bodies of water due to the warm tailings discharged into the ponds. Ingestion of bitumen from the surface water or settled in the sediment, from oiled feathers by swimming or diving into the tailings ponds or from contaminated prey including invertebrates and fish are sources of dietary PAC exposure to migratory birds (40–42). PAC concentrations linked to oil sand operations found in moose and wolf scat also suggest exposure to PACs through diet as moose largely feed on vegetation along river margins and banks, and wolves can be exposed through PAC-burdened prey (43). Furthermore, concentrations of PACs have been detected in both predator and prey species across AOSR food webs suggesting bioaccumulation. For example, Xia et al. (2019) detected PACs in the livers of predators such as northern pike (12.5 ng/g) and river otter (5.5 ng/g), and in species they prey upon such as snails (170.5 ng/g) and lake whitefish (16.3 ng/g). However, the higher concentrations in prey compared to predators suggest that, although there is bioaccumulation, PACs do not seem to magnify through the food web (44).

1.2 Toxicity of Polycyclic Aromatic Compounds

1.2.1 PAC Toxicity to AOSR Wildlife

PAC exposure is associated with various toxic effects in wildlife species in the oil sands. PACs have been shown to accumulate in vertebrate terrestrial animals, but there is limited study of PACs potential toxicity to these animals in the AOSR (45). However, PACs from the AOSR have been shown to cause effects in small mammals. Mice exposed to 40 μg/kg of a mixture of three-ringed alkylated PACs, which were dominate in melted snow found near an AOSR upgrading facility, had organs (i.e., spleen, liver, and kidney) that were 9-20% larger compared to controls. In addition, PAC exposure also altered the murine levels of hepatic vitamins A and E and oxidative stress biomarkers (e.g., glutathione redox status) in their testes (46). Avian toxicological studies on PAC exposure effects in the AOSR are predominantly based on tree swallows and reveal effects to the endocrine system, metabolism, and development. Increased thyroid hormone levels (47), and suppressed T cell response and higher ethoxyresorufin-*O*-deethylase (EROD; induction of detoxification enzymes in the liver) activity (48) were observed in tree swallow nestlings from reclaimed wetlands (contaminated with tailings slurry) and nestlings situated in nest boxes within 5 km of active oil sands industrial sites, respectively. Increased EROD activity and cytochrome P450 1A4 (CYP1A4) messenger RNA expression was also shown in avian hepatocytes exposed to petcoke (49) and extracts from passive sampling devices deployed near bitumen upgrading facilities (50). In addition, tree swallow growth (body weight and fledging production) and reproductive success have been shown to be influenced by PACs in the AOSR in combination with multiple stressors such as diet, sex, hatch date, which are or can be influenced by PAC disposition and exposure to these birds (51). Aside from tree swallows, juvenile mallard ducklings, placed on reclaimed wetlands in the AOSR, had decreased

body mass and skeletal size (length of tarsus, bill length and depth) indicating potential effects to the survival of juvenile waterfowl residing on wetlands contaminated with PACs (52). Similarly, wood frog tadpoles exposed to PAC-burdened wetlands showed delayed metamorphosis and thyroid hormone disruption in the AOSR (53).

There are several studies showing that PACs elicit embryo toxicity when exposed to fish at early-life stages. Effects include impacts to development, survival, and the circulatory system; for example larval mortality, delayed hatching, formation of edemas, hemorrhages, and fin, skeletal, craniofacial, and eye malformations (30,54,55). In the AOSR specifically, there are some studies that show comparable PAC effects to fish. For example, high PAC concentrations from the lower Athabasca river sediment impacted hatching success in fathead minnows (56). Jaw deformities and decreased growth and survival were also observed in fathead minnow embryos exposed to bitumen containing sediment from the AOSR (57). Malformations such as the lack of an inflated swim bladder, yolk sac and pericardial edemas in Japanese medaka embryos (58), and presence of yolk sac and pericardial edemas in zebrafish (59) were induced when exposed to diluted bitumen (routinely used to transport oil through pipelines). Similarly, western clawed frog embryos exposed to high PAC concentrations in diluted bitumen exhibited gut and craniofacial malformations, edemas, and shorter body lengths (60). Taken together, toxic effects of PAC exposure include impaired reproduction and growth, deformities, endocrine disruption, and embryotoxicity in wildlife from the AOSR.

1.2.2 PAC Genotoxicity

Several PACs are known to be genotoxic and are classified as carcinogens (e.g., B[*a*]P) (32). Many of these PACs elicit their genotoxic effects when metabolized by cytochrome P 450 enzymes (CYP450), which results in the production of reactive metabolites and oxidative stress

that cause DNA damage. Usually, phase I metabolism enzymes, such as CYP450, transform xenobiotic compounds into more polar and hydrophilic metabolites that can be excreted (61). However, genotoxic PACs are activated by CYP450 into three types of reactive metabolites: diol epoxides, PAC radical cations, and quinones. Epoxides and radical cations can bind to DNA, changing the structure, and form DNA adducts or induce oxidative stress. DNA adducts, if not properly controlled by DNA repair mechanisms, can lead to strand breaks and mutations. Oxidative stress also provokes the formation of mutations through the production of reactive oxygen species (ROS). Quinones, the third type of reactive metabolites, can cause the production of ROS as well. ROS are highly reactive chemical species that contain oxygen and contribute to oxidative stress in organisms. They react with DNA, causing point mutations, oxidized bases, and DNA-strand breaks (62–64). Often, genetic damage from PACs will result in cell death. However, when non-lethal mutations occur in key genes it can lead to genetic consequences such as tumor formation and cancer (64,65). Further, if DNA damage occurs in germ cells, it could lead to mutations that cause heritable diseases (66).

PAC exposures have exhibited genotoxic damage in both laboratory organisms and wildlife. For instance, coal tar, another complex mixture of hydrocarbons, induced DNA damage (e.g. caused DNA adducts) and point mutations in mice (67), and promoted murine skin tumor formation in combination with other PAC mixtures, diesel particulate and cigarette smoke condensate (68). In the Great lakes, gulls and cormorants had elevated mini- and microsatellite mutations rates (mutations resulting in the loss or gain of DNA nucleotides) that were associated with exposure to PACs released from industrial steel operations (69,70). Though it was not significant, there is also evidence of increased microsatellite mutation rates in fathead minnows exposed to PAH contaminated sediment (71). Additionally, DNA-adducts were detected in the

liver of fish collected near aluminum smelters in British Columbia (72). Clearly, exposure to ecologically relevant PAC mixtures have been associated with genomic health effects; however, whether PACs found in the AOSR can cause mutations in wildlife at environmentally relevant concentrations remains uncertain.

1.3 Measuring Exposure-Induced Mutations

1.3.1 Microsatellite Mutation Assays

A mutation is a change in the nucleotide sequence of DNA. These changes can lead to the production of a different or malfunctional protein, the wrong amount of protein, or no protein. Gene mutations can be classified as either hereditary or somatic. Hereditary or germline mutations (present in the egg or sperm) are inherited from a parent and present throughout an individual's life in all cells of the body. Somatic or acquired mutations are caused by environmental factors, such as from radiation or chemicals, or through errors in cell division, are only present in certain cells, and cannot be passed on to progeny (73–75). Detecting *in vivo* mutation induction can be challenging. Most mutation types have very low background frequency making it very difficult to detect even moderate increases. For example, the single nucleotide mutation rate for somatic and germline tissue in humans has been estimated to be approximately 3×10^{-9} and 3×10^{-11} mutations per base pair per mitosis, respectively (76). Until recently, identifying *in vivo* germline genotoxic/mutagenic effects was limited as detection methods required massive numbers of animals, and was time consuming (77). For example, gold standards for germ cell testing were the mouse heritable translocation (HTT) and mouse specific locus tests (SLT), which detected genome wide chromosomal rearrangements and mutations that cause non-functional protein or no protein product in the offspring of treated mice, respectively. HTT involved cytogenetic or fertility analysis to detect translocation heterozygosity and required

500 first generation male mice per dose group (77,78). SLT detects heritable point mutations, which uses a mutant mouse strain carrying visible markers that is no longer housed in any laboratory (77,79). Several methods have emerged to improve detection of *in vivo* germline mutations including tandem repeat mutation analysis and transgenic rodent mutation assays. Due to the high spontaneous mutation rate of microsatellites and expanded simple tandem repeats (ESTR) loci, assays that measure these loci were popular and have been used to analyze mutation induction in the germline of mice exposed to various chemicals and pollutants. However, even though these loci can be measured using polymerase chain reaction technology, pedigree-based approaches where the DNA samples from dozens to hundreds of parents/offspring trios were still required to detect mutations (77). For example, Yauk et al. (2000) required ~1000 animals to detect induced germline minisatellite mutations in herring gulls living near steel mills using DNA fingerprinting (69). In addition, minisatellites and ESTRs are long DNA sequences (range up to 20 kb), which makes scoring of novel fragments difficult due to high GC rich repetitive regions and the detection of band length differences that are subjective when using long agarose gels or southern blotting. On the other hand, microsatellites, another class of tandem repeats, are more useful in detection of *in vivo* mutations due to their small size (total length < 1 kb), which makes them better suited to higher-throughput approaches than ESTRs (77).

Microsatellites, short DNA sequences comprising tandem repeats of one to six base pairs, are genetic markers that can be used for mutation detection. Microsatellites are common in the genome of most eukaryotes and tend to occur in non-coding regions of DNA (80–82). They are highly polymorphic and co-dominant genetic markers (83), so heterozygotes can be easily distinguished from homozygotes based on differences in allele size (84). Due to their repetitive sequences, microsatellites are prone to DNA replication and repair errors (accidental gain or loss

of a repeat unit) (80) and are therefore sensitive to mutation induction by DNA-damaging chemicals, such as PACs (70,85). Because of their high polymorphism, microsatellites are very useful for investigating population genetics (80) and thus, many microsatellite alleles have been characterized in a large number of species (86). Because of their high instability and ubiquitous characterization, microsatellites can be useful for measuring mutation induction in wildlife species, even ones that do not have well-characterized and annotated genome assemblies.

Another important advance in mutation detection is the emergence of single-molecule detection methods. Single molecule-PCR (SM-PCR) is a more sensitive approach developed to measure microsatellite mutation rates in individual animals circumventing the requirement for large samples sizes (85). SM-PCR is like standard PCR that results in exponential amplification of a selected region of DNA, but it amplifies single target molecules. Samples are diluted to contain, on average, one target molecule per reaction. Hundreds of reactions are then conducted per sample to determine the size of individual microsatellite copies. This type of PCR allows the mutation frequency of microsatellites to be determined by counting the occurrence of microsatellite length variants in individual target molecules (87). Since mutation rates can be detected in individual animals (as opposed to families of animals), far fewer samples are required to establish exposure-related effects, making it a suitable method for laboratory or field studies involving rare or difficult to obtain samples. For example, Rowan-Carroll et al. (2017) detected a significant increase in germline mutations using SM-PCR by quantifying microsatellite mutations in the sperm of B[*a*]P exposed mice using only 4-7 mice per dose group (88).

1.3.2 Transgenic Rodent Models

The transgenic rodent (TGR) gene mutation reporter assay enables efficient and effective screening of chemically induced mutations. The TGR assay is an internationally endorsed

laboratory method (89) used to detect, quantify, and characterize the *in vivo* mutagenicity of chemicals and mixtures in both somatic and germ cells (67,90). A variety of rodent models can be used for this assay, the most common of which are MutaMouse and BigBlue rat/mouse. Each of these models harbor a mutation-reporting transgene chromosomally integrated into their genome via a plasmid or viral vector. The vector carrying the transgene is easily recoverable from virtually any tissue in the rodent and induced mutations can be detected by analyzing the phenotype of the reporter gene in a bacterial host (91). Mutagenicity of a compound can be determined qualitatively by comparing mutant frequencies between control and exposed animals; however, quantitative estimates of mutagenic potency through dose-response modeling of mutant frequency across many exposure groups is becoming more common (92,93). Another benefit of the TGR assay is that the recovered reporter transgenes can be sequenced to discover the types of mutations induced providing additional information about the mutational mechanism of a chemical. With next generation sequencing technology, a high-resolution mutation spectrum of the transgene can be generated associating a mutational "fingerprint" to the chemical of interest, which then can be used as an environmental biomarker to detect exposure (90,94).

In light of efforts to reduce and replace animals for toxicity testing, the US EPA plans to eliminate all mammalian study funding by 2035 (95,96). In response, the field of mutation research is undergoing a shift from large-scale *in vivo* animal experiments to small-sized *in vitro* studies (97). As a result, the development of a number of TGR cell lines have been established: Big Blue mouse embryonic fibroblast (98), rat mammary epithelial and fibroblast carcinoma cell lines (99,100) as well as a MutaMouse FE1 epithelial cell line derived from lung tissue (101). The MutaMouse FE1 and BigBlue embryonic fibroblast cell lines have proved to be useful tools for mutagenicity testing (102,103). However, BigBlue embryonic fibroblasts were immortalized

and transformed by repeated mutagen exposure, by x-ray irradiation and B[*a*]P, and are known to be cytogenetically unstable, poorly differentiated, and pleomorphic (101,104). In addition, the embryonic cells have no capacity to identify mutagens that require metabolic activation to elicit genotoxic effects (105). The MutaMouse FE1 cell mutagenicity assay, on the other hand, has been used more routinely (> 30 agents) and is currently undergoing validation to be used as a standard *in vitro* mammalian mutagenicity assay (105). FE1 cells have proven useful in identifying mutagens that require metabolic activation, such as PACs, including B[*a*]P (101,106). The mutagenicity of PAC mixtures such as carbon black (102,107), diesel exhaust (108), and soil from PAH-contaminated industrial sites in Sweden (109) have also been evaluated using FE1 cells. Thus, the FE1 *in vitro* TGR model can be a useful animal-free alternative for rapidly characterizing the mutagenicity of compounds and mixtures.

1.4 Thesis Objectives and Hypothesis

PACs are increasing in the Alberta oil sands region because of mining operations in the area. The PACs in the Alberta oil sands environment are a diverse mixture of high and low molecular weight and alkylated and parent compounds, most of which have uncharacterized toxicity. Many structurally diverse PACs have been shown to induce toxicity via a variety of mechanisms, including genotoxicity; and some PACs are very potent mutagens. Thus, I hypothesize that PACs from anthropogenic sources in the Alberta oils sands are mutagenic.

The experimental chapters of my thesis will investigate two testable predictions of this hypothesis: (1) Chapter 2 tests the prediction that wildlife with elevated exposure to anthropogenic sources of PACs will also have elevated mutation burdens. I will test this by evaluating whether elevated PAC exposure in the AOSR is associated with increased microsatellite mutation frequency in river otters that reside in the Athabasca River. I predict that

there will be a positive correlation between microsatellite mutation frequencies and PAC tissue burdens in river otters. (2) Chapter 3 tests the prediction that PACs from anthropogenic sources will be mutagenic in controlled laboratory tests. I will test this by determining the mutagenic potential of PACs extracted from tailings pond bitumen using a dose-response approach with the *in vitro* mammalian mutagenicity assay – FE1 MutaMouse lung epithelial cell line (FE1 cells). I predict that PAC mixtures derived from the Athabasca oil sands tailings will induce *lacZ* mutations in FE1 cells.

This proposed research will provide much needed data on the toxicity of PAC contaminants in the AOSR and will improve our ability to assess the impacts of human mining activities in the AOSR on wildlife health.

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Chapter 2. Microsatellite Mutation Frequencies in River Otters (*Lontra Canadensis*) from the Athabasca Oil Sands Region are Correlated to Polycyclic Aromatic Compound Tissue Burden

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2.1 Abstract

Oil sand mining activities in the Athabasca oil sands region (AOSR) have contributed to increased deposition of pollutants such as polycyclic aromatic compounds (PACs) to the surrounding environment. However, the majority of PACs found in the AOSR, and the combined effects of PAC mixtures have not been evaluated for genotoxicity in wildlife. Here, we examine whether mutation frequencies in AOSR river otters are correlated to PAC tissue burdens. We used single-molecule polymerase chain reaction (SM-PCR) to measure the mutant frequency of unstable DNA microsatellite loci in the bone marrow of wild river otters (n=11) from the AOSR. Microsatellite mutation frequencies were regressed against liver PAC burden (total, low/high molecular weight (LMW/HMW), and parent/alkylated PACs), and to the distances from where the samples were collected to nearby bitumen upgraders. We found that microsatellite mutation frequency was positively correlated with total liver PAC burden. LMW and alkylated PACs were detected at higher levels and had a stronger positive relationship with mutation frequency than HMW (alkylated and parent) PACs. There were no significant relationships detected between mutation frequency and LMW parent PACs or the distance from bitumen upgraders. Furthermore, pyrogenic and petrogenic signatures suggest that PACs in animals with high mutation frequencies were associated with combustion processes; although further investigation is warranted, due to limitations of diagnostic ratio determination with biotic models. Our findings support the hypothesis that PACs found in the AOSR increase mutation frequency in wildlife. Further investigation is required to determine if the elevated PAC levels associated with higher mutation frequencies are due to natural exposure or elevated human activity.

2.2 Introduction

Oil sands are a vital source of energy for transportation, residential and/or industrial purposes. Canada has the third largest oil reserves in the world where 98% of the reserves are in the Northern Alberta oil sands (1,2). In 2018, Alberta averaged 3 million barrels of raw bitumen production per day and this is anticipated to increase to 4.3 million barrels per day by 2035 (1,3). Consequently, mining activities in the Athabasca oil sands region (AOSR) have altered and polluted the surrounding environment with various petroleum processing by-products, including polycyclic aromatic compounds (PACs) (4,5). PAC is a general term for a group of compounds comprising polycyclic aromatic hydrocarbons (PAHs) and various alkylated and heterocyclic congeners (6). PACs have been found throughout the AOSR environment within lake/river sediment (5,7,8), air (9), snow (5,10), and terrestrial (11,12) and aquatic wildlife (13).

Certain PACs are known to be genotoxic (i.e., can cause mutations) and carcinogenic (e.g., benzo[*a*]pyrene (B[*a*]P) (14)), and exposure to PAC mixtures has been shown to elicit genotoxic consequences in both laboratory animals and in wildlife. For instance, laboratory exposure to coal tar has been shown to induce DNA damage (i.e., cause DNA adducts) and mutations in mice (15) as well as promote murine skin tumor formation when mixed with other PAC mixtures diesel particulate and cigarette smoke condensate (16). In the Great lakes, herring gulls and cormorants had elevated mutation rates that were associated with PACs released from industrial operations (17,18). There is also evidence of increased mutation rates in fathead minnows exposed to PAC-contaminated sediment, though these observations were not statistically significant (19). Exposure to ecologically relevant PAC mixtures have thus been associated with genomic health effects; however, evidence that PACs found in the AOSR can cause mutations in wildlife at environmentally relevant concentrations remains elusive.

Single-molecule PCR (SM-PCR) amplification of DNA microsatellites is a technique that may address many of the challenges associated with detecting chemically induced mutations in wildlife at environmentally relevant concentrations. Due to their repetitive sequences, DNA microsatellites are prone to replication and repair errors (20), and have been shown to be responsive to chemical exposure, including PACs, in the laboratory and in wildlife (18,21). Importantly, microsatellite mutation analysis does not require a well-characterized and annotated genome assembly, as is often the case with wildlife species. Traditional approaches to measuring microsatellite mutation rates used family pedigrees and required very large sample sizes. For example, Yauk et al. required ~1000 animals to detect induced germline mutations in herring gulls living near steel mills using DNA fingerprinting (17). However, SM-PCR can quantify germline and somatic microsatellite mutations in individuals (21,22), circumventing the requirement to genotype large sets of parent-offspring trios. These qualities make microsatellite SM-PCR a practical tool for determining mutation frequencies in wildlife.

