ASSESSMENT OF POLYCYCLIC AROMATIC HYDROCARBON BIOAVAILABILITY FROM SOIL USING THE JUVENILE SWINE MODEL

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By

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are common soil contaminants due to their lipophilic nature which limits partitioning to water or air. Soil properties such as organic carbon can affect PAH release from soil, and thus affect PAH bioavailability of ingested soil. Risk assessment of PAHs in soil generally assumes equal bioavailability of PAHs ingested in soil compared to PAHs ingested in reference dose media, leading to environmental cleanup guidelines that are potentially too conservative. This research intended to use the juvenile swine model to assess PAH bioavailability from impacted soil to better inform bioavailability estimates for risk assessment. This was done by assessing PAH bioavailability from single and repeated exposure to PAHs in different spiked exposure media, assessing PAH bioavailability from soil collected from PAH impacted sites, and assessing biomarkers of exposure and effect following PAH exposure.

The effect of exposure duration on bioavailability was assessed because people are usually chronically exposed to PAHs, rather than acutely exposed, as most bioavailability studies are performed, and chronic exposure may lead to increases in xenobiotic metabolizing enzymes and transporters which may affect bioavailability. This research found that exposure duration did not significantly affect anthracene and benzo[a]pyrene bioavailability ($p>0.075$), but exposure media did ($p<0.004$). These results suggest that exposure medium has a more important effect on bioavailability than exposure duration, and also bioavailability calculated from a single exposure is appropriate for use in risk assessment.

Bioavailability from 24 naturally impacted soils was assessed to determine which soil characteristics had the greatest effect on PAH bioavailability. Area under the curve (AUC) measurements for benzo[a]pyrene (BaP) and anthracene in swine blood after oral exposure from

a soil matrix for benzo[a]pyrene and anthracene in soils had s very poor relationship with soil concentrations in soils collected from impacted sites (r^2<0.15), but a very strong relationship with soil concentrations from spiked artificial soils (r^2<0.95). As spiked soils had much higher concentrations of PAH, these results suggest there is a point of departure in soil concentrations where internal exposure becomes linearly related to soil concentration. Point of departure modeling indicates that this point occurs at soil PAH concentrations greater than 1,900 mg kg^{-1}. Thus, risk assessment can assume a constant exposure to PAHs at soil concentrations lower than the point of departure. Comparison of terminal rate constants from intravenous (IV) exposure to PAHs and oral exposure to PAHs in a soilmatrix suggest that flip-flop kinetics occur in swine, where absorption occurs at a slower rate than elimination. Flip-flop kinetics likely explains the lack of relationship between real world soil concentrations and area under the curve measurements as absorption is the rate limiting step of elimination.

Biomarkers of exposure and effect were assessed in swine liver and ileum tissue, as well as blood following single and subchronic exposure to PAHs to determine if relationships could be drawn between exposure magnitude and duration and biomarker formation. Biomarkers included cytochrome P450 (P450) 1A1, 1A2, and 1B1 expression and activity as biomarkers of exposure and DNA adducts, carbonylated proteins, and micronucleated reticulocytes as biomarkers of effect. Biomarkers of exposure were not affected by exposure magnitude or duration, indicating that they would serve best as exposure markers rather than indicators of bioavailability or other effects. However, DNA adduct and protein carbonyl formation was significantly affected by exposure duration ($p<0.045$), but micronuclei formation was not. The micronuclei results suggest the liver was effective at clearing PAHs to non-toxic metabolites at the study doses, while tissue biomarkers of effect may correlate more effectively with exposure

length and magnitude of dose. This work indicates that PAH bioavailability from soil is lower than 100%, but additional work needs to be done to determine soil characteristics that affect bioavailability and to determine a bioavailability value relative to reference material.