River otters (*Lontra canadensis*) are known as sensitive indicators of ecosystem health and are thus suitable bioindicators (sentinels) (23,24). Intensive trapping of river otters in the nineteenth and early twentieth century as well as habitat destruction through industrial development (e.g., mining, oil and gas development, pulp and paper), and subsequent water pollution led to the decline and local extirpation of some populations in regions of North America (25–27). River otters are sensitive to environmental disturbances because of their status as top predators in aquatic environments. As an apex mammalian predator, they can be susceptible to negative health consequences resulting from the bioaccumulation/magnification of persistent environmental pollutants through their diet (28–31). River otters that reside in the Athabasca area are exposed to pollutants from nearby industrial operations including PACs from

oil sands activities. As ecosystem sentinels within the AOSR, the river otter represents an ideal species to better understand the effects of PACs on the genomic health of wildlife.

With evidence that PACs are increasing in the AOSR due to mining operations, and that PACs have the potential to cause genotoxic effects, we investigated whether PACs from the AOSR are associated with increased mutation frequencies in wildlife. To do this, we evaluated whether microsatellite mutation frequencies in the bone marrow of AOSR river otters were correlated to PAC liver tissue burden (as a proxy measure of exposure) or to the distance of where the otters were captured (i.e., centroid of trapline) relative to the nearest bitumen upgrading facility.

2.3 Materials and Methods

2.3.1 Study Area, Sample Collection & Preparation

The study areas included four locations from the AOSR: Saline Lake, Opportunity No. 17, Peace-Athabasca Delta (PAD) and Gordon Lake. Saline Lake is in the center of the surface mineable area (SMA) and Opportunity No. 17, a remote municipal district, is 140 km west of the SMA. The PAD is located downstream of the Athabasca River inside Wood Buffalo National Park, a UNESCO World Heritage Site. The fourth site, Gordon Lake, is upstream of the SMA within the Gipsy Lake Wildland Provincial Park (Figure 2.1). These sites include varying degrees of anthropogenic disturbances and were identified by traditional land users as being important for the practice of cultural and recreational activities (e.g., hunting, fishing, and trapping).

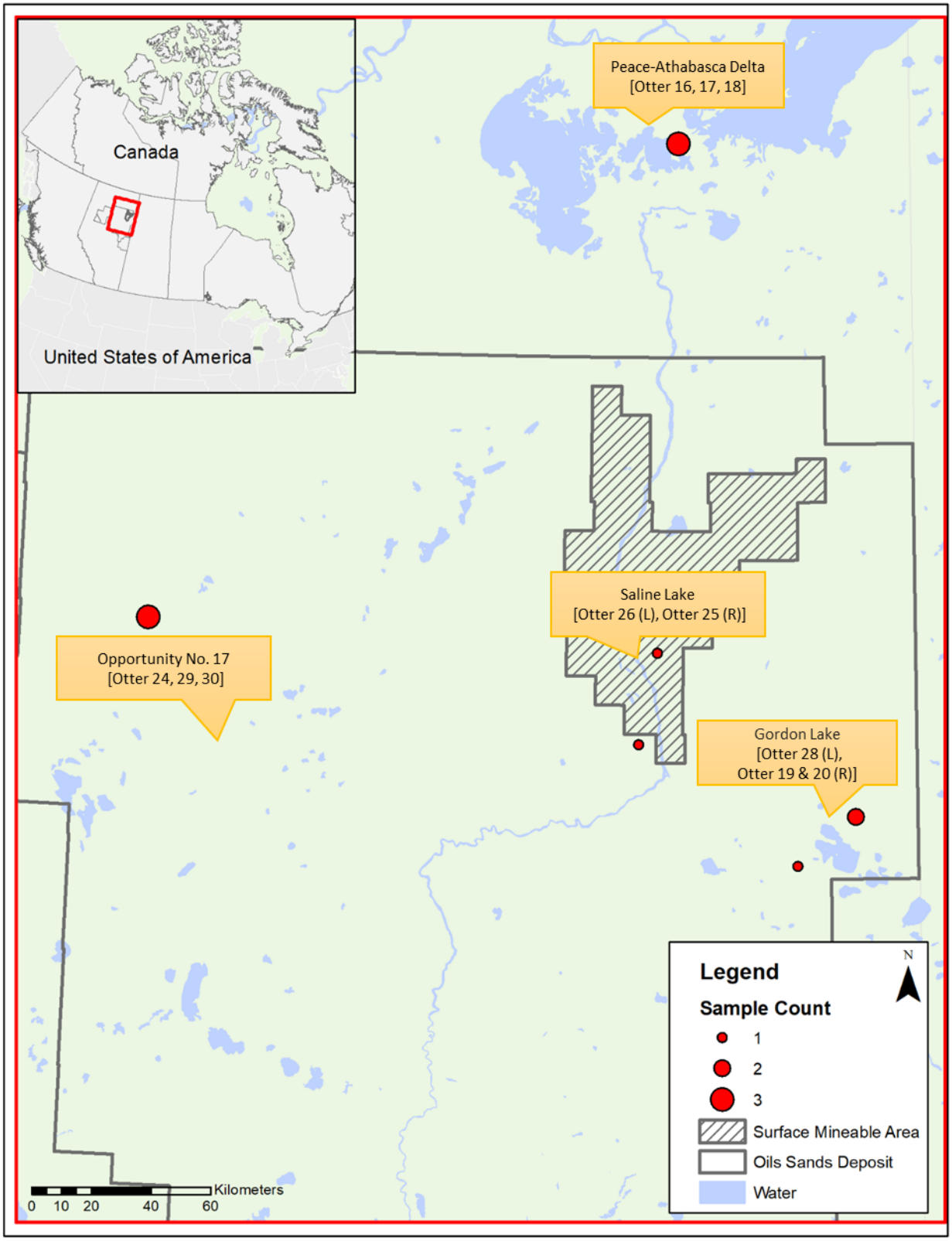

Figure 2.1. Map of the Athabasca oil sands region (AOSR). Boundaries of the oil sands development area as well as sampling locations and sample counts are presented.

The river otter samples were collected by Northern Alberta commercial trappers recruited through the Alberta Trappers Association (Westlock, Alberta) under permit following the Alberta Code for Responsible Trapping and the Agreement on International Humane Trapping Standards (AIHTS). Otters were frozen (-20℃) and shipped to the National Wildlife Research Center (NWRC; Ottawa, ON). At the NWRC post-mortem evaluations and gross necropsies were performed for each otter. Otters were categorically aged using tooth wear evaluations and dental pulp measures of canine teeth. The ratio of pulp cavity width (mm) to tooth width (mm; percent pulp) from canine teeth was determined by radiography and each individual was assigned to juvenile or adult age classes (32). Livers were dissected and stored at -20℃ for chemical analysis. Hind leg femurs were dissected, transversely cut using a Dremel rotary tool and approximately 15-30 mg of bone marrow was collected using a microspatula and stored at -20°C for DNA extraction.

2.3.2 Chemical Analysis

PAC concentrations within aliquots of homogenized whole liver were determined by the Centre of Oil and Gas Research and Development (COGRAD, University of Manitoba, Winnipeg, Canada). Briefly, ~5 g wet weight (ww) of liver homogenate was accurately weighed on a Sartorius Cubis MSE225P-100-DI (Fisher Scientific) and mixed with diatomaceous earth (DE). The mixture was loaded in an accelerated solvent extraction (ASE) cell, spiked with C-13 labelled internal standards and the dead volume of the cell filled with Ottawa sand. Procedural blanks, standard reference material (SRM) from the National Institutes of Standards and Technology (NIST), NIST SRM 2974a, and duplicates were included in each sample run. Samples were extracted under high pressure conditions with dichloromethane (DCM) used as the extraction solvent. ASE conditions are outlined in Idowu et al. (2018) (33). The extract volume

was transferred to a rotary evaporator, evaporated down to 2.6 mL under ultra-high-purity (UHP) nitrogen, and mixed with hexane [1:1 volume/volume (v/v)] to a final volume of 5.2 mL. 200 µl of this volume was used to gravimetrically determine lipid composition. Removal of lipids from the extracts was achieved using automated gel permeation chromatography (GPC; J2-scientific AccuPrepMPS™, Columbia, Missouri, USA). The final extract was again reduced to 1 mL under UHP nitrogen and loaded on a silica/alumina column for final clean-up by adsorption chromatography. The PAC containing fraction was eluted with 25 mL DCM/Hexane (1:1, v/v), and reduced in volume by rotary evaporation and UHP nitrogen. The final extracts were stored at 4ºC in amber glass gas chromatography vials prior to instrumental analysis.

An Agilent 7890 gas chromatograph coupled with a triple quadrupole mass spectrometer (GC/MS/MS) fitted with an electron ionization (EI) source was used. Separations were performed on an Agilent J&W HP-5 ms ultra inert 30 m × 0.25 mm × 0.25 µm column with helium as the carrier gas at a constant flow rate of 1.2 mL per minute. Sample (1 µL) was injected with a PAL RSI 85 autosampler at 250ºC in splitless mode. Further GC/MS/MS and multiple-reaction monitoring (MRM) conditions are outlined in Idowu et al. (2018) (33).

To distinguish pyrogenic versus the petrogenic PACs found in the liver, multiple diagnostic ratios were adopted from Tobiszewski and Namieśnik 2012 (34).

2.3.3 DNA Isolation & Single Molecule PCR Microsatellite Mutation Assay

DNA was extracted from river otter bone marrow (n=11) using DNeasy Blood and Tissue Kits (Qiagen) following the total DNA from Animal Tissues protocol. The concentration and purity of the DNA was determined by measuring absorbance at 230, 260 and 280 nm using a Nanodrop 2000 spectrophotometer (Thermo Scientific). All samples had 260/280 ratio > 1.8 and 260/230 > 2.0.

Two microsatellites, RIO06 (Genbank accession: AY268056.1) and RIO18 (GenBank accession: AY833269.1) were selected based on their repeat sequence length and their high size polymorphism within the otter population (35,36). Because our capillary electrophoresis instrument had a minimum resolution of 2-3 bp units, we selected microsatellites with tetranucleotide repeat units to ensure allele variants and mutations could be accurately resolved. The sequence of custom forward and reverse PCR primers for the two target microsatellites are shown in Supplementary Table 2.1. Both microsatellites were amplified in multiplexed PCRs using the Type-it Microsatellite PCR Kit (Qiagen). Briefly, each 10µL reaction contained 2µL Rnase-free water, 5µL 2X Type-it Multiplex PCR Master Mix (2µM), 0.5µL RIO06 Primer Mix (1.5µM), 0.5µL RIO18 Primer Mix (2.5µM), 1 µL of Q-solution (5µM), and finally 1uL of diluted DNA. Thermal cycler conditions were as follows: 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 59.5°C for 90 s and 72°C for 30 seconds, and a final extension at 60°C for 30 min.

DNA samples were serially diluted to determine the target concentration that gave positive PCR amplifications in ~40-70% of reactions (Supplementary Figure 2.1.). This success rate ensured that, on average, each PCR product was amplified from a single molecule of DNA. Once the single-molecule concentrations were determined for each sample, SM-PCRs were replicated on two to three 96-well plates per individual otter. The success rate and size of the 96-well SM-PCR products were determined by capillary electrophoresis on a QIAxcel instrument using QIAxcel DNA high resolution kits and ScreenGel software (Qiagen), following the manufacturer's protocols. All electropherograms were manually verified by two blinded technicians to minimize false positive or false negative peak detection (Supplementary Figure 2.2).

2.3.4 Determining Microsatellite Mutation Frequencies & Data Analysis

The total number of PCR template molecules was estimated from the PCR success rate (positive/negative PCR products) using a Poisson distribution. Each otter was genotyped based on the most frequently observed PCR product sizes (Supplementary Figure 2.3), and mutants were identified as PCR products that were at least +/- one repeat unit (i.e., 4 bp) difference in size compared to the wildtype genotype product sizes and occurred on the electropherogram as single peaks. Stutter peaks (i.e., very small peaks of varying size that co-occur with the main PCR product peak), were presumed to be artifacts caused by replication slippage that occurred during PCR amplification and were omitted from the analysis (Supplementary Figure 2.4). Mutant frequencies were calculated by dividing the number of mutants scored by the estimated total number of template molecules scored per individual. A clonal correction was applied, which assumed that all mutants of equal size originated from a single mutation event, to determine the microsatellite mutation frequency for each otter.

Microsatellite mutation frequencies were compared to the level of PAC tissue burden (total, low/ high molecular weight (LMW/HMW), and parent/alkylated PACs) and to the distance of where the animals were captured relative to the nearest bitumen upgrading facility using generalized linear models assuming a Poisson distribution. The model included the estimated total number of template molecules as the offset.

2.4 Results

2.4.1 River Otters & PAC Burden

Eleven river otters were collected from the AOSR and subjected to chemical and mutation assay analyses. All otters were determined to be older adult individuals between the ages of three-seven years old. 6/11 (55%) of otters were male, 5/11 (45%) were female. Due to the low representation of each class, both sexes were binned together for analysis.

A total of 51 PACs were measured in the liver of the river otters (Supplementary Table 2.2). The PAC profiles were summarized into various groupings such as LMW/HMW and parent/alkylated congeners (Table 2.1). The individual with the highest PAC burden (Otter 16) had a total concentration of 48.0 ng/g lipid weight (lw) in the liver, while the individual with the lowest concentration (Otter 19) had a total of 2.0 ng/g lw. Most PACs (80.9% on average) measured in the animals were alkylated LMW PACs, whereas parent and alkylated HMW PACs were less abundant (average of 5.1% and 4.7%, respectively). River otters that resided in the same area had dissimilar PAC burden concentrations, and there was no significant relationship between hepatic PAC burden and distance to bitumen upgraders (Figure 2.1, Table 2.1, & Supplementary Figure 2.5).

Table 2.1. River otter (*Lontra canadensis*; n=11) hepatic polycyclic aromatic compound (PAC) burden, organized into various classifications (%)[a] and distances to the nearest upgrader (km).

Animal ID	Total Hepatic PAC burden (ng/g lw)[b]	% Priority PACs[c]	% Parent PACs	% Alkylated PACs	% LMW PACs	% HMW PACs	% PLMW PACs	% ALMW PACs	% PHMW PACs	% AHMW PACs	Distance to Upgrader (km)
Otter 16	48.0	7.4	7.5	92.5	90.2	9.8	2.2	87.9	5.3	4.6	146.2
Otter 17	20.4	17.9	18.0	82.0	94.7	5.3	17.1	77.6	0.9	4.4	146.2
Otter 18	4.1	15.6	16.0	84.0	84.1	15.9	4.3	79.8	11.7	4.3	146.2
Otter 19	2.0	8.3	8.3	91.7	97.7	2.3	6.9	90.9	1.5	0.8	80.6
Otter 20	26.9	8.4	8.6	91.4	91.7	8.3	4.9	86.8	3.6	4.7	80.6
Otter 24	2.4	6.6	7.2	92.8	90.3	9.7	1.1	89.1	6.0	3.6	149.3
Otter 25	9.9	2.6	3.8	96.2	97.7	2.3	3.1	94.6	0.6	1.7	11.2
Otter 26	2.4	0.0	0.4	99.6	87.5	12.5	0.4	87.1	0.0	12.5	20.1
Otter 28	18.2	28.8	28.8	71.2	67.9	32.1	7.7	60.3	21.1	10.9	77.6
Otter 29	11.3	32.5	32.8	67.2	99.6	0.4	32.8	66.8	0.0	0.4	149.3
Otter 30	5.8	26.9	27.5	72.5	90.8	9.2	22.3	68.5	5.2	4.0	149.3
Average	**13.8**	**14.1**	**14.4**	**85.6**	**90.2**	**9.8**	**9.3**	**80.9**	**5.1**	**4.7**	**105.2**

[a] LMW= low molecular weight; HMW=high molecular weight; PLMW = parent lower molecular weight; ALMW = alkylated lower molecular weight; PHMW = parent higher molecular weight; AHMW = alkylated higher molecular weight

[b] lw= lipid weight

[c]Priority PACs include: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, benzo[*ghi*]perylene, indeno[*1,2,3-c,d*]pyrene, and dibenz[*a,h*]anthracene

2.4.2 Microsatellite Selection & Prioritization

Two different microsatellite loci, RIO06 and RIO18, were initially selected for multiplex PCRs to detect the microsatellite frequency (Supplementary Table 2.1). PCR conditions were optimized for both microsatellite loci, but the PCR success was unstable for RIO18. Thus, only PCR products for RIO06 were considered for determining mutation frequency (i.e., RIO18 primers were included in the PCRs to maintain optimal amplification of RIO06, but RIO18 products were not scored).

2.4.3 SM-PCR & Mutation Frequency

Microsatellite RIO06 mutation frequency was determined in bone marrow DNA by SM-PCR. The target single molecule concentration ranged between 6 and 16 pg/uL for all individuals with an average success rate of ~61% (Supplementary Table 2.3). An average of 196.7 SM-PCR

products were scored per individual (Table 2.2). Otters were genotyped based on the frequency of the most common PCR product sizes (Supplementary Figure 2.3). Seven individuals were found to be heterozygous, with the most frequent PCR products between 147-149 base pairs (bp) for the first allele, and 149-153 bp for the second allele. Four individuals were homozygous with the most frequent product between 143-149 bp (Supplementary Figure 2.3 & Supplementary Table 2.3). The overall mutation spectrum of microsatellite RIO06, before and after the clonal correction, is shown in Figure 2.2. Insertions were the most common mutation type and generally ranged from 1 to 4 repeat unit insertions. Deletions were generally only 1 repeat unit in size. We also observed one larger insertion (11 repeat units) and one larger deletion (6 repeat units).

SM-PCR products were screened for microsatellite size mutants to determine the mutation frequency of each individual (Table 2.2). At least 1 mutant was detected for all river otters, except for two. Mutant frequency was corrected for clonal expansion by assuming that all mutants of the same size originated from a single mutation event. The clonally corrected mutation frequency was highest in Otter 16 (0.034) and lowest for Otters 17 and 19 (0). The confidence interval for every combination of the number of mutants scored in this study, from 0 to 6 mutants, and a range of alleles scored from 50 to 500 was calculated and a good confidence to allele ratio was found with an average of ~200 alleles increasing our confidence with the mutation frequencies we observed (Supplementary Figure 2.6.).

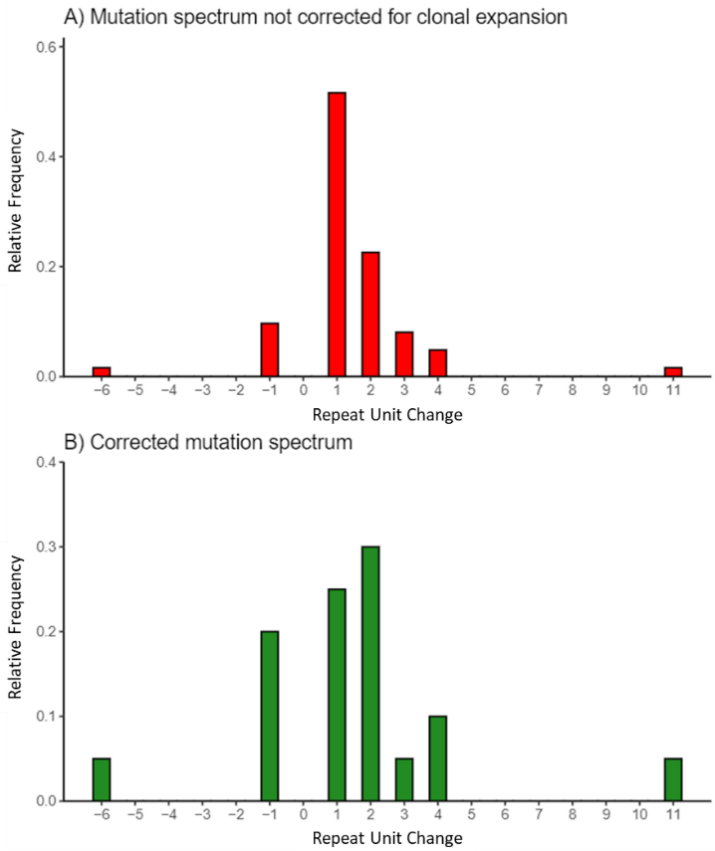

Figure 2.2. Frequency of the number of repeats lost (-) or gained (+) of microsatellite RIO06 before (a) and after (b) clonal correction.

Table 2.2. Summary table of the total number of alleles and mutants detected for each river otter (*Lontra canadensis*), as well as the bone marrow mutation frequency after clonal correction.

Animal ID	Alleles Scored	Mutants Scored	Mutant Frequency (uncorrected)	Unique Mutations (clonal correction)	Mutation Frequency (corrected)
Otter 16	177	23	0.13	6	0.034
Otter 17	171	0	0	0	0
Otter 18	296	4	0.014	2	0.007
Otter 19	195	0	0	0	0
Otter 20	110	7	0.064	3	0.027
Otter 24	176	11	0.062	1	0.006
Otter 25	254	1	0.004	1	0.004
Otter 26	241	1	0.004	1	0.004
Otter 28	214	5	0.023	3	0.014
Otter 29	190	2	0.011	1	0.005
Otter 30	140	8	0.057	2	0.014
AVERAGE	**196.7**	**5.6**	**0.034**	**1.8**	**0.010**

Mutation frequencies were regressed against the hepatic burden of total PACs (Figure 2.3), the eight different classifications of PACs (Supplementary Figure 2.7), and the distance of the registered fur management area (i.e., trapline) centroid to the nearest bitumen upgrading facility (Supplementary Figure 2.8). There were significant positive relationships between mutation frequency and hepatic concentration of total PACs ($p<0.003$, $R^2=0.56$, Figure 2.3) and each of the PAC classifications except for the parent low molecular weight PACs (PLMW) (Figure S2.6 & Table 2.3). Among the PAC classifications, the strongest relationship with mutation frequency was with the alkylated LMW PACs ($p<0.005$, $R^2 \geq 0.5$), and the weakest relationships were found with both the parent, and the parent high molecular weight (PHMW) PACs ($R^2=0.12$ & 0.34, respectively) (Figure S2.6 & Table 2.3). There was no significant relationship found between mutation frequency and the distance where the river otters were captured (centroid of the trapline) relative to bitumen upgraders (Supplementary Figure 2.8).

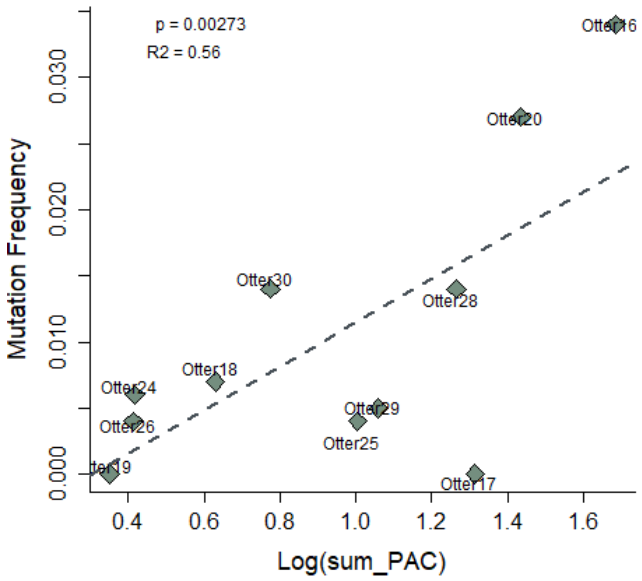

Figure 2.3. Relationship between mutation frequency of river otter (*Lontra canadensis*; n=11) microsatellites in bone marrow and PAC tissue burden in liver tissue (log transformed) ($p<0.05$; Poisson regression).

Table 2.3. Regression statistics ($p<0.05$; Poisson regression) for comparison of microsatellite mutation frequencies in the bone marrow of river otters (*Lontra canadensis*; n=11) from the Athabasca oil sands region (AOSR) to the different types of polycyclic aromatic compounds (PACs) measured in in the liver tissue.

PAC Category[a]	p-value	R^2	Slope
Total PACs	0.003	0.56	1.68
Total Priority (PEF)[b]	0.017	0.07	0.42
Parent	0.041	0.12	0.78
Alkylated	0.002	0.57	1.65
LMW	0.004	0.5	1.62
HMW	0.002	0.46	1.16
PLMW	0.132	-0.08	0.48
ALMW	0.002	0.55	1.63
PHMW	0.019	0.34	0.58
AHMW	0.003	0.46	1.23

[a] LMW= low molecular weight; HMW=high molecular weight; PLMW = parent lower molecular weight; ALMW = alkylated lower molecular weight; PHMW = parent higher molecular weight; AHMW = alkylated higher molecular weight

[b] Sum of the priority PACs based on their benzo[*a*]pyrene potency equivalent factors (PEF). The PACs and (PEFs) are benz[*a*]anthracene (0.1), benzo[*a*]pyrene (1), benzo[*b,j,k*]fluoranthene (0.1), chrysene (0.01), benzo[*ghi*]perylene (0.01), dibenz[*a,h*]anthreacene (1), and indeno[*1,2,3-c,d*]pyrene (0.1)

2.4.4 Diagnostic Ratios: Petrogenic vs Pyrogenic PACs

Diagnostic ratios were used to distinguish between petrogenic and pyrogenic PAC sources. We computed 11 different types of diagnostic ratios (Supplementary Table 2.4) for each individual to which the microsatellite mutation frequencies were compared. Out of the 11 different diagnostic ratios, two – ANT/(ANT/PHE) *[Anthracene/(Anthracene + Phenanthrene)]* and IcdP/(IcdP + BghiP) *[Indeno[1,2,3-cd] pyrene/ Indeno[1,2,3-cd] pyrene + Benzo[ghi] perylene]* – showed a significant correlation with the mutation frequency (i.e., $p<0.05$, $R^2>0.1$; Supplementary Table 2.5), and thus were used to make a cross-plot for source identification (Figure 2.4). ANT(ANT/PHE) and IcdP/(IcdP + BghiP) ratios could not be computed for Otter 26 as the PACs in these ratios were not detected in this animal (see Table S2 & Table S5). ANT/(ANT/PHE) ratio distinguishes between petrogenic (<0.1) and pyrogenic (>0.1) sources, and IcdP/(IcdP + BghiP) ratio organizes the values into petrogenic (<0.2), petroleum combustion

(0.2-0.5), and grass, wood, and coal combustion (>0.5) sources. From the cross-plot, the river otter samples with lower mutation frequencies tended to cluster in the petrogenic PAC areas (bottom left) whereas the higher mutation frequencies were more situated to the pyrogenic region (top right) on the plot (Figure 2.4).

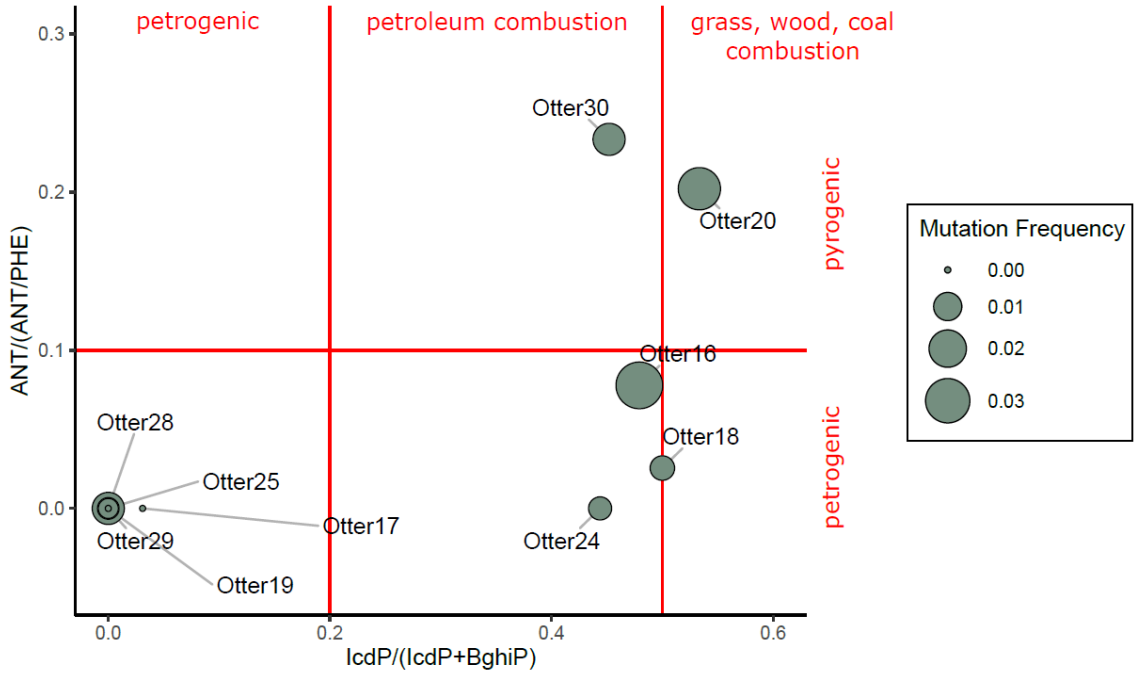

Figure 2.4. Diagnostic ratio cross-plot for river otter (*Lontra canadensis*) hepatic PACs showcasing IcdP/(IcdP + BghiP *[Indeno[1,2,3-cd] pyrene/ Indeno[1,2,3-cd] pyrene + Benzo[ghi] perylene]* vs ANT/ANT/PHE *[Anthracene/(Anthracene + Phenanthrene]*. River otters (n=10; Otter 26 not included as PACs in the ratios herein were not detected) are indicated with green dots with point size scaling of mutation frequency.

2.5 Discussion

To assess the impact of environmental PACs on the genomic health of wildlife in the AOSR, we compared microsatellite mutation frequencies to liver PAC burdens in river otters captured in the region. Mutation frequencies were determined in the bone marrow DNA of individual otters using SM-PCR. We observed a highly significant correlation between

microsatellite frequency and liver PAC burden. The correlation seemed to be largely driven by the alkylated low molecular weight PACs, which where the most abundant class of PACs detected. Significant correlations were also observed for other classes of PACs such as the parent high molecular weight congeners. These results support the hypothesis that PACs in the AOSR may increase mutation frequencies in wildlife.

2.5.1 River Otters & PAC Burden

By looking at the levels of PAC contaminants in liver tissue samples, we were able to approximate the potential level of contaminant exposure on an individual basis. The greatest number of PACs detected in the river otter samples were alkylated and LMW compounds. The abundance of alkylated PACs was also observed in traditional foods (i.e., wild plants and animals) consumed by an indigenous community in Alberta, leading to suggestions to include alkylated PACs when measuring PAC levels and conducting PAC exposure assessments in the AOSR (37). Alkylated PACs are more hydrophobic than parent PACs, and have greater biota-sediment accumulation factors, which could account for the higher levels detected within the river otter tissues (38,39). Consequently, parent and HMW PACs were detected at low levels. However, these low levels do not necessarily mean that the animals were not exposed to parent HMW PACs at high levels. B[*a*]P, a class 1 carcinogenic parent HMW PAC, becomes mutagenic when metabolized by cytochrome P450 enzymes (CYP450) (14). Thus, the low levels could reflect metabolism of the parent HMW PACs into DNA-reactive metabolites, which could be an integral factor for the mutation frequencies we observed (discussed below). Further investigation is therefore essential to discern how residual un-metabolized parent compounds detected in tissues are quantitatively related to actual exposure levels.

2.5.2 Microsatellites & SM-PCR

Microsatellite mutation frequencies vary depending on numerous factors, including the loci, number of repeats, repeat length of the motif, and species. When designing the SM-PCR experiments for river otter microsatellites, we chose loci that had a high average number of repeat units (10-13 repeats per loci) and heterozygosity in the otter population (35,36). Given that the insertions were more common than deletions, and that the wildtype alleles of most heterozygotes were very close in size (approximately one repeat unit difference), it is possible that the mutation frequency of the smaller allele in heterozygotes was underestimated. For example, insertions in the smaller allele may have either been misidentified as wildtype large alleles, or larger insertions may also have been misidentified as insertion mutations to the large allele. The methodology that we employed is unable to distinguish these instances and biases of the mutation scoring to the large allele when insertions are the most common mutation type. However, as previously discussed, animals that were homozygous for the small allele generally had lower mutation frequencies that were less responsive to PACs, which suggests that this underestimation would be low and not likely to have a major impact on the trends we observed. On average, we observed a microsatellite mutation frequency of 1×10^{-2} with a tetra-nucleotide microsatellite, microsatellite RIO06. In general, the microsatellite mutation frequency range has not been established for mammals in somatic tissues including within the bone marrow. However, the mammalian microsatellite mutation germline rate ranges from 10^{-5}-10^{-2} events per locus per generation (40). It is important to note that to maintain optimal amplification of RIO06, the SM-PCR conditions were optimized for multiplex PCRs including two different microsatellite loci, RIO06 and RIO18. PCR success was inconsistent for RIO18. Thus, to determine the river otter microsatellite mutation frequencies we scored PCR products for RIO06

only. Regardless, we demonstrate that the mutation frequency of RIO06 in a sentinel species, the North American river otter, measured using SM-PCR, is comparable with observed frequencies within mammals, and may be useful to screen wildlife exposure to mutagenic substances.

2.5.3 Mutation Frequency

When comparing the mutation frequency relative to the different types of PACs, there was a stronger relationship between LMW and alkylated PACs with mutation frequency, than with HMW and parent PACs. Numerous studies have shown that alkylated PACs can be more toxic than their unsubstituted counterparts. For example, tumorigenic 7,12-dimethylbenz[*a*]anthracene (DMBA) has a greater DNA-binding affinity than anthracene (parent) and B[*a*]P (41,42). In addition, sediment mixtures spiked with crude oil, containing mostly alkylated and LMW PACs, were more toxic to early-life stages of Japanese medaka fish (i.e. elicited genotoxic effects [increase in DNA damage] and developmental abnormalities [e.g., delayed hatching]) than sediment contaminated with more parent and HMW PACs (43). The abundance of alkylated PACs we detected in the river otter livers, and their strong correlation with mutation frequency, suggests that the alkylated PACs may be, at least partially, responsible for the elevated microsatellite mutation frequencies we observed in some individuals. Another explanation is that the correlation was due to co-exposure with non-alkylated PAC parent compounds to which have a well-characterized genotoxicity and can induce mutations through the production of reactive intermediate metabolites.

Scientists have largely used the 16 priority PAHs, established by the United States Environmental Protection Agency (US EPA), as the standard suite measured in environmental genotoxicity studies (44,45). This priority list identifies parent PACs that are environmentally concerning due to their potential genotoxicity and their prevalent and persistent nature in the

environment (46). Accordingly, we compared the measured mutation frequencies to the sum of the priority PACs, based on their B[*a*]P potency equivalent factors (47). The priority PACs had very low concentrations in the otter livers (Table 2.1) and had a weaker relationship ($p<0.02$, $R^2<0.07$) with mutation frequency compared to the alkylated congeners (Table 2.3 & Supplementary Figure 2.9). It is important to note that the PAC concentrations detected in the otter livers do not reflect actual exposure, as they do not account for metabolic processes. It is well-established that the mutagenicity of most PACs is due to DNA-reactive metabolites. Thus, it is very possible that the priority PACs are driving the elevated microsatellite mutations in some individuals but are not correlated due to metabolic removal of the parent compounds. As previously mentioned, understanding the quantitative relationship between exposure and residual PAC tissue burdens will be critical to clarifying the role of parent compounds in the strong correlation with mutation frequency. Given our results, genotoxicity assessments based on the tissue burden of priority PACs alone fail to account for original exposure levels, or the potential genotoxicity of alkylated compounds, which have high concentrations in oil-contaminated sites. Regardless of which congeners are responsible, the strong correlation between microsatellite mutation frequency and alkylated and low molecular weight PACs in the liver of river otters is indicative (i.e., a good biomarker) of exposure to a mutagenic substance.

2.5.4 Diagnostic Ratios: Source Determination

PAC source identification using diagnostic ratios inferred that the higher mutation frequencies were more correlated to PACs that are from pyrogenic sources (i.e., generated from incomplete combustion of fossil-fuels and organic matter [e.g., wood]). Pyrogenic sources are typically dominated by parent and HMW PACs whereas petrogenic substances (i.e., petroleum-based products such as crude oil) have greater alkylated and LMW PAC levels (44). This

contradicts our finding that mutation frequency is correlated with alkylated and LMW PACs from our previous analysis. However, diagnostic ratios are typically used for distinguishing petrogenic versus pyrogenic PACs in abiotic media including the atmosphere (48), sediments (8), and waterways (49). Furthermore, diagnostic ratios are sensitive to changes from environmental or biological processes, so estimated corrective factors must be applied to correct for phase transport or degradation that could occur, which is not available for processes that take place within tissues (34). For instance, similarly to the PAC chemical analysis, the diagnostic ratios do not take metabolism into consideration, thus reducing the diagnostic potential of this tool. The lack of accounting for metabolic processes is a probable explanation for contradictory results from this analysis.

Conversely, we found PAC tissue burden and mutation frequencies were poorly correlated with distance from the nearest upgraders (which use combustion processes to transform bitumen into synthetic crude oil). Likewise, PAC concentrations found within wood frog tadpoles did not vary spatially according to their position relative to the nearest upgraders in the AOSR, despite the fact that concentrations detected by passive samplers, immersed in shallow surface waters, declined with distance from oil sands activities (50). This led us to suggest that the river otters' trapline distance to the upgraders did not have an impact on the observed elevated mutation frequency. However, it is important to note that the location of where the river otters were captured relative to the upgraders does not necessarily mean that exposure was local. Free-ranging wildlife, such as river otters, move over their home range and integrate chemicals across the whole range, making these distance, exposure, and effect relationships more challenging to model. This is more evident as river otters captured in the same area did not have similar PAC concentrations. Given that the oil sands is a vital source of energy,

further investigation is warranted to discern if pollution from the industrial activities is indeed a source of mutagenic PACs for the surrounding environment and wildlife.

2.6 Limitations and Future Considerations

Overall, our findings support the hypothesis that exposure to PACs in the AOSR increases mutation frequency in wildlife species. Although the sample size of the study is small, strong correlations and relationships were evident between mutation frequency and PAC liver burden. It would be ideal to have a larger sample size; however, the process for detecting microsatellite mutations at the single-molecule level was resource intensive. We recognize that the relationships described herein were largely driven by three individuals (i.e., Otter 16, 20 and 28; Figure 2.3). Nonetheless, we found that alkylated PAC tissue burden was a good indicator of mutagenic contaminant exposure, as microsatellite mutation frequency increased with increasing alkylated PAC concentrations in river otter livers. Elevated mutation frequencies were also associated with pyrogenic contaminants, yet increasing concentrations of PACs were not directly linked to anthropogenic activities involving combustion processes. However, with the inconclusive source identification analysis in biota, further investigations of the abiotic environment (including aquatic sediment, air and water), where there are less biotransformation processes, are required to determine whether elevated mutation frequencies are due to either natural or anthropogenic PAC sources at these collection sites. These data might be relevant for Environmental Effects Monitoring (EEM) programs that require such biological data to set environmental triggers that would lead to closer investigations of cause. Furthermore, a dose-response analysis, using a larger number of samples would strengthen the evidence and be beneficial to further resolve the findings presented here. Developing the assay to measure mutations from non-invasive matrices (such as hair, scat, or fresh blood) may also prove useful.

Another an important consideration for future studies would involve determining to what extent environmental mixtures of PACs in the AOSR (e.g., from anthropogenic sources like tailings ponds and petroleum coke [petcoke] or from naturally occurring bitumen) can induce mutations in lab exposures (i.e., under controlled experimental conditions).

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Chapter 3. Polycyclic Aromatic Compound Extracts from Athabasca Oil Sands Tailings Pond Bitumen Do Not Induce *LacZ* Mutations in FE1 MutaMouse Lung Epithelial Cells

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3.1 Abstract

Oil sands mining activities in the Athabasca oil sands region (AOSR) have polluted the surrounding environment with polycyclic aromatic compounds (PACs). However, the effects of most PACs and PAC mixtures in the AOSR have not been evaluated for genotoxicity in wildlife. One route of PAC exposure to AOSR wildlife are tailings ponds, which contain by-products of bitumen extraction processes. Previously, PACs in the AOSR were shown to be correlated to elevated mutation frequency in wildlife. Here, we investigate the cytotoxicity and mutagenicity of PAC mixture extracts derived from bitumen, originating from tailings ponds, in an *in vitro* mammalian mutagenicity assay using the FE1 MutaMouse lung epithelial cell line (FE1). FE1 cells were exposed to serial dilutions of three PAC extracts prepared from different tailings pond bitumen. Cytotoxicity was evaluated by relative increase in cell counts (RICC), and the mutant frequency was determined in the *lacZ* transgene. With increasing concentration of each bitumen extract we observed an increase in cytotoxicity. The maximum tolerable concentration of each bitumen extract tested resulted in >90% cytotoxicity. In terms of the mutagenicity assay, none of the three bitumen extracts investigated increased *lacZ* mutant frequency. Follow up studies using RNA sequencing will be conducted to determine the toxicity pathways involved with the observed cytotoxicity. Overall, our results do not support the hypothesis that PACs from AOSR tailings pond bitumen is mutagenic.

3.2 Introduction

Bitumen mining operations are a source of polycyclic aromatic compound (PAC) exposure to wildlife in the Athabasca oil sands region (AOSR). Tailings, a mixture of sand, clay, and residual bitumen and process-affected water, are a by-product of bitumen extraction when bitumen is separated from the ore. Tailings are discharged and stored in engineered dam and dyke reservoirs that enable water to be recycled back into the extraction process (1). Aquatic wildlife, mammals, and migratory birds are exposed to PACs in tailings ponds via various routes, which has been associated with several toxicological effects. Aquatic wildlife are exposed to PACs that settle in the sediment of tailings ponds. Exposure to oil sand tailings pond sediment was shown to decrease survival rates and induce deformities (i.e., edemas and cranial and spinal malformations) in early-life stage fish native to the Athabasca river (2–4). Mammals, such as river otters, wolves, and moose, can also be exposed to PACs found in the tailings ponds through diet as they feed on PAC-burdened prey or vegetation (5,6). Migratory birds are directly exposed to PACs at mining sites through contact with bitumen floating on the surface of tailings ponds (oil slicks) or settled in the sediment, effectively oiling the feathers of the exposed birds (7,8). Birds can dive, float, wade, and swim in these ponds resulting in several effects that affect their survival and fitness for migration. For example, oiled feathers can decrease flight and swimming performance, result in birds sinking to the bottom of the tailings ponds, lead to ingestion of bitumen from preening their feathers and from oiled prey, and result in inhalation of volatile components from the tailings (7,9). Tailings ponds, a source of PACs in the avian food chain, can also serve as pre-migratory fueling sites and affect the ability of migratory birds to store energy for long-distance migration flights (fueling ability). Insufficient fueling can impact duration at fueling sites, migration speed and distance of impacted birds (10).

PAC mixture exposure has been shown to cause genotoxic effects (e.g., DNA damage and mutations) in both laboratory animals and wildlife (11–15). Previously, we showed that microsatellite mutation frequency was highly correlated to PAC tissue burdens in river otters from the Athabasca area. PAC characterization from that study revealed a strong positive correlation between mutation frequency and alkylated PACs, which most likely originate from a petrogenic source (i.e., crude oil). With evidence that PAC exposure to wildlife is occurring as a result of mining operations in the AOSR, and that PACs have been shown to cause genotoxic effects in wildlife, it is important to investigate whether an anthropogenic source of petrogenic PACs, tailings pond bitumen, could be mutagenic.

The *in vitro* transgene mutation assay in FE1 MutaMouse lung epithelial cells is an emerging animal-free method for testing the mutagenicity of compounds. FE1 is a transgenic cell line that harbors a *lacZ* mutation reporter gene. The reporter gene can be easily recovered after exposure to a suspected mutagen and the induction of mutations can be quantified. In addition, the cells are from a mammalian model, whereas other *in vitro* models are bacterial (e.g., Ames assay), and are metabolically competent (16) making the screening approach more relevant to *in vivo* conditions. Furthermore, the mutagenicity of various PACs and PAC mixtures have previously been tested in FE1 cells, including benzo[*a*]pyrene (B[*a*]P) (16,17), carbon black (95,100), PAC-contaminated soil (20), and diesel exhaust (21).

Given the toxicological effects observed in wildlife exposed to tailings ponds waste, the potential for genotoxicity of complex PAC mixtures, and our previously reported evidence that wildlife mutation frequency correlate with PAC burdens in the AOSR, we hypothesize that tailings pond bitumen, an anthropogenic source of PACs in the AOSR, is mutagenic. In the

present study, we test this hypothesis using a dose-response approach to determine the mutagenic potential of PAC mixtures extracted from tailings pond bitumen in FE1 cells.

3.3 Materials and Methods

3.3.1 Bitumen Extracts & PAC Analysis

Three bitumen samples, Bit A, Bit B, and Bit C, were collected off ducks' feathers from 3 different tailings ponds in the Athabasca area (exact locations of the tailings ponds have been kept confidential). Ducks collected dead or moribund by oil sands operators from each tailings pond were submitted to Environment and Climate Change Canada (ECCC) for necropsy evaluations. Bitumen was collected from the feathers of each duck (n=1/tailing) with chemically cleaned spatulas, weighed, and stored in amber glass jars at 4°C.

PACs were extracted from bitumen using accelerated solvent extraction (ASE), isolation and purification methods as reported in Yang et al. (2011) (22). Briefly, approximately 0.1 – 0.2 g bitumen samples were dissolved in 5 mL of dichloromethane (DCM). 0.5 mL of each sample was evaporated to dryness to determine the total solvent extractable mass (TSEM). For subsequent sample cleanup and fractionation, bitumen solution volumes equivalent to 16 mg TSEM were solvent exchanged into 1 mL of *n*-hexanes. PACs were fractionated using 3 g silica gel columns (Grade 923, 100 – 200 mesh, EMD Millipore, Billerica, MA, USA) adapted from Yang et al. (2011) (22). Fractionations were repeated for the remainder of each bitumen solution, creating one composite fraction of PAC elution per individual bitumen sample. PAC fractions were evaporated to 1 mL, then 0.5 mL DCM was added for a final volume of 1.5 mL hexane:DCM [2:1, volume/volume (v/v)]. A composite method blank was created following the same procedure. Final samples were subsampled for dilutions, spiked with *p*-terphenyl-d_{14} (Cambridge Isotope Laboratories, Tewksbury, MA, USA) as an internal standard, and analyzed

by gas-chromatography (Agilent 7890B)-mass spectrometry (Agilent 5977B). Injections were made in pulsed splitless mode at 290 °C on an HP-5MS UI 60 m x 0.25 µm x 250 µm column. An initial oven temperature of 100 °C was held for 5 min, then increased at a rate of 6 °C per min to 300 °C and held for 12 min. A constant flow rate of 32 cm per second of helium was used for a total run time of 50 min. The mass spectrometer was set to have a transfer line temperature of 300 °C, with a source temperature of 310 °C and quadrupole temperature of 150 °C. The characterized PACs included unsubstituted polycyclic aromatic compounds (parent PACs) and their alkylated homologues, including both the low and high molecular weight (LMW/HMW) PACs. Parent PACs included the 16 priority PACs established by the United States Environmental Protection Agency (US EPA).

Dosing solutions were prepared for each bitumen extract by a 1:1 solvent exchange from 2:1 hexane: dichloromethane (DCM) to dimethyl sulfoxide (DMSO) using a Nitrogen Evaporator III (Organomation Associates, Inc.). A standard reference material (SRM) of PACs derived from a coal-tar and petroleum mixture, purchased from the National Institutes of Standards and Technology (NIST), NIST SRM 1991, was also solvent exchanged (1:1) from DCM to DMSO and used as a positive control. Finally, B[*a*]P (Supelco, 50-32-8) was also dissolved in DMSO and used as a positive control (0.1 µg/mL).

3.3.2 Cell Culture & Exposure

The development and characterization of the FE1-MutaMouse lung epithelial cell line has been described previously (16). Cells were thawed and maintained in Dulbecco's modified eagle medium/Nutrient mixture F-12 (DMEM/F-12) with L-glutamine (Gibco, 11320082) supplemented with 2% fetal bovine serum (FBS) (Gibco, 12483020), 100 U/mL of penicillin, 100 µg/mL of streptomycin (Gibco, 15140122), and 1 ng/mL of human epidermal growth factor

recombinant protein (Gibco, PHG0311L**)** at 37°C with 5% CO2. Cells were grown in 10 cm cell culture plates (Greiner Bio-One, 82050-916) and were passaged when ~70% confluent (approximately 72 hours). Cells were detached from the plate by the addition of 0.5 mL trypsin-EDTA for 1 min, followed by centrifugation at 200 x g for 4 min, and the resulting pellet was distributed into two plates in 10 mL of fresh medium. The cells were passaged twice into the desired number of plates before being used for assays. The day before the exposure, approximately 300,000 cells were seeded per plate. On exposure day, 100 uL of the appropriate chemical dilution (dissolved in DMSO) was prepared for each test chemical in 9.9 mL of exposure medium (culture medium prepared without FBS). The 10 mL of treatment medium was split between two replicate plates (i.e., 5 mL per plate). For the final study, the concentrations for each extract, Bit A-Bit C, were a 6-point 2-fold dilution series with the maximum tolerable dose as the highest dose determined by cytotoxicity of the extracts (see below). Positive controls, 0.1 µg/ mL B[*a*]P and 362.1 µg/mL NIST SRM 1991, and negative controls, DMSO and method blank were run concurrently in duplicate. Exposure time was 6 hours at 37°C, 95% humidity and 5% CO_2 in an externally vented incubator. Following exposure, the media was replaced with 10 mL of fresh media and the cells were incubated for an additional 72 hours for the mutations to be fixed. Following incubation, the medium was removed from the plates, cells were washed with Dulbecco's phosphate buffered saline (DPBS), and 3 mL of freshly prepared lysis buffer was added. The plates were then incubated overnight (~16-18 hours) and total genomic DNA was isolated by phenol-chloroform extraction and precipitated in ethanol as previously described (16).

A pilot study with the bitumen extracts and NIST SRM 1991 were conducted with and without the exogenous metabolic activation mixture, MUTAZYME™ S9 Mix, containing

Aroclor induced Sprague Dawley rat liver S9 (TRINOVA, 11-403L). There was no significant difference in mutant frequency observed with the addition of S9 for any of the bitumen samples or NIST SRM 1991, and therefore, exposures for the final study were performed without S9.

3.3.3 Cytotoxicity

A pilot exposure study was performed to identify the maximum tolerable dose(s) of the bitumen extracts. Cytotoxicity was evaluated for each bitumen extract and NIST SRM 1991. Four dilutions (0.8%, 4%, 20%, 100 % [stock]) for each extract, Bit A-Bit C, were tested in addition to the DMSO, method blank, and NIST SRM 1991 controls. B[*a*]P was not included in the cytotoxicity experiments as a recommended concentration, 0.1 µg/mL B[*a*]P, was previously established for the FE1 cells (16). On the day of exposure, cells from the "initial count" plate (cells plated before exposure) were counted. Following exposure, cytotoxicity was determined by measuring the relative increase in cell counts (RICC). Cell counts from cells incubated with DMSO (control) were compared to those exposed to the method blank, bitumen extracts, and NIST SRM 1991: *[RICC= (# of cells (treated) – initial cell count before exposure) / (# of cells (control) – initial cell count before exposure)]*. For *in vitro* mammalian studies the recommended maximum concentration should result in cytotoxicity between 80-90%; Cytotoxicity= 100%-RICC (%) (23,24).

3.3.4 LacZ Mutation Assay

The mutant frequency of the *lacZ* transgene from exposed FE1 cells was determined using the phenyl-β-D-galactopyranoside (P-Gal) positive selection assay as described by Gossen et al. (1992) (25). Briefly, *lacZ* transgenes are recovered from the cells by packaging the DNA into λ Phage using the Transpack lambda packaging system from Agilent (Agilent Technologies, Mississauga, ON, Canada). E.coli (Escherichia coli *LacZ*−, galE−, recA−, pAA119 with galT

and galK), infected with the phages containing the *lacZ* transgenes, were grown on agar plates overnight at 37°C containing P-Gal to detect mutant plaques, or on agar without P-Gal to determine the total number of plaque forming units (pfu).The mutant frequency was calculated as the number of mutant plaques divided by the total number of pfus.

3.3.5 Data Analysis

LacZ mutant frequencies were compared between treated and DMSO control groups using a generalized linear model with a quasi-binomial distribution to account for non-constant variance, using the *glm* function in R (Ver R-4.0.2). The resulting p-values were adjusted using the Bonferroni method. Only group mutant frequencies with $p < 0.05$ were considered significant.

3.4 Results and Discussion

3.4.1 Bitumen Extracts Contained Mostly Alkylated PACs

The majority of PACs in the bitumen extracts were alkylated (>90%) (Table 3.1, Supplementary Table 3.1) and had various alkyl group (methyl, ethyl, etc.) substitutions compared to their respective parent PAC. The extracts also had high levels of high molecular weight PACs (mostly alkylated), which made up >50% of the PACs in two of the three extracts (Bit A and Bit C). Alkylated PACs are abundant in petrogenic sources of PACs (i.e. originate from petroleum) such as bitumen, (26–28) and persist for a longer time in the environment than parent PACs (29,30). Some alkylated PACs have been found to have greater carcinogenicity compared to their parent structures, depending on the location and type of the alkyl substitutions (31,32). For example, in a review from Baird et al. (2007), it was observed that methylation at the meso-anthracenic position (the middle benzene ring of three benzenes linked linearly), at the "bay-region" (a pocket formed by linearly connected benzene rings), or opposite the bay-region were associated with the formation of tumors (31). Despite the abundance of alkylated PACs in the environment, they have been less studied than their parent counterparts and the capacity of alkylated PACs to impact the genomic health of wildlife is unclear. Over 90% of bitumen is alkylated yet the majority of studies focus on the US EPA 16 priority parent PACs, resulting in concerns that the toxicity and mutagenicity of PAC mixtures from petrogenic sources have, to date, been underestimated (33).

The total PACs in the NIST SRM 1991, a known carcinogenic standard reference material, was on average three times more concentrated than the bitumen extracts and had a different profile (Table 3.1). This sample comprised ~25% priority PACs and ~70% alkylated PACs. It also had a much lower proportion of high molecular weight PACs, only 16% (with 4%

alkylated high molecular weight). Based on the priority PAC profile alone, it would be assumed that the NIST SRM 1991 would be more hazardous, due to mutagenicity, than the bitumen extracts.

Table 3.1. PAC composition (%) of the method blank, bitumen extracts and NIST SRM -1991 summarized into various classifications[a]. Detailed profiles of the individual PACs are shown in Supplementary Table 3.1.

Sample	Total PACs (µg/mL)	% Priority PACs[b]	% Parent	% Alkylated	% LMW	% HMW	% PLMW	% ALMW	% PHMW	% AHMW
Method Blank	0.10	33.7	33.7	66.3	59.2	40.8	32.4	26.8	1.3	39.5
Bit A	115.2	3.4	4.6	95.4	43.5	56.5	1.1	42.4	3.6	52.9
Bit B	165.7	4.5	5.9	94.1	60.6	39.4	2.6	58.0	3.3	36.1
Bit C	74.3	3.3	4.4	95.6	37.5	62.5	0.2	37.3	4.1	58.3
NIST SRM 1991	362.1	24.9	31.8	68.2	84.0	16.0	19.7	64.3	12.1	3.8

[a] LMW= low molecular weight; HMW=high molecular weight; PLMW = parent lower molecular weight; ALMW = alkylated lower molecular weight; PHMW = parent higher molecular weight; AHMW = alkylated higher molecular weight

[b] Priority PACs include: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, benzo[*ghi*]perylene, indeno[*1,2,3-c,d*]pyrene, and dibenz[*a,h*]anthracene

3.4.2 Bitumen Extracts were Cytotoxic

With increasing concentration of each bitumen extract, we observed increasing cytotoxicity. Cytotoxicity was evaluated and measured using RICC (Table 3.2). An average of four cell counts per sample, including the method blank, top four concentrations of each bitumen extract, and NIST SRM 1991, were compared to the average of four cell counts of the DMSO negative control. We performed a series of cytotoxicity range finding experiments and the average cell count for the "initial count" was 4.98 x 10^4 cells/mL. The average cytotoxicity from the experiments for the controls, DMSO, NIST SRM 1991, and method blank are presented in Table 3.2. The recommended cytotoxicity of the maximum tolerable dose for *in vitro* mammalian assays is between 80-90% (23,24). We exposed the cells to a serial dilution from

0.8% to 100% concentration of each bitumen extract (Table 3.2). Based on these results, we selected 50% (Bit A and Bit B) and 66.7 % (Bit C) as the top doses to result in a cytotoxicity value closer to the recommended cytotoxicity range for the maximum dose.

Table 3.2. Relative increase in cell counts (RICC) for the negative controls, DMSO and Method Blank, serial dilutions of each bitumen extract, Bit A, Bit B and Bit C, and the positive control, NIST SRM 1991, as well as the resulting cytotoxicity[a]. The top dosed selected for the mutagenicity tests are shown in bold.

Sample	Dose (µg/mL)	RICC[b] (%)	Cytotoxicity[c] (%)
DMSO	NA	100.0	0
Method Blank	NA	98.6	1.4
Bit A_0.8%	0.9	46.8	53.2
Bit A_4%	4.6	41.4	58.6
Bit A_6.3%	7.2	73.9	26.1
Bit A_12.5%	14.4	60.0	40.0
Bit A_20%	23	35.2	64.8
Bit A_25%	28.8	1.5	98.5
Bit A_50%	**57.6**	**0.3**	**99.7**
Bit A_100%	115.2	-6.0	106.0
Bit B_0.8%	1.3	70.4	29.6
Bit B_4%	6.6	46.3	53.7
Bit B_6.3%	10.4	69.8	30.2
Bit B_12.5%	20.7	46.0	54.0
Bit B_20%	33.1	27.9	72.1
Bit B_25%	41.4	4.5	95.5
Bit B_50%	**82.8**	**0.4**	**99.6**
Bit B_100%	165.7	-6.2	106.2
Bit C_0.8%	0.6	65.5	34.5
Bit C_4%	3.0	68.3	31.7
Bit C_8.3%	6.2	62.7	37.3
Bit C_16.7%	12.4	54.5	45.5
Bit C_20%	14.9	49.4	50.6
Bit C_33.3%	24.8	47.1	52.9
Bit C_66.7%	**49.5**	**0.8**	**99.2**
Bit C_100%	74.3	-6.2	106.2
NIST SRM 1991	362.1	51.1	48.9

[a] FE1 cells were exposed to each sample for 6 hours, the media was replaced, and the cells were incubated for 72 hours before they were collected and counted

[b]RICC (%) = (# of cells (treated) – initial cell count before exposure) / (# of cells (control) – initial cell count before exposure)

[c]Cytotoxicity = 100% -RICC (%)

3.4.3 Bitumen Extracts Did Not Induce LacZ Mutations

PAC mixtures derived from AOSR tailings pond bitumen did not induce *lacZ* mutations in FE1 cells. FE1 cells were exposed to a 6-point dilution series of each bitumen extract with a concentration range for each extract as follows: Bit A (1.8-57.6 µg/mL), Bit B (2.6-82.8 µg/mL), and Bit C (1.5-49.5 µg/mL) (Table 3.3). Over 100, 000 pfus were scored for all doses except the maximum dose and the *lacZ* mutant frequencies in bitumen exposed FE1 cells are summarized in Table 3.3 and visualized in Figure 3.1. DMSO control and method blank average mutant frequencies ($x10^5$) were 80.4 and 73.0, respectively. The average range of mutant frequencies for each bitumen extract was 52.2-80.5 ($x10^5$) for Bit A, 69.4-75.4 ($x10^5$) for Bit B, and 68.1-83.8 ($x10^5$) for Bit C. None of these changes were significantly different compared to the DMSO control or method blank. A power analysis was performed to determine how many pfus should be analyzed to be confident on the obtained mutant frequencies. The power was calculated for pfu counts ranging from 50,000 to 200,000 with the effect size of 2-fold over the control levels [i.e., DMSO control mutation frequency= ~$8x10^{-4}$ (127 avg. mutants counted /156,224 avg. plaques tested)]. From the analysis, 100,000 plaques resulted in a power level >0.8 (typically the desired level) (Supplementary Figure 3.1) thus providing confidence with the mutant frequencies observed. In addition, generally, it is recommended to score at least 100,000 plaques per sample (34). The maximum dose for each bitumen extract was too cytotoxic for a mutant frequency to be determined, as indicated by the low number of tested plaques in these samples. These top dose samples had <30,000 plaques. The positive controls, NIST SRM 1991 and B[*a*]P, both had significant effects on mutant frequencies, with a fold-increase of 2.01 and 4.58, respectively (Table 3.3), indicating that the assay worked properly. Therefore, the bitumen extracts were negative for *lacZ* mutation induction in FE1 cells.

These findings do not support our hypothesis that bitumen is a source of mutagenic PACs in the AOSR. Further, they suggest that PACs originating from bitumen may not be responsible for the elevated mutation frequencies that were previously reported in AOSR otters (Gyasi et al. *manuscript in preparation)*. A possible explanation for the disparity between the lack of *lacZ* mutations and the correlation we observed between mutations and PAC tissue burden in AOSR otters, is that the profile of PACs in the bitumen extracts is not representative of the PAC profile to which the otters were exposed. On average, the otter livers had a greater relative proportion of priority PACs (~14%; which are known to be mutagenic) and a much higher relative proportion of low molecular weight PACs (~90%) in their livers. In comparison, the bitumen extracts had only ~4% priority PACs and ~50% low molecular weight PACs (Table 3.3). If the priority and low molecular weight PACs are mutagenic, they may not have been concentrated enough in the bitumen extracts to induce detectable effects. It is also important to note that bioaccumulation, biomagnification, and metabolism through the food web can change the PAC profile from the source to ultimate point of exposure, which can make comparisons of PACs profiles between wildlife and emissions sources a challenge. However, it is interesting to note that the NIST SRM 1991 sample, which was positive for *lacZ* mutation induction, had a PAC profile that was more similar to the profile observed in otters: it had 25% priority and 84% low molecular weight PACs (Table 3.3).

Another important consideration is the difference in metabolic processes between wildlife and the FE1 cell line. Although FE1 cells have been shown to have an inducible CYP response (i.e., they are metabolically competent), it is unclear if they have sufficient metabolic capacity to activate alkylated PACs compared to an intact liver (assuming that liver can even render alkylated PACs mutagenic). In preliminary experiments, we tested the use of exogenous S9 to

augment metabolism and it did not change the observed mutant frequency compared to the control groups (data not shown), which furthers the narrative that the mixture of PACs in the bitumen extracts are not mutagenic. Future experiments that use mixtures with congener profiles that are more representative of the PACs in wildlife tissue might help clarify whether bioaccumulation and metabolic processes alter the mutagenicity of PAC mixtures.

One limitation of the *lacZ* assay is that it primarily detects point mutations, small insertions/deletions of gene nucleotides (indels), and may not detect other forms of genotoxicity such as DNA strand breaks and cytogenetic effects (35). Further, it cannot detect mutations in tandem repeat sequences such as microsatellites. Thus, these detection limitations may explain why the negative result of this study did not agree with previously reported findings of elevated microsatellite mutations, which were correlated with alkylated PAC liver burden in AOSR river otters (Gyasi et al. *manuscript in preparation*). Measuring clastogenic and/or aneugenic DNA damage endpoints and microsatellite mutations frequencies in the exposed FE1 cells would help resolve this possibility. For example, to detect DNA strand breaks, the comet assay or analysis of γH2AX foci immediately after the end of exposure could provide evidence for the induction of DNA damage by bitumen.

Table 3.3. *LacZ* Mutant frequency in FE1 MutaMouse lung epithelial cells exposed to Bit A (orange), Bit B (blue) and Bit C (green). Negative controls include DMSO and method blank (MB). Positive controls include NIST SRM 1991and benzo[*a*]pyrene (B[*a*]P). Average plaques tested, standard deviation, fold change, p-values and Bonferroni adjusted p-values are also presented.

Group	Dose	Average Mutants Counted	Average Plaques Tested	Average Mutant Frequency ($x10^5$)	Standard Deviation	Fold Change	P-Value	Adjusted P-Value[a]
DMSO	0	127	156,224	80.4	22.62	1	1	1
MB	0	108	145,083	73.0	8.77	0.91	0.44	1
Bit A_1.6%	1.8 µg/mL	84	103,873	80.5	5.31	1	0.94	1
Bit A_3.1%	3.6 µg/mL	78	115,304	66.2	10.85	0.82	0.26	1
Bit A_6.3%	7.2 µg/mL	76	135,184	52.2	38.87	0.65	0.03	0.61
Bit A_12.5%	14.4 µg/mL	112	146,449	77.4	17.24	0.96	0.67	1
Bit A_25%	28.8 µg/mL	143	213,586	66.6	5.72	0.83	0.09	1
Bit A_50%	57.6 µg/mL	11	15,241	NA	NA	NA	NA	NA
Bit B_1.6%	2.6 µg/mL	140	187,700	73.9	2.19	0.92	0.52	1
Bit B_3.1%	5.2 µg/mL	146	195,155	74.5	3.33	0.93	0.53	1
Bit B_6.3%	10.4 µg/mL	109	145,207	75.4	5.41	0.94	0.57	1
Bit B_12.5%	20.7 µg/mL	157	220,502	71.9	2.19	0.89	0.32	1
Bit B_25%	41.4 µg/mL	115	167,696	69.4	8.84	0.86	0.15	1
Bit B_50%	82.8 µg/mL	10	25,181	NA	NA	NA	NA	NA
Bit C_2.1%	1.5 µg/mL	127	175,275	73.6	11.74	0.91	0.41	1
Bit C_4.2%	3.1 µg/mL	131	193,996	68.9	15.32	0.86	0.19	1
Bit C_8.3%	6.2 µg/mL	143	210,314	68.1	2.03	0.85	0.18	1
Bit C_16.7%	12.4 µg/mL	196	232,845	83.8	4.05	1.04	0.79	1
Bit C_33.3%	24.8 µg/mL	177	229,366	77.4	5.65	0.96	0.62	1
Bit C_66.7%	49.5 µg/mL	29	29,157	NA	NA	NA	NA	NA
NIST SRM 1991	362.1 µg/mL	287	181,488	162.0	13.71	2.01	1.30E-07	**2.33E-06**
B[*a*]P	0.1 µg/mL	492	131,622	368.2	35.63	4.58	6.46E-16	**1.16E-14**

[a]Bonferroni corrected p-value; significant $p<0.05$ (**bolded**)

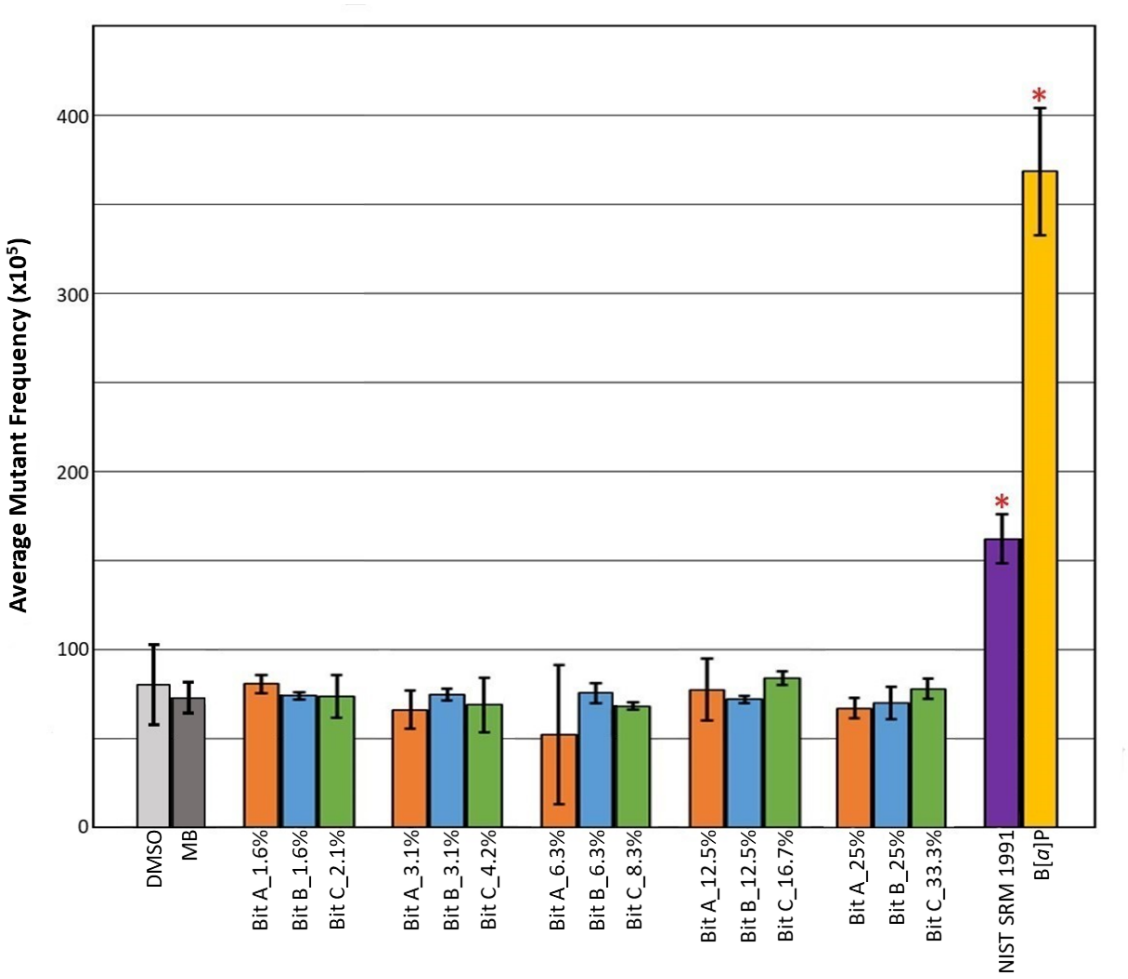

Figure 3.1. Average *LacZ* mutant frequency in FE1 MutaMouse lung epithelial cells exposed to serial dilutions (%) of Bit A (orange), Bit B (blue) and Bit C (green). Negative controls include DMSO (light grey) and method blank (MB; dark grey). Positive controls include NIST SRM 1991 (purple) and benzo[*a*]pyrene (B[*a*]P; gold). Error bars represent standard deviation *Significant results ($p<0.05$; Bonferroni).

3.5 Conclusions and Future Considerations

In summary, we did not detect an increase in *lacZ* mutation frequency in FE1 cells exposed to PAC mixtures extracted from bitumen samples collected from wildlife found in AOSR tailings ponds. These results do not support the hypothesis that this anthropogenic source of PACs, tailings pond bitumen, is mutagenic. Although the bitumen extracts did not induce *lacZ* mutations, we did observe cytotoxic effects. Studies have shown PACs , including alkylated PACs, have immune, cardiovascular, and endocrine system disruption properties (36–38). Therefore, we propose that future studies investigating bitumen PAC toxicity should investigate non-genotoxic hazards and pathways. For example, through investigation of specific pathways (e.g., endocrine disruption, immunotoxicity) or systems biology approaches (e.g., whole transcriptome sequencing).

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Chapter 4. General Conclusions

4.1 Thesis Summary

Since the onset of oil sands development in the Athabasca oil sands region (AOSR; Alberta, Canada), the deposition and concentration of polycyclic aromatic compounds (PACs) have been monitored throughout the environment. However, the toxicity of PAC mixtures from the AOSR is not well characterized to date. Some PACs are known to elicit mutagenic effects, but there is a lack of understanding regarding the impact that complex mixtures of PACs can have on the genomic health of wildlife in the AOSR. Given the increased concentrations of PACs associated with the oil sands development, and knowledge of the mutagenic potential of some PACs and mixtures, I hypothesized that PACs from anthropogenic sources in the Alberta oils sands would be mutagenic to wildlife. The experimental chapters of my thesis investigated two testable predictions of this hypothesis:

(1) Wildlife (river otters) with elevated exposure to anthropogenic PACs will have elevated mutations (Chapter 2)

(2) Anthropogenic sources of PACs (tailings pond bitumen) will be mutagenic in controlled laboratory tests (Chapter 3)

In Chapter 2, the aim was to determine whether elevated PAC exposure was associated with increased microsatellite mutation frequency in river otters from the AOSR. We also investigated whether any effect on microsatellite mutation frequency was related to any particular PAC class. Mutation frequencies in the bone marrow DNA of river otters were found to be correlated to PAC liver concentration. High concentrations of low molecular weight (LMW) and alkylated PACs, usually found in petrogenic sources of PACs (e.g., crude oil), were detected and had a stronger positive relationship with mutation frequency than the other types of

PACs measured. We also detected low concentrations of priority parent PACs in the otter livers. Such low concentrations could suggest that the otters were not exposed to a high concentration of priority PACs, or alternatively, the priority PACs were rapidly metabolized. If the priority PACs were metabolized, activation of DNA damaging metabolites could explain the observed elevated mutation frequency. Uncertain source determination raised questions about whether the increase in mutation frequency was due to petrogenic or pyrogenic PACs. Source determination was conducted using diagnostic ratios, which are better suited for abiotic material, where processes like metabolism do not need to be considered to determine whether the PACs are from a petrogenic or pyrogenic source. PAC profile comparisons between the river otters' food source, mainly fish, the liver profiles characterized herein, and environment samples (i.e., sediment, air, or water) will provide more insight on the types of PACs exposed to the otters. PAC profile comparisons may also confirm or identify other PACs that could be correlated to the elevated mutations frequencies in otters.

Before we can examine whether anthropogenic sources of PACs are mutagenic, we determined whether the high concentrations of PACs from the AOSR cause a significant mutational effect. Accordingly, results from chapter 2 demonstrate a strong correlation between elevated PAC tissue burdens in river otters and mutation frequency, thereby providing evidence to support my hypothesis that anthropogenic sources of PACs from the Alberta oil sands are mutagenic.

Next, in Chapter 3, I investigated whether a mixture of PACs of anthropogenic origin from the AOSR was mutagenic in a controlled lab setting. Using an *in vitro* mammalian mutagenicity assay, three PAC extracts from bitumen samples collected from different tailings ponds, an anthropogenic source of petrogenic PACs, were prepared and administered to FE1

cells. Results from this study revealed that the extracts did not induce *lacZ* mutations. However, increasing cytotoxicity was observed with increasing concentrations of each bitumen extract, suggesting other mechanisms of toxicity. From this study, the tailings pond bitumen extracts did not induce mutations and thus this source of anthropogenic petrogenic PACs from the Alberta oil sands was not mutagenic.

4.2 Discussion of Results and Future Considerations

4.2.1 PAC Profiles

Given the contradictory empirical results from Chapter 2 and 3, with regards to the mutagenic potential of anthropogenic sources of PAC mixtures in the Alberta oil sands region, it is important to reflect on the possible reasons for these differences. One of the most likely reasons behind the inconsistent findings could be the difference in PAC profiles between the river otter tissue burden and bitumen extracts (Table 4.1 & Figure 4.1). The otter tissues had a higher relative proportion of priority PACs (~14% average), and alkylated lower molecular weight (ALMW) PACs (~80% average) than the bitumen extracts [~4% priority, and 46% ALMW)]. The PAC profiles were visually compared using a heatmap, along with the NIST SRM 1991 positive control from chapter 3 (Figure 4.1). The PAC profiles for each mixture type were measured using different methods, and therefore a different suite of specific PACs is reported for each mixture. For this reason, only 33 PACs that were measured in all three sample types were considered during this comparison. Hierarchical clustering was performed on log-transformed relative proportions based on Euclidean distance and centroid linkage. All the otter samples cluster away from the bitumen extracts, and interestingly, the three otter samples that had the highest microsatellite mutation frequency, Otter 16, 20 & 30, clustered with NIST SRM 1991, which was positive in the *lacZ* assay, and is a known carcinogen. The clustering also revealed

some interesting differences in the relative abundance of these 33 PACs. The bitumen extracts had a higher proportion of certain alkylated and high molecular weight (AHMW) PACs (Figure 4.1, bracket A; also shown in Table 4.1). In addition, C2-naphthalenes and a group of priority parent LMW (PLMW) PACs (Figure 4.1, bracket B) were very low in bitumen compared to the NIST SRM 1991 and otter clusters. The similarity with consisting of priority PLMW and certain ALMW PACs in the NIST SRM 1991 and otter samples with the highest microsatellite mutations, might suggest these PACs are related to elevated otter mutation frequencies, but given the small sample size for the otter samples, we can't make any conclusive inferences. It is also interesting to note that NIST SRM 1991 is derived from a mixture of pyrogenic and petrogenic PACs (i.e., from coal tar and petroleum, respectively). The difference of the NIST SRM 1991 to the bitumen samples, and its similarity to some otter samples, suggests that the PAC profiles in the otters might also be partially of pyrogenic origin. However, it is important to note that when comparing the bitumen extracts and the river otters, as stated previously, biological processes like bioaccumulation through the food web and xenobiotic metabolism could alter the PAC composition when comparing the source to the exposed wildlife. Thus, the exposure PAC profile to the otters could be different to what was detected in their livers, and so possibly it was a set of PACs that the river otters were exposed to rather than what was detected in the livers that caused the elevated mutations. Therefore, an imperative consideration for future study would be to test the river otter PAC profiles in mutagenicity assays (e.g., the FE1 assay) to help identify whether biological processes like metabolism can alter the mutagenicity of PAC mixtures, and to test different sources of PACs found in the AOSR (discussed further below).

Table 4.1. PAC composition (%) of river otter liver tissues, bitumen extracts and NIST SRM 1991 summarized into various classifications[a]. Detailed concentrations of the PAC groupings are shown in Supplementary Table 4.1.

Sample	% Priority[b]	% Parent	% Alkylated	% LMW	% HMW	% PLMW	% ALMW	% PHMW	% AHMW
Otter16	7.4	7.5	92.5	90.2	9.8	2.2	87.9	5.3	4.6
Otter17	17.9	18.0	82.0	94.7	5.3	17.1	77.6	0.9	4.4
Otter18	15.6	16.0	84.0	84.1	15.9	4.3	79.8	11.7	4.3
Otter19	8.3	8.3	91.7	97.8	2.2	6.8	90.9	1.4	0.8
Otter20	8.4	8.6	91.4	91.7	8.3	4.9	86.8	3.6	4.7
Otter24	6.6	7.2	92.8	90.3	9.7	1.1	89.2	6.1	3.7
Otter25	2.6	3.7	96.3	97.7	2.3	3.1	94.6	0.6	1.7
Otter26	0.0	0.4	99.6	87.5	12.5	0.4	87.1	0.0	12.5
Otter28	28.8	28.8	71.2	67.9	32.1	7.7	60.3	21.1	10.9
Otter29	32.5	32.8	67.2	99.6	0.4	32.8	66.8	0.0	0.4
Otter30	26.9	27.5	72.5	90.8	9.2	22.3	68.5	5.2	4.0
BitA	3.4	4.6	95.4	43.5	56.5	1.1	42.4	3.6	52.9
BitB	4.5	5.9	94.1	60.6	39.4	2.6	58.0	3.3	36.1
BitC	3.3	4.4	95.6	37.5	62.5	0.2	37.3	4.1	58.3
NIST SRM 1991	24.9	31.8	68.2	84.0	16.0	19.7	64.3	12.1	3.8

[a] LMW= low molecular weight; HMW=high molecular weight; PLMW = parent lower molecular weight; ALMW = alkylated lower molecular weight; PHMW = parent higher molecular weight; AHMW = alkylated higher molecular weight

[b]Priority PACs include: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, benzo[*ghi*]perylene, indeno[*1,2,3-c,d*]pyrene, and dibenz[*a,h*]anthracene

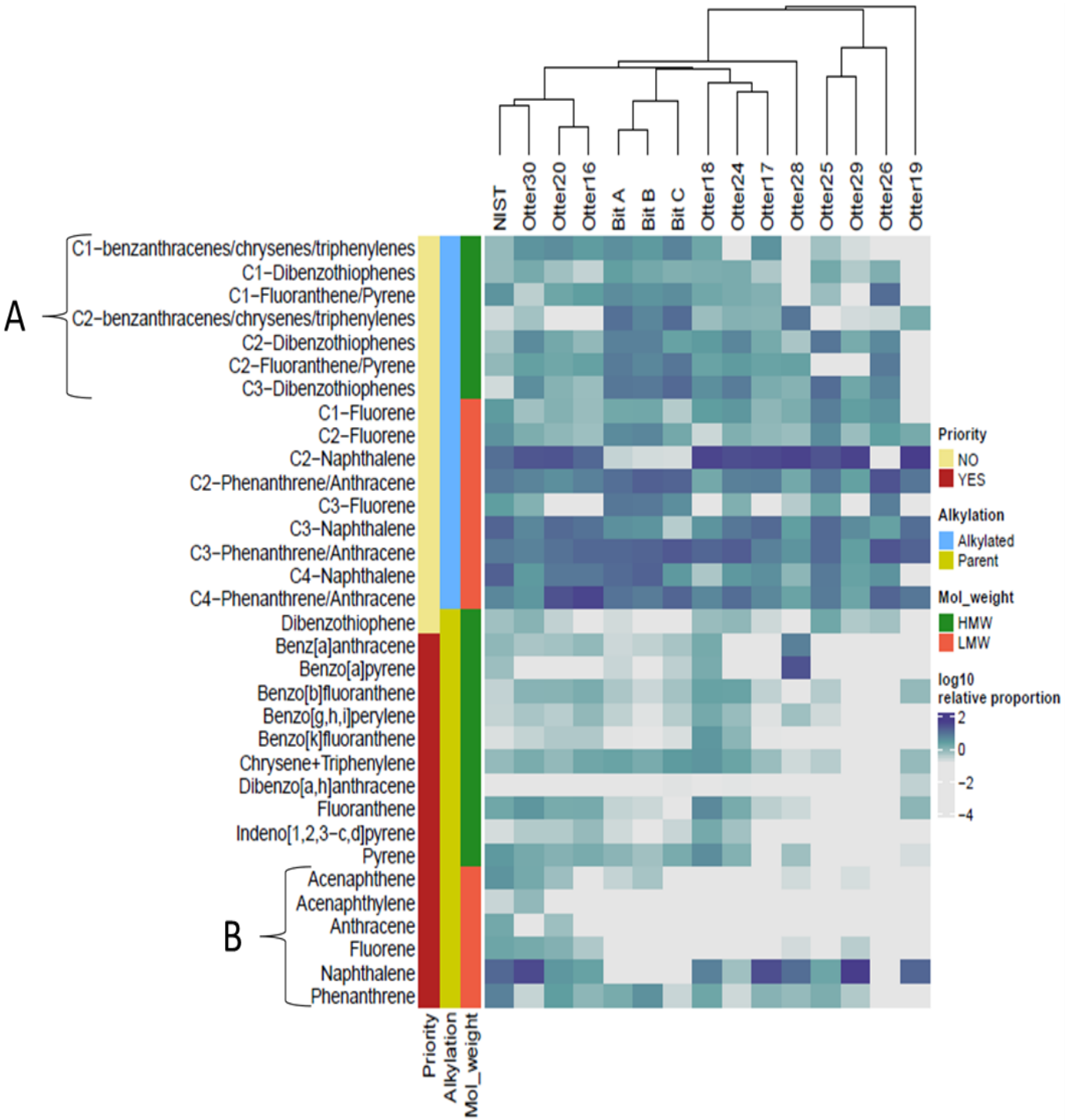

Figure 4.1. Heatmap demonstrating the PAC composition of river otter liver tissues, bitumen extracts and NIST SRM-1991. Each PAC is classified by priority, alkylation, and molecular weight. The scale represents the log10 transformed percent PAC value in each sample. PACs that were not measured across all samples were not included. A) Grouping of alkylated higher molecular weight PACs and B) Grouping of priority parent and lower molecular weight PACs.

4.2.2 Cell Cycle, Detection Method, & Mutation Type

The different cell cycle duration between the otter bone marrow and mouse FE1 cells could be a reason for the discrepancy between the two assay results. Bone marrow has high cell proliferation rate, and the microsatellite mutations were observed after 3-7 years of PAC exposure to the otters. On the other hand, the FE1 cells have a doubling time of ~19 hours and would have only gone through ~4 cell cycles, with a sampling time of ~72 hours, post exposure to the bitumen extracts. Thus, it is possible we did not observe DNA damage that may be occurring at a lower frequency or that the maximum mutant frequency was not reached to observe a response in the FE1 cells. Measuring the distribution of cell populations in the cell cycle phases of the FE1 cells after bitumen extract exposure would reveal the bitumen extracts effects on cell cycle progression and help mitigate the results.

The difference in mutation detection between the two techniques used for each study could also help explain the contradictory results. In Chapter 2, we used a single molecule microsatellite amplification method, single molecule polymerase chain reaction (SM-PCR), to identify mutations in tandem repeat sequences in wild otters. In contrast, in Chapter 3, we used an *in vitro lacZ* assay in FE1 cells that primarily detects point mutations. The mechanism by which PACs can induce microsatellite and point mutations are not the same. The primary mechanism of microsatellite mutations is DNA polymerase slippage, which is dependent on a variety of factors including microsatellite sequence, repeat number, flanking sequence, and mismatch repair errors (1). During replication, microsatellite mutations can be caused by slippage of the polymerase due to bulky adducts (formed by binding to DNA) or intercalated compounds (2,3). If PACs were responsible for the elevated microsatellite mutations in river otters, they would likely have to be able to either form DNA adducts and/or intercalate with

DNA resulting in the gain or loss of microsatellite repeat units (one-six nucleotides) if not repaired. PACs can also cause point mutations through adduct formation, but would alter the structure of single nucleotides, resulting in different base pairing during replication (4). Without efficient repair of the adducts or the differently structured nucleotides, DNA reactive PACs would be most responsible for producing the point mutations we observed in the FE1 *lacZ* assay. Larger mutations (e.g., microsatellite mutations) or damage on the chromosomal level are not identified in the *lacZ* transgene assay. However, just as they can be detected in any genome, microsatellite mutations can be measured in the genomic DNA in almost any mouse cell, including FE1s. For example, Beal et al. (2015) measured both microsatellite and *lacZ* mutations in the sperm of B[*a*]P exposed MutaMouse males (5,6). Thus, microsatellite mutation detection assays, such as SM-PCR, could be utilized to determine if the bitumen PAC extracts induced these types of mutations in the FE1s. Measuring other DNA damage endpoints such as DNA adduct formation or aneugenic/clastogenic damage in both river otter tissue and FEl cells would also help mitigate discrepancies between the two assays and provide additional clarification of the potential mutagenicity of PACs found in the river otters and the tailings pond bitumen extracts.

4.2.3 Other Sources of PACs in the AOSR

In this thesis, I investigated one source of PACs, tailings pond bitumen; however, there are other sources, both anthropogenic and natural, in the AOSR that could be evaluated for mutagenicity. One major source of anthropogenic PACs in the AOSR is petcoke. Petcoke, a by-product of the bitumen upgrading process, is stockpiled on mine sites where it is susceptible to wind/weather erosion leading to the contamination of the surrounding environment. Petcoke dust was identified as a major source of airborne PACs in the AOSR, with PAC concentrations

reported to be 25000−28000 ng/g dry weight of petcoke (7). In addition, within 25 km of a surface oil sand facility, petcoke was identified as the most abundant source of PACs in the area; over half the deposition was explained by the petcoke PACs using a source apportionment model based on the concentrations in collected lichen (8) and radiocarbon isotope analysis of lake snowpack (9). Clearly, petcoke is responsible for a large proportion of PAC contamination in the AOSR, but petcoke has a different PAC composition compared to bitumen. Using a chemical mass burden model, Zhang et al. (2016) reported concentrations of 19 target PACs (which included four alkylated PACs) within different PAC sources including petcoke, tailings, oil sands ore, and natural bitumen exposed along the Athabasca River (7). When comparing PAC profiles, the total PAC concentration was greater in petcoke (25000-28000 ng/g) than tailings (11900 ng/g), oil sands ore (4900 ng/g), and naturally exposed bitumen (2630 ng/g) and comprised a lower proportion (%) of alkylated PACs: petcoke (6%), tailings (60%), oil sands ore (28%), and exposed bitumen (16%). Furthermore, uptake of PACs from water, sediment and food sources can be different and/or transformed through the food web. PAC profiles within trout-perch, a forage fish species found in the Athabasca River, were compared to those in water and sediments documented from the JOSM program and PAC profiles were different (10). For example, trout-perch had a dominant signature of naphthalene and alkylated naphthalenes, which were also significant components in water but not sediment, whereas dibenzothiophene was a minor component in fish but a major constituent in sediment and water. Given the varying profiles and deposition from the different sources of PACs in the AOSR, an important consideration for future studies will be to test the mutagenicity of PACs from sources such as, petcoke, sediment, and PAC profiles found in wildlife diets in the AOSR.

4.2.4. Non-Genotoxic Mechanisms

Although the bitumen PAC extracts did not induce *lacZ* mutations in FE1, we did observe cytotoxic effects indicating that non-genotoxic pathways should be considered when investigating the toxicity of tailings pond bitumen. In the laboratory, PAC and PAC mixtures have been shown to elicit effects to the immune, endocrine, and cardiovascular system. The immunotoxicity of PACs has been extensively evaluated using murine as well as human and mouse cell models. Through these studies, PACs and their metabolites have been shown to cause immunosuppressive effects like inhibition of cytokine production, disruption to lymphocyte and myeloid cell development, and suppression and apoptosis of immune cells (i.e., B and T cell) (11,12). In addition, PACs from numerous environmental mixtures, including river/bay sediments, diesel exhaust and PAC-contaminated soils, have showcased both estrogenic and anti-estrogenic effects through a variety of bioassays (13). For example, the endocrine disruptive potential of PACs frequently found in crude oil have been reported by Lee et al. (2017) (14). Lee et al. (2017) showcased the estrogenic potency of five PACs (i.e., naphthalene, fluorene, phenanthrene, dibenzothiophene and chrysene) and their alkylated congeners to the estrogen receptor (14). 1-methychrysene followed by phenanthrene and its alkylated congeners were the most potent agonists. PAC effects on steroidogenesis were also evaluated by Lee et al. (2017) using an adrenal cell line. They found that the production of sex hormones depended on the type of PAC and alkylation status. For example, estradiol production was only induced by chrysene and not its alkylated congeners. However, among phenanthrenes, unsubstituted phenanthrenes and those with over three alkyl groups elicited greater estradiol production (14). Furthermore, mainly through fish exposure studies, PACs have been shown to impact the cardiovascular system. Zebrafish embryos exposed to phenanthrene, a reference PAH

found in oil, exhibited pericardial edema and bradycardia (15). Cardiac defects (i.e., dysregulation of cells responsible for the rhythmic beating of the heart and formation of edemas), craniofacial malformations, shorter body lengths, and impacts to ocular development were also observed in fish embryos exposed to crude oil (16–18).

Some of the PAC effects shown in lab have also been observed in AOSR wildlife. For example, metabolism (EROD), thyroid hormone regulation, and the development of tree swallows and amphibians have been influenced by PAC exposure in the AOSR environment (19–24). In addition, embryo toxicity within early-life stages of fish populations that affected survival, development, and circulatory system, in the AOSR is well documented (25–30).

Given the role PACs can have in non-genotoxic pathways and the cytotoxicity observed by the tailings pond bitumen PAC extracts, future work measuring specific pathways (e.g., immune, endocrine, cardiovascular, development) or using systems biology approaches (e.g., RNA sequencing) will aide to characterize the toxicity and further elucidate the mechanism(s) of action of PACs from the tailings pond bitumen.

4.3 Overall Conclusion Statements

In summary, the results from my thesis analyzed the mutagenic potential of a complex mixture of PACs from the AOSR in wildlife and cultured mammalian cells. Due to the difference in PAC profiles of the samples investigated and the difference in mutation detection techniques, we cannot make definitive conclusions about the mutagenicity of the PAC mixtures found in the AOSR without additional studies. Elucidating the mutagenic potential of other sources of PACs in the area could help further our understanding (e.g., testing extracts from sediment and petcoke, and PAC profiles found in wildlife diets). Nonetheless, my results act as a steppingstone to further investigate the toxicity of PACs found in the AOSR. Results from the river otter work

show microsatellite instability assessment by single molecule PCR analysis of genomic DNA in a wildlife species, which has not been done before. The river otter mutation results could lead to future studies that include a similar study with a larger number of samples or using more advanced sequencing technologies to further characterize the mutagenicity observed. In addition, exploring non-invasive methods (e.g., DNA from fresh blood, hair, or scat) is recommended in order to reduce reliance on lethal sampling. From the *in vitro* work, the bitumen samples in FE1 cells study did not induce mutations although, we did observe cytotoxic effects. Future considerations would be to investigate non-genotoxic pathways (e.g., effects to endocrine, immune, and cardiovascular systems, and development) when studying bitumen PAC toxicity. Overall, my thesis provides novel scientific contributions in terms of potential mutagenic effects of PAC mixtures and serves to aide in the assessment of the toxicity of these mixtures that are found in a priority area of Canada, the Athabasca oil sands region.

4.4 References

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Appendix

Supplemental Information

A1. Chapter 2

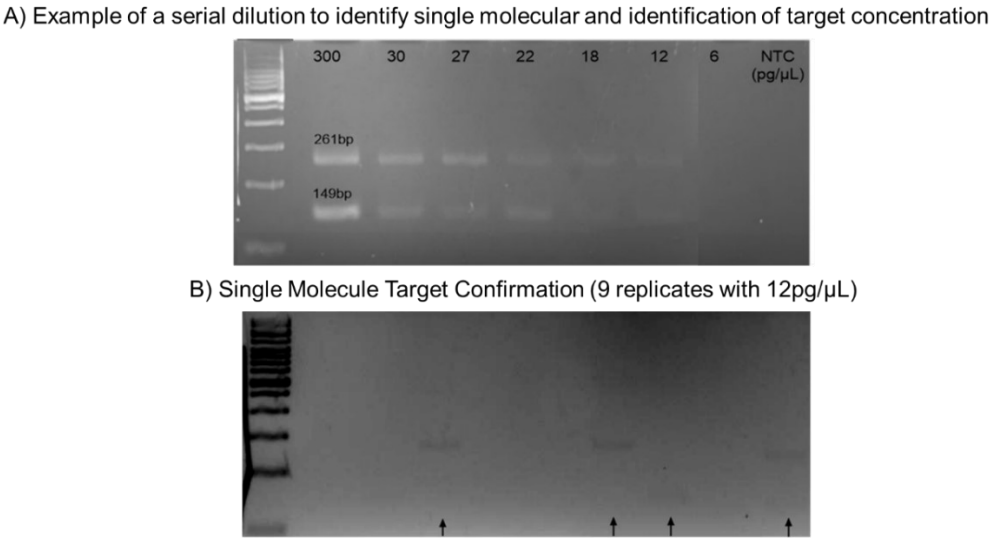

Figure S2.1. A) Example of a serial dilution to identify single molecule concentrations on a 4% agarose mini-gel. In this example, 12 pg/ul was identified as the single molecule concentration. B) Example of a subsequent visual confirmation of single molecule dilution. In this example, repeat PCR reactions of a template diluted to 12 pg/ul produced 4 positive reactions (indicated by small black arrows below each gel lane) out of 9 replicates.

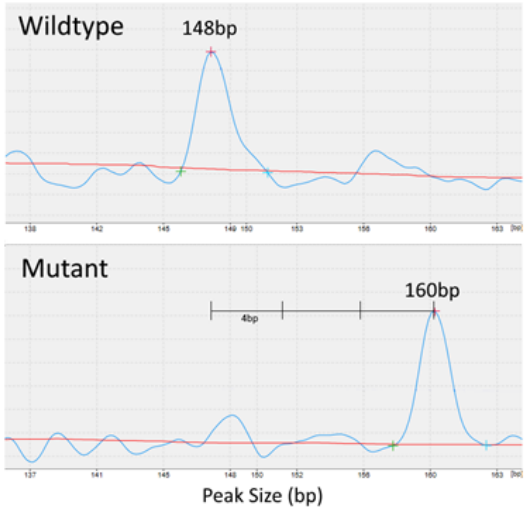

Figure S2.2. Representative example of a capillary electropherogram with SM-PCR amplified DNA microsatellite RIO06 in Otter 16 showing a wildtype allele (top) and mutant allele (+4-unit repeat-insertion mutation; bottom)

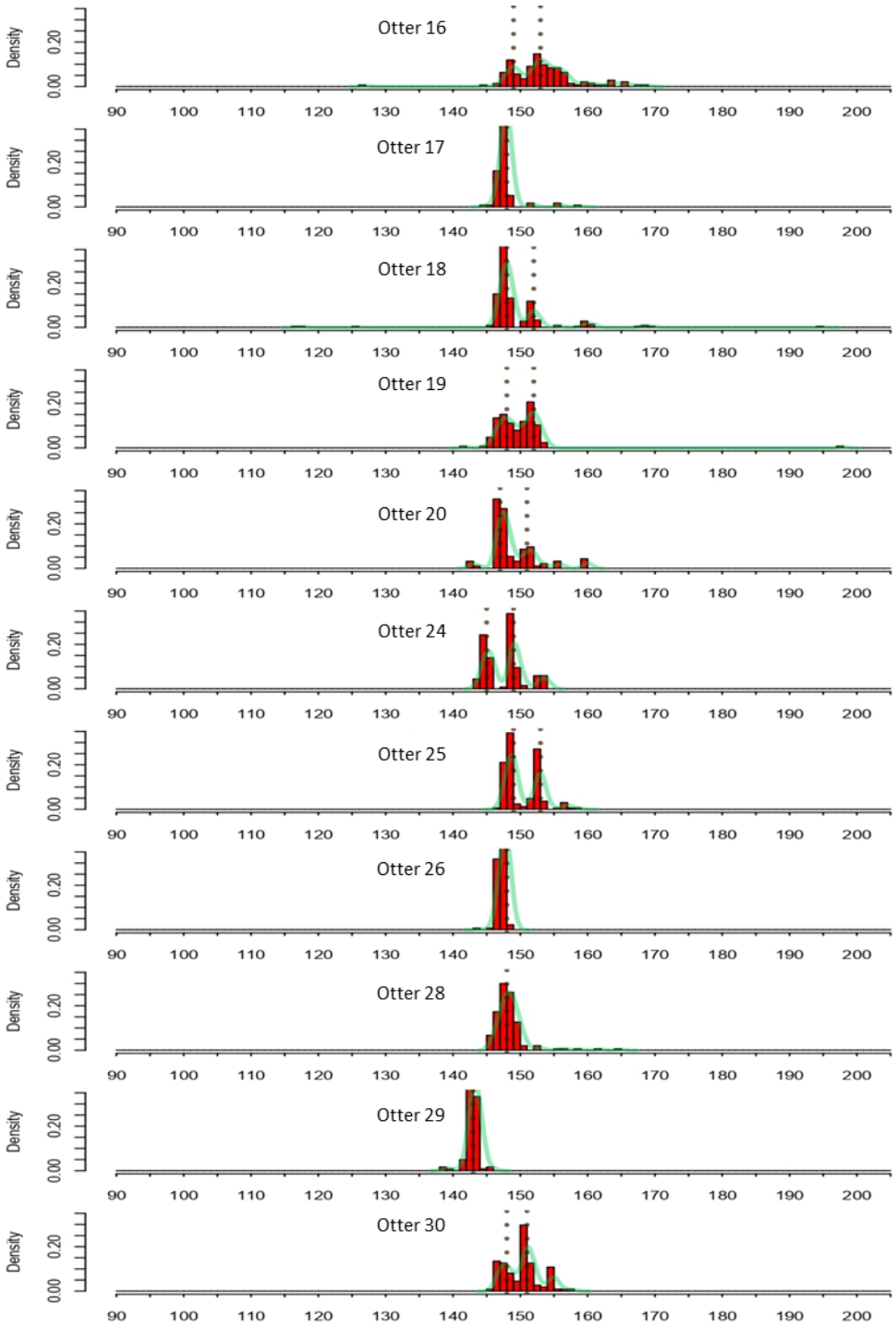

Figure S2.3. Frequency of all allele sizes scored per river otter. Homozygous individuals are indicated by one dashed line and heterozygous individuals are indicated with two.

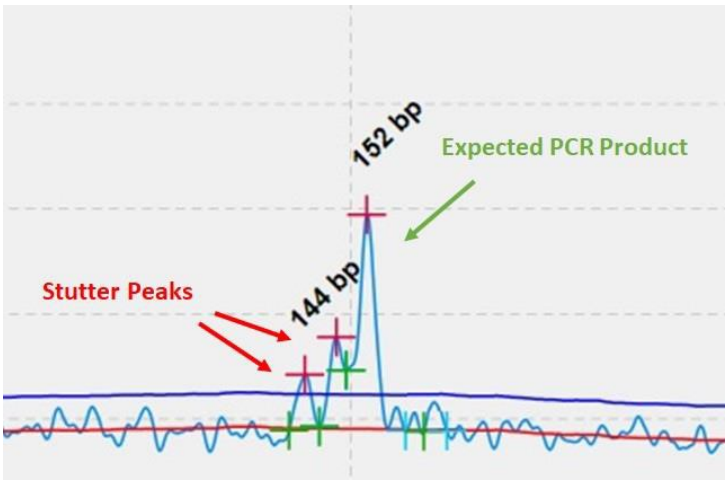

Figure S2.4. Example of a ScreenGel electropherogram detecting stutter peaks before the expected peak.

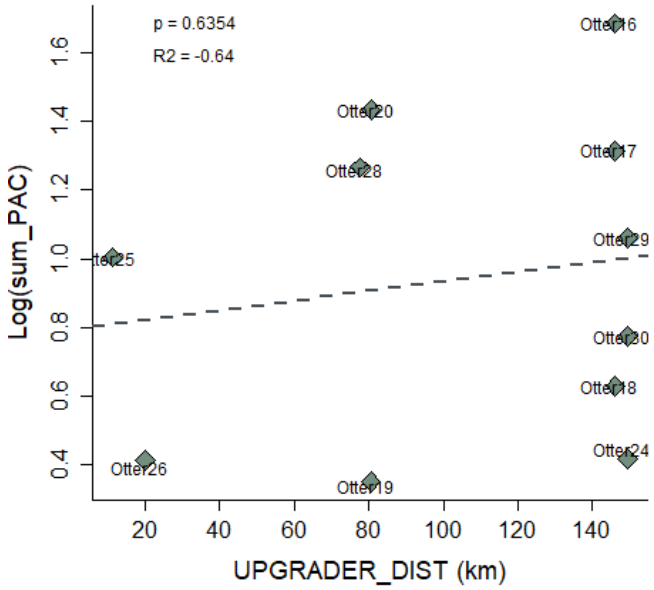

Figure S2.5. Relationship between the river otter (*Lontra canadensis*; n=11) PAC tissue burden and their distance to the nearest upgrader in kilometers.

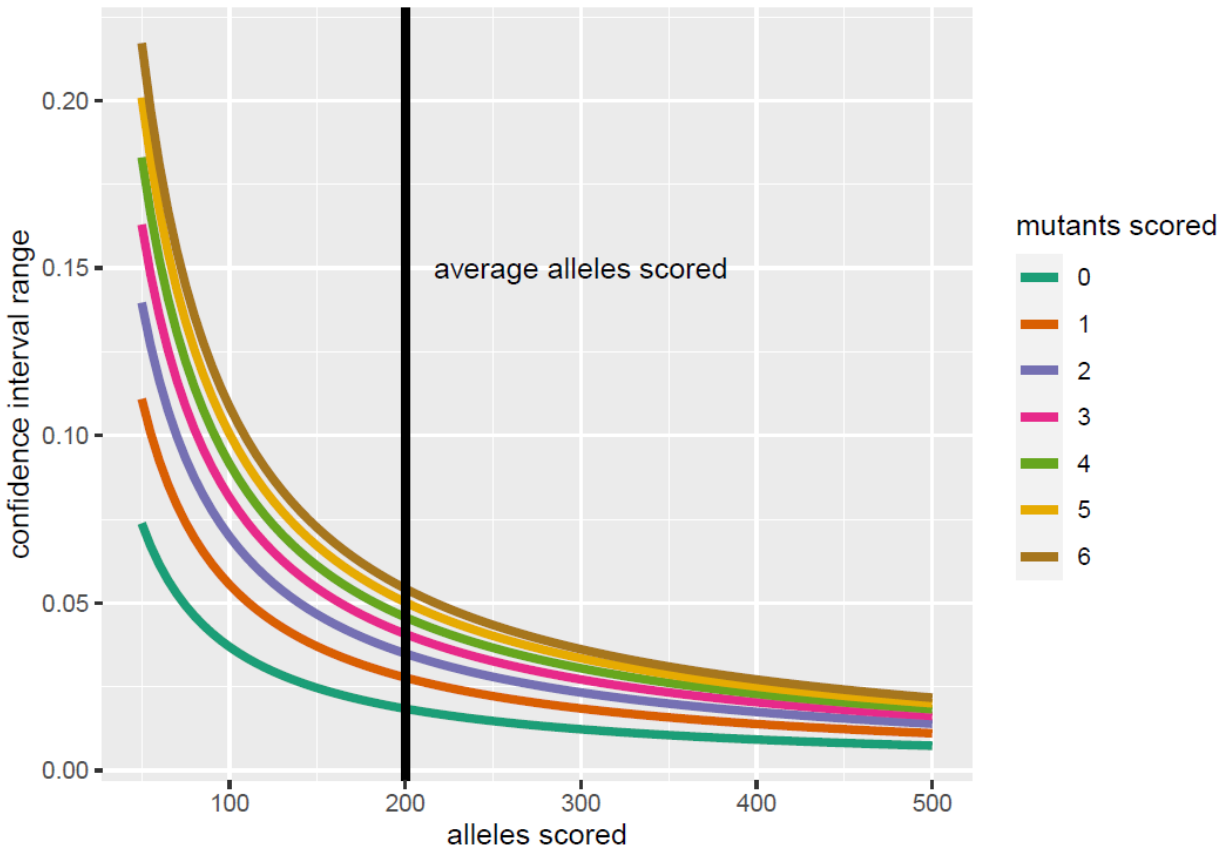

Figure S2.6. Relationship between the number of alleles scored, mutants scored, and confidence interval range associated.

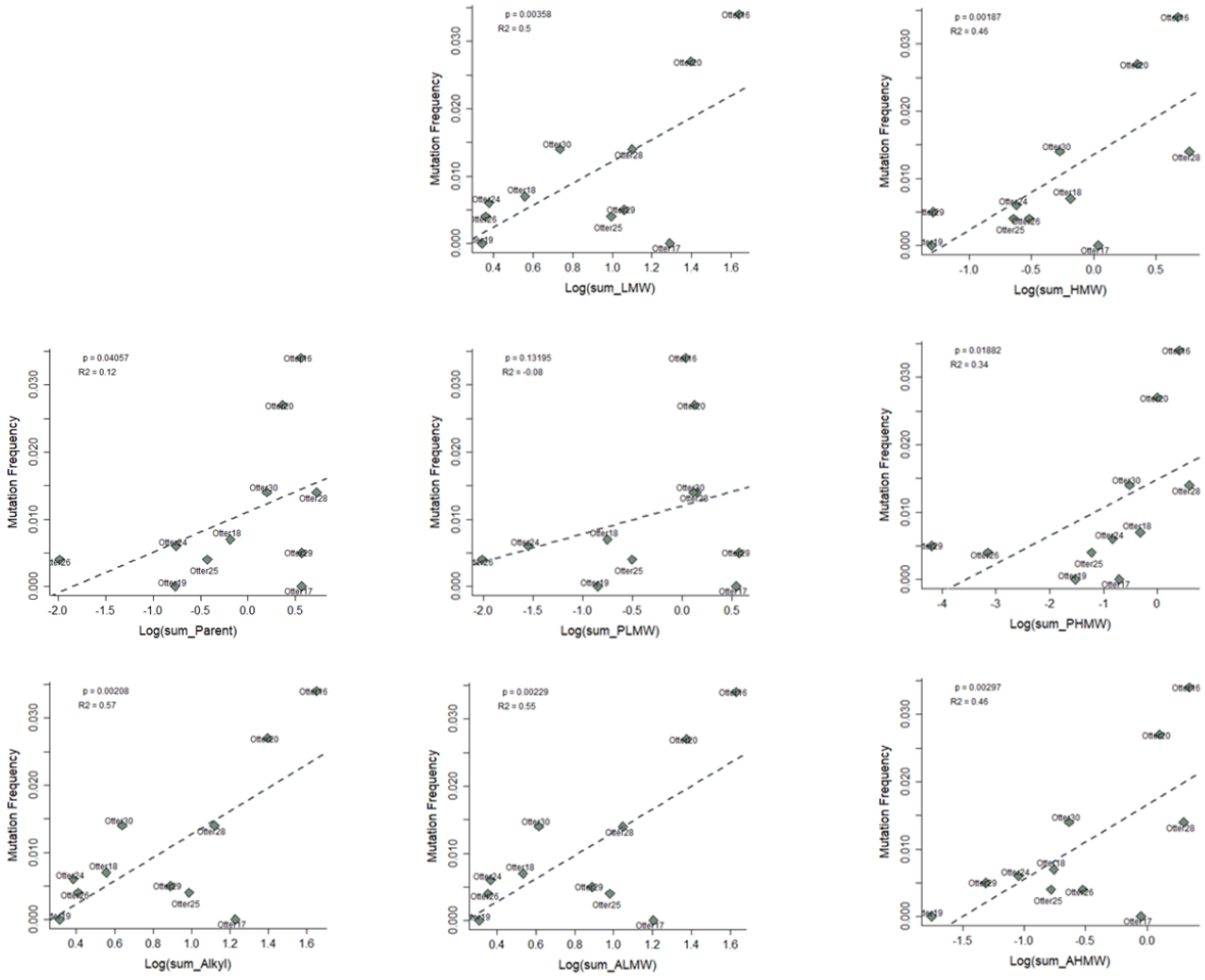

Figure S2.7. Relationship between the river otter microsatellite mutation frequencies (*Lontra canadensis*; n=11) and the different PAC types found in the liver tissue (log transformed) ($p<0.05$; Poisson regression).

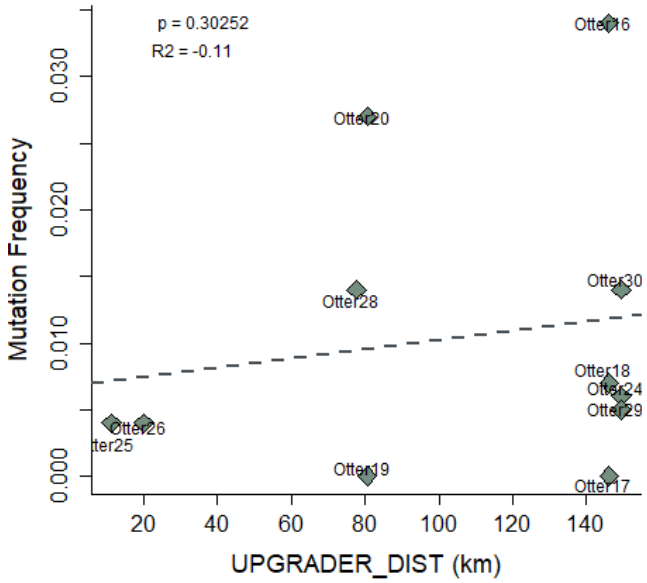

Figure S2.8. Relationship between microsatellite mutation frequency of river otters (*Lontra canadensis*; n=11) in bone marrow and distance (in km) to the nearest upgrader in the Athabasca oil sands region ($p<0.05$; Poisson regression)

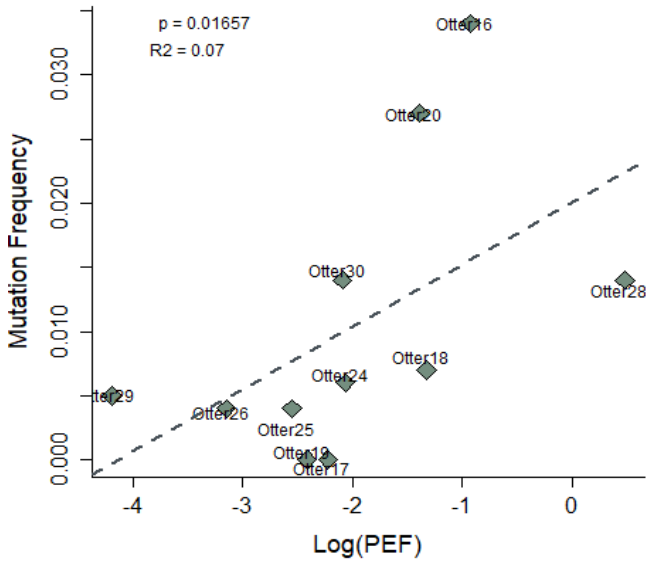

Figure S2.9. Relationship between mutation frequency of river otter (*Lontra canadensis*; n=11) microsatellites in bone marrow and sum of the priority PACs based on their benzo[*a*]pyrene potency equivalent factors (PEF) (log transformed) ($p<0.05$; Poisson regression).

Table S2.1. Characterization of 2 microsatellite loci in river otter DNA.

Microsatellite	Locus	GenBank ID	Primer Sequence	Motif	PCR Product (bp)
Microsatellite RIO06	RIO06	AY268056.1	F: CCAAGATGGCAACTACTCCTG R: CTTGACCTACTTGATAACCAGAAA	$(TCTA)_2$ $(TCTA)_8$	149
Microsatellite RIO18	RIO18	AY833269.1	F: TTCCATTGTCTCTTGGCTTG R: CTGGACTCGGCTTTAGTTCT	$(CTAT)_{10}$ $(CTAT)_3$	261

Table S2.2. PACs identified in the liver of the river otters (ng/g lipid weight) and their compound type

PAC	Compound Type[a]	Otter 16	Otter 17	Otter 18	Otter 19	Otter 20	Otter 24	Otter 25	Otter 26	Otter 28	Otter 29	Otter 30
1,7-Dimethylphenanthrene	ALMW	0.16	0.10	0	0.01	0.07	0.02	0.06	0.09	0.02	0.01	0.04
1,8-Dimethylphenanthrene	ALMW	0.62	0.24	0	0	0.23	0.002	0.01	0.05	0.01	0.00	0.02
1-Methylnaphthalene	ALMW	1.40	2.30	0.11	0.11	1.39	0.13	0.59	0	1.36	0.92	0.54
1-Methylphenanthrene	ALMW	0.26	0.13	0.001	0.01	0.21	0.01	0.07	0.04	0.02	0.02	0.02
2,6-Dimethylphenanthrene	ALMW	0.05	0.03	0.01	0.01	0.04	0.01	0.04	0.03	0.00	0.01	0.01
2-Methylnaphthalene	ALMW	1.69	3.97	1.58	0.72	2.05	0.15	1.25	0.01	2.74	1.82	1.07
2-Methylphenanthrene	ALMW	0.15	0.13	0	0.02	0.17	0.01	0.11	0.08	0.04	0.02	0.02
3,6-Dimethylphenanthrene	ALMW	0.09	0.05	0.002	0.01	0.04	0.01	0.06	0.03	0.02	0.02	0.02
3-Methylphenanthrene	ALMW	0.09	0.08	0	0.01	0.12	0.01	0.09	0.04	0.04	0.12	0.02
9/4-Methylphenanthrene	ALMW	0.13	0.08	0	0.01	0.15	0.01	0.07	0.04	0.03	0.02	0.02
Acenaphthene	PLMW	0.03	0	0	0	0.10	0	0	0	0.02	0.01	0.05
Acenaphthylene*	PLMW	0	0	0	0	0	0	0	0	0.01	0	0.03
Anthracene*	PLMW	0.02	0	0.001	0	0.10	0	0	0	0	0	0.003
Benz[*a*]anthracene*	PHMW	0.23	0	0.03	0	0.09	0.001	0	0	0.72	0	0.02
Benzo[*a*]pyrene*	PHMW	0	0	0.03	0	0	0	0	0	2.93	0	0
Benzo[*b*]fluoranthene*	PHMW	0.39	0.03	0.04	0.008	0.16	0.03	0.02	0	0.00	0	0.03
Benzo[*ghi*]perylene	PHMW	0.23	0.02	0.03	0	0.06	0.01	0.01	0	0.07	0	0.02
Benzo[*k*]fluoranthene*	PHMW	0.12	0.02	0.06	0	0.07	0.02	0	0	0.00	0	0.01
C1-Benzanthracenes/ Chrysenes/Triphenylenes	AHMW	0.76	0.40	0.04	0	0.67	0	0.04	0	0	0.01	0.12
C1-Benzo[*a*]pyrene	AHMW	0.08	0.00	0	0	0.001	0	0	0	0	0	0
C1-Dibenzothiophenes	ALHET	0.08	0.05	0.03	0	0.10	0.02	0.10	0.02	0.01	0.03	0.05
C1-Fluoranthene/Pyrene	AHMW	0.71	0.13	0.03	0	0.29	0.02	0.04	0.18	0.01	0.00	0.01
C1-Fluorene	ALMW	0.20	0.10	0.05	0	0.18	0.05	0.41	0.05	0.15	0.17	0.02
C2-Benzanthracenes/ Chrysenes/Triphenylenes	AHMW	0.00	0.12	0.01	0.01	0.01	0.02	0	0.00	0.98	0.01	0.02
C2-Benzo[*a*]pyrene	AHMW	0.00	0.00	0	0	0.0003	0.001	0.004	0	0.01	0.001	0.00

C2-Dibenzothiophenes	ALHET	0.23	0.18	0.06	0	0.26	0.08	0.52	0.07	0.05	0.11	0.15
C2-Fluoranthene/Pyrene	AHMW	0.63	0.23	0.04	0	0.28	0.04	0	0.12	0.25	0	0.08
C2-Fluorene	ALMW	0.18	0.10	0.00	0.02	0.14	0.02	0.27	0.04	0.08	0.05	0.05
C2-Naphthalene	ALMW	3.65	3.84	0.77	0.49	3.93	0.48	1.50	0	4.76	3.02	0.79
C2-Phenanthrene/ Anthracene	ALMW	1.71	0.77	0.03	0.08	0.63	0.10	0.50	0.39	0.19	0.17	0.19
C3-Benz[*a*]anthracene/ Chrysene	AHMW	0.01	0	0	0	0.004	0	0	0	0.62	0.01	0
C3-Dibenzothiophenes	ALHET	0.19	0.16	0.08	0	0.17	0.11	0.70	0.08	0.12	0.13	0.14
C3-Fluoranthene/Pyrene	AHMW	0	0	0	0	0	0	0.005	0	0.002	0.001	0.00
C3-Fluorene	ALMW	0.01	0.01	0	0	0.01	0.02	0.12	0.10	0.04	0.0001	0.04
C3-Naphthalene	ALMW	2.74	1.39	0.07	0.11	1.97	0.13	0.77	0.03	0.28	0.32	0.17
C3-Phenanthrene/ Anthracene	ALMW	3.64	0.70	0.28	0.15	1.52	0.30	0.76	0.37	0.39	0.17	0.20
C4-Benz[*a*]anthracene/ Chrysene	AHMW	0	0	0.05	0.001	0	0	0.06	0	0.11	0.001	0
C4-Dibenzothiophenes	ALHET	0.11	0.12	0.05	0	0.07	0.10	0.27	0.08	0.17	0.05	0.11
C4-Fluoranthene/Pyrene	AHMW	0.00	0.001	0.00	0	0.001	0	0.01	0	0.01	0.001	0
C4-Naphthalene	ALMW	2.22	0.45	0.01	0	1.26	0.05	0.47	0.05	0.11	0.16	0.10
C4-Phenanthrene/ Anthracene	ALMW	11.04	0.55	0.10	0.08	4.19	0.18	0.45	0.22	0.23	0.14	0.10
Chrysene+Triphenylene*	PHMW	0.50	0.08	0.07	0.01	0.13	0.03	0.02	0	0.02	0	0.05
Dibenzo[*a,h*]anthracene*	PHMW	0.02	0	0	0.003	0	0.002	0	0.001	0.002	0	0
Dibenzothiophene	PLHET	0.03	0.03	0.01	0	0.05	0.01	0.11	0.01	0	0.03	0.04
Fluoranthene*	PHMW	0.42	0.04	0.10	0.009	0.23	0.02	0	0	0.03	0	0.11
Fluorene*	PLMW	0.12	0	0	0	0.18	0	0	0	0.02	0.03	0.05
Indeno[*1,2,3-c,d*]pyrene*	PHMW	0.21	0	0.03	0	0.06	0.01	0	0	0	0	0.01
Naphthalene*	PLMW	0.61	3.34	0.12	0.14	0.49	0.01	0.12	0	1.25	3.60	1.11
Phenanthrene*	PLMW	0.26	0.12	0.04	0	0.40	0.004	0.08	0	0.09	0.03	0.01
Pyrene*	PHMW	0.40	0.01	0.08	0.002	0.18	0.02	0	0	0.07	0	0.05
Retene	ALMW	11.60	0.29	0.00	0.02	4.42	0.13	0.16	0.14	0.11	0.07	0.08

*Denotes priority PAC

[a]LMW= low molecular weight; HMW=high molecular weight; PLMW = parent lower molecular weight; PHMW = parent higher molecular weight; ALMW = alkylated lower molecular weight; AHMW = alkylated higher molecular weight; PLHET= parent lower molecular weight heterocyclic aromatic compound; ALHET= alkylated lower molecular weight heterocyclic aromatic compound

Table S2.3. Target DNA concentrations used for single-molecule PCR reactions, resulting PCR success rates and individual wild-type allele sizes.

Animal	DNA Target Concentration (pg/g)	Number of Positive Reactions (% PCR Success)	Wildtype Allele Sizes (Genotype)[a]
Otter 16	13	47	149 & 153 (HE)
Otter17	6	59.9	148 (HO)
Otter18	16	67.4	148 & 152 (HE)
Otter19	12	72.7	148 & 152 (HE)
Otter 20	8	44.6	147 & 152 (HE)
Otter 24	9	59.5	145 & 149 (HE)
Otter 25	12	72.8	149 & 153(HE)
Otter 26	12	72.6	148 (HO)
Otter 28	15	56.9	148 (HO)
Otter 29	8	64	143 (HO)
Otter 30	10	50.8	147 & 151 (HE)

[a]HE= heterozygous; HO=homozygous

Table S2.4. PAC Diagnostic Ratios computed for source identification[a]

Animal ID	LMW HMW	FLAPYR	ANTPHE	IcdPBghiP	BaACHR	FLPYR	BaPBghiP	BbFBkF	2Methyl-naphthalenePHE	Sum_ MePhePHE	CombParent
Otter 16	0.13	0.51	0.08	0.48	0.32	0.23	0	3.25	6.46	5.92	0.70
Otter 17	0.13	0.76	0	0.03	0	0.15	0	2.19	32.66	6.92	0.05
Otter 18	0.19	0.54	0.03	0.50	0.32	0	0.92	0.68	43.26	0.43	0.73
Otter 19	0.06	0.84	0	0	0	0	0	0	0	0	0.16
Otter 20	0.11	0.57	0.20	0.53	0.41	0.51	0	2.45	5.13	2.61	0.43
Otter 24	0.10	0.56	0	0.44	0.02	0	0	2.13	40.80	25.26	0.83
Otter 25	0.03	0	0	0	0	0	0	0	14.94	6.24	0.16
Otter 26	0.07	0	0	0	0	0	0	0	0	0	0
Otter 28	0.44	0.27	0	0	0.97	0.24	41.96	0.52	31.17	2.05	0.73
Otter 29	0.20	0	0	0	0	1.00	0	0	60.13	7.08	0
Otter 30	0.23	0.67	0.23	0.45	0.25	0.50	0	3.72	120.03	19.56	0.19

[a] LMWHMW=ΣLMW/ΣHMW; FLAPYR=FLA/(FLA + PYR); ANTPHE=ANT/(ANT + PHE); IcdPBghiP=IcdP/(IcdP + BghiP); BaACHR= BaA/(BaA + CHR); FLPYR= FL/(FL + PYR); BaPBghiP=BaP/BghiP; BbFBkF=BbF/BkF; 2MethylnaphthalenePHE= 2-methylnaphthalene/PHE; Sum_MePhePHE= ΣMePHE/PHE; CombParent= ΣCOMB/ΣPAHs [ΣCOMB – (FLA, PYR, BaA, CHR, BkF, BbF, BaP, IcdP and BghiP); ΣPAHs – sum of total non-alkylated PACs]
FLA= Fluoranthene; PYR= Pyrene; IcdP=Indeno[*1,2,3-c,d*]pyrene; BaA= Benzo[*a*]anthracene; CHR= Chrysene; FL= Fluorene; BaP= Benzo[*a*]pyrene; BghiP=Benzo[*ghi*]perylene; PHE= Phenanthrene; BkF= Benzo[*k*]fluoranthene; BbF=Benzo[*b*]fluoranthene

Table S2.4. Regression statistics for comparison of microsatellite mutation frequencies to PAC diagnostic ratios[a]

Diagnostic Ratio	p-value	R^2
LMWHMW	0.24	-0.09
FLAPYR	0.93	0.27
ANTPHE	0.01	0.43
IcdPBghiP	0.02	0.41
BaACHR	0.02	-0.03
FLPYR	0.32	0.28
BaPBghiP	0.53	0.3
BbFBkF	0.07	0.58
MethylnaphthalenePHE	0.29	0.42
Sum_MePhePHE	0.61	0.34
CombParent	0.04	0.01

[a] LMWHMW=ΣLMW/ΣHMW; FLAPYR=FLA/(FLA + PYR); ANTPHE=ANT/(ANT + PHE); IcdPBghiP=IcdP/(IcdP + BghiP); BaACHR= BaA/(BaA + CHR); FLPYR= FL/(FL + PYR); BaPBghiP=BaP/BghiP; BbFBkF=BbF/BkF; 2MethylnaphthalenePHE= 2-methylnaphthalene/PHE; Sum_MePhePHE= ΣMePHE/PHE; CombParent= ΣCOMB/ΣPAHs [ΣCOMB – (FLA, PYR, BaA, CHR, BkF, BbF, BaP, IcdP and BghiP); ΣPAHs – sum of total non-alkylated PACs]

FLA= Fluoranthene; PYR= Pyrene; IcdP=Indeno[*1,2,3-c,d*]pyrene; BaA= Benzo[*a*]anthracene; CHR= Chrysene; FL= Fluorene; BaP= Benzo[*a*]pyrene; BghiP=Benzo[*ghi*]perylene; PHE= Phenanthrene; BkF= Benzo[*k*]fluoranthene; BbF=Benzo[*b*]fluoranthene

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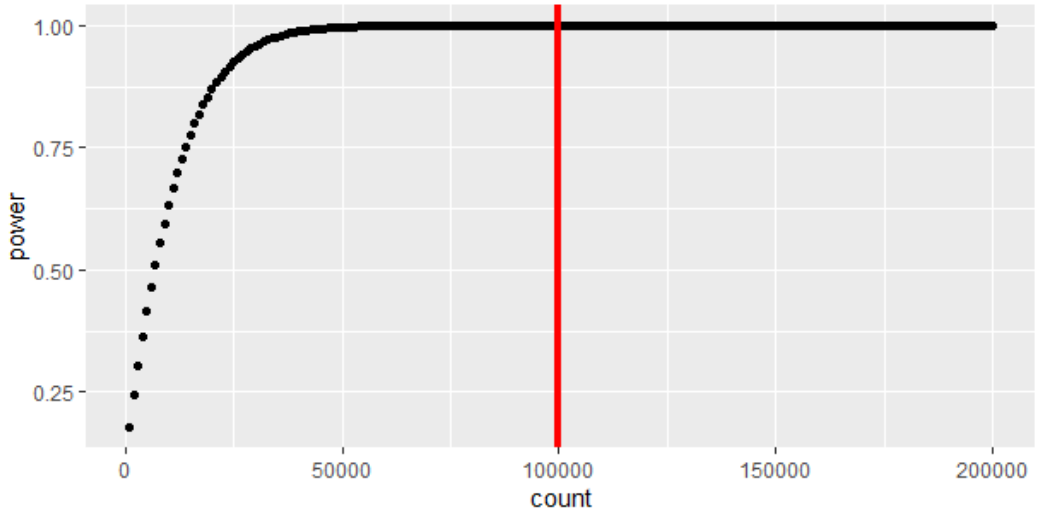

Figure S3.1. Power analysis for doubling effect when control mutation frequency is $8x10^{-4}$ Relationship between the number of plaque forming units and power level associated.

Table S3.1. Concentrations (µg/mL).and compound type of PACs identified in the method blank, bitumen extracts and NIST SMR 1991[a].

PAC	Compound Type[b]	Method Blank	Bit A	Bit B	Bit C	NIST SRM 1991
1,7-Dimethylphenanthrene	ALMW	NM	NM	NM	NM	0.67
1-Methylnaphthalene	ALMW	NM	NM	NM	NM	10.67
1-Methylphenanthrene	ALMW	NM	NM	NM	NM	2.05
2-Methylanthracene	ALMW	NM	NM	NM	NM	1.80
2-Methylnaphthalene	ALMW	NM	NM	NM	NM	15.56
2-Methylphenanthrene	ALMW	NM	NM	NM	NM	2.66
3-Methylphenanthrene	ALMW	NM	NM	NM	NM	3.19
4H-Cyclopenta[*def*]phenanthrene	PHMW	NM	NM	NM	NM	2.39
9/4-Methylphenanthrene	ALMW	NM	NM	NM	NM	1.56
Acenaphthene*	PLMW	0	0.15	0.49	0.03	9.08
Acenaphthylene*	PLMW	0	0	0	0	0.67
Anthanthrene	PHMW	NM	NM	NM	NM	0.29
Anthracene*	PLMW	0	0	0.057	0.030	4.43
Benz[*a*]anthracene*	PHMW	0	0.13	0.28	0.17	2.38
Benzo[*a*]fluoranthene	PHMW	NM	NM	NM	NM	2.39
Benzo[*a*]pyrene*	PHMW	0	0.14	0.10	0.11	1.77
Benzo[*b*]chyrsene	PHMW	NM	NM	NM	NM	0.16
Benzo[*b*]fluoranthene*	PHMW	0	0.26	0.23	0.19	0.75
Benzo[*b*]naphtho[*2,1-d*]thiophene	PHHET	0	0.32	0.46	0.29	NM

Benzo[*b*]naphthothiophenes (*2,3-d* and *1,2-d*)	PHHET	0	0.49	1.37	0.12	NM
Benzo[*c*]phenanthrene	PHMW	NM	NM	NM	NM	0.45
Benzo[*e*]pyrene	PHMW	0	0.47	0.42	0.37	0.59
Benzo[*ghi*]perylene*	PHMW	0	0.17	0.14	0.13	0.73
Benzo[*ghi*]fluoranthene	PHMW	NM	NM	NM	NM	0.87
Benzo[*j*]fluoranthene	PHMW	NM	NM	NM	NM	0.39
Benzo[*k*]fluoranthene*	PHMW	0	0.08	0.07	0.06	0.45
Biphenyl	PLMW	NM	NM	NM	NM	0.64
C1-Benzanthracenes/ Chrysenes/Triphenylenes	AHMW	0	2.27	2.73	1.99	2.05
C1-BNTs	AHHET	0	6.53	8.74	5.75	NM
C1-Dibenzothiophenes	ALHET	0	1.36	1.56	0.43	2.03
C1-Fluoranthene/Pyrene	AHMW	0	2.44	3.23	1.49	9.12
C1-Fluorene	ALMW	0.0004	0.96	1.66	0.13	7.47
C1-Naphthalenes	ALMW	0.0093	0.010	0.013	0.007	NM
C1-Phenanthrenes	ALMW	0	2.77	10.38	0.65	2.70
C2-Benzanthracenes/ Chrysenes/Triphenylenes	AHMW	0	5.77	4.93	3.97	0.59
C2-BNTs	AHHET	0.029	14.83	12.19	9.51	NM
C2-Dibenzothiophenes	ALHET	0	3.44	5.84	0.74	1.41
C2-Fluoranthene/Pyrene	AHMW	0	4.11	4.89	2.95	2.22
C2-Fluorene	ALMW	0.0009	2.54	5.03	0.57	9.58
C2-Naphthalene	ALMW	0	0.15	0.19	0.06	28.33
C2-Phenanthrene/Anthracene	ALMW	0.005	5.94	16.13	5.19	20.62
C3-Benz[*a*]anthracene/Chrysene	AHMW	0	4.57	3.82	3.35	NM
C3-BNTs	AHHET	0.009	8.08	7.52	5.89	NM
C3-Dibenzothiophenes	ALHET	0.002	4.71	7.31	4.38	0.55
C3-Fluoranthene/Pyrene	AHMW	0	4.74	5.10	3.36	NM
C3-Fluorene	ALMW	0.005	4.69	7.34	1.41	6.86
C3-Naphthalene	ALMW	0.001	2.18	3.29	0.13	39.24
C3-Phenanthrene/Anthracene	ALMW	0.001	7.47	14.11	7.21	20.08
C4-BNTs	AHHET	0	3.84	3.12	2.67	NM
C4-Fluoranthene/Pyrene	AHMW	0	3.75	3.60	2.39	NM
C4-Naphthalene	ALMW	0	6.83	15.24	1.02	44.95
C4-Phenanthrene/Anthracene	ALMW	0.001	5.25	6.80	5.08	13.57
Chrysene+Triphenylene*	PHMW	0.001	1.17	1.39	0.98	2.09
Coronene	PHMW	NM	NM	NM	NM	0.24
Cyclopenta[*cd*]pyrene	PHMW	NM	NM	NM	NM	0.15
Dibenz[*a,j*]anthracene	PHMW	NM	NM	NM	NM	0.16
Dibenz[*a*]anthracene	PHMW	NM	NM	NM	NM	0.16
Dibenzo[*a,e*]pyrene	PHMW	NM	NM	NM	NM	0.09
Dibenzo[*a,h*]anthracene*	PHMW	0	0.07	0.05	0.05	0.17
Dibenzo[*a,h*]pyrene	PHMW	NM	NM	NM	NM	0.14
Dibenzo[*b,k*]fluoranthene	PHMW	NM	NM	NM	NM	0.04

Dibenzothiophene	PLHET	0	0.1	0.1	0	1.60
Fluoranthene*	PHMW	0	0.2	0.2	0	4.71
Fluorene*	PLMW	0	0.1	0.1	0	5.05
Indeno[*1,2,3-c,d*]pyrene*	PHMW	0	0.1	0.1	0.08	0.51
Naphthalene*	PLMW	0.03	0.03	0.03	0.03	34.58
Perylene	PHMW	NM	NM	NM	NM	14.23
Phenanthrene*	PLMW	0.001	0.84	3.62	0.09	16.09
Picene	PHMW	NM	NM	NM	NM	0.27
Pyrene*	PHMW	0.0006	0.55	0.56	0.51	7.86
Retene	ALMW	0.0008	0.61	1.19	0.69	NM

*Denotes priority PAC

[a]NM= PACs that were not measured due to different detection methods for the bitumen samples and NIST SRM 1991

[b]LMW= low molecular weight; HMW=high molecular weight; PLMW = parent lower molecular weight; PHMW = parent higher molecular weight; ALMW = alkylated lower molecular weight; AHMW = alkylated higher molecular weight; PLHET= parent lower molecular weight heterocyclic aromatic compound; PHHET= parent higher molecular weight heterocyclic aromatic compound ; AHHET= alkylated higher molecular weight heterocyclic aromatic compound; ALHET= alkylated lower molecular weight heterocyclic aromatic compound

A3. Chapter 4

Table S4.1. PAC composition of the river otter liver PAC burdens (ng/g lipid weight) bitumen extracts (µg/mL) and NIST SRM -1991 (µg/mL) summarized into various classifications[a].

Sample	Total PAC	Total Priority [b]	Total Parent	Total Alkyl -ated	Total LMW	Total HMW	Total PLMW	Total ALMW	Total PHMW	Total AHMW
Otter16	48.0	3.6	3.6	44.4	43.3	4.7	1.1	42.2	2.5	2.2
Otter17	20.4	3.7	3.7	16.7	19.3	1.1	3.5	15.8	0.2	0.9
Otter18	4.1	0.6	0.6	3.4	3.4	0.6	0.2	3.2	0.5	0.2
Otter19	2.0	0.2	0.2	1.9	2.0	0.0	0.1	1.9	0.0	0.0
Otter20	26.9	2.3	2.3	24.6	24.7	2.2	1.3	23.3	1.0	1.3
Otter24	2.4	0.2	0.2	2.2	2.2	0.2	0.0	2.2	0.1	0.1
Otter25	9.9	0.3	0.4	9.5	9.7	0.2	0.3	9.4	0.1	0.2
Otter26	2.4	0.0	0.0	2.4	2.1	0.3	0.0	2.1	0.0	0.3
Otter28	18.2	5.2	5.2	13.0	12.4	5.8	1.4	11.0	3.9	2.0
Otter29	11.3	3.7	3.7	7.6	11.2	0.0	3.7	7.5	0.0	0.0
Otter30	5.8	1.5	1.6	4.2	5.2	0.5	1.3	3.9	0.3	0.2
BitA	115.2	4.0	5.4	109.8	50.1	65.1	1.2	48.9	4.1	60.9
BitB	165.7	7.4	9.7	155.9	100.4	65.3	4.3	96.1	5.4	59.9
BitC	74.3	2.5	3.3	71.0	27.9	46.4	0.2	27.7	3.1	43.3
NIST	362.1	90.3	115.3	246.8	304.3	57.8	71.3	233.0	44.0	13.8

[a] LMW= low molecular weight; HMW=high molecular weight; PLMW = parent lower molecular weight; ALMW = alkylated lower molecular weight; PHMW = parent higher molecular weight; AHMW = alkylated higher molecular weight

[b] Priority PACs include: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, benzo[*ghi*]perylene, indeno[*1,2,3-c,d*]pyrene, and dibenz[*a,h*]anthracene