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1-OHP	1-hydroxypyrene
AF_G	Relative Gastrointestinal Absorption Factor
AF_L	Lung Absorption Factor
AF_S	Skin Absorption Factor
AhR	Aryl Hydrocarbon Receptor
ANOVA	Analysis of Variance
ASE	Accelerated Solvent Extraction
ATSDR	Agency of Toxic Substances and Disease Registry
AUC	Area under the Plasma Concentration versus Time Curve
BA	Bioavailability
BaP	Benzo[a]pyrene
BGS	British Geological Survey
BMD	Benchmark Dose
BMDS	Benchmark Dose Software
BPDE	7,8-diol, 9,10-epoxide Benzo[a]pyrene
BSC	Background Soil Concentration
BW	Body Weight
CCME	Canadian Council of Ministers of the Environment
COT	Communities of Tomorrow
$_{\Delta\Delta}C_T$	Comparative Threshold Cycle
CSF	Cancer Slope Factor
CYP	Cytochrome P450

DNA	Deoxyribonucleic Acid
ELISA	Enzyme-linked Immunosorbant Assay
EROD	Ethoxyresorufin-O-deethylase
G6PD	Glucose-6-phosphate dehydrogenase
GW5	Gas Works Soil #5
HPLC-FD	High Performance Liquid Chromatography – Fluorescence Detection
ILCR	Incremental Lifetime Cancer Risk
IR_S	Soil Inhalation Rate
IV	Intravenous
k_a	Absorption rate constant
k_e	Elimination rate constant
MN	Micronuclei
MN-RET	Micronucleated Reticulocyte
mRNA	Messenger Ribonucleic Acid
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
OM	Organic Matter
p53	Tumor Promoter p53
PAH	Polycyclic Aromatic Hydrocarbon
PBPK	Physiologically Based Pharmacokinetic
POD	Point of Departure
qPCR	Quantitative Polymerase Chain Reaction
ROS	Reactive Oxygen Species

RSD	Risk Specific Dose
SF	Soil Allocation Factor
SIR	Soil Ingestion Rate
SPE	Solid Phase Extraction
SQG_{HH}	Soil Quality Guideline for Human Health
SR	Soil Dermal Contact Rate
T_d	Threshold Dose
TDI	Tolerable Daily Intake
US EPA	United States Environmental Protection Agency
WP1	Wood Preservation Soil #1

1 INTRODUCTION

Human health risk assessments of polycyclic aromatic hydrocarbon (PAH) impacted soils generally use default bioavailability assumptions to estimate human exposure to these compounds. PAHs are compounds of toxicological interest because they are suspected human carcinogens, and as such, soil quality guidelines are quite stringent. However, oral exposure to PAHs in soil likely does not result in complete absorption as PAHs bind to soil particles which limits absorption. The default assumption of equal bioavailability from soil and exposure media used to derive toxicological reference values is likely too conservative, and would lead to the over-estimation of risk to humans and too stringent cleanup guidelines. Additionally, people are often repeatedly exposed to PAHs, which may influence systemic absorption of PAHs, as well as biomarkers of PAH exposure and effect, like enzyme induction and DNA adduct formation, and characterizing these changes will help derive more accurate bioavailability estimates.

In order to move past the default bioavailability assumption, a number of data gaps must be addressed. The first data gap is determining if repeated exposure to PAHs influences bioavailability and toxicity of compounds. People undergo repeated exposure to PAHs, and changes in cellular processes induced by PAH exposure, like enzyme activity, can influence bioavailability. The second data gap is determining if PAH bioavailability is consistent across PAH soil concentrations, or if soil characteristics change bioavailability in a predictable manner. PAHs are lipophilic compounds, and are expected to associate with organic matter present in soil; thus organic matter content in soil could influence PAH bioavailability. The research detailed in this PhD thesis is intended to address these data gaps.

1.1 Objectives and Hypotheses

The overall objective of this research is to assess the appropriateness of the current default PAH bioavailability estimate of 100%, that is, equal bioavailability from soil and toxicological reference dose material, and recommend modifications to this value if warranted. This objective has been tested by assessing whether repeated exposure to PAHs affects bioavailability, and if soil PAH concentrations or other soil characteristics can be used to predict PAH bioavailability. Additionally, we tested if changes in biomarkers of exposure and effect could determine if biomarkers of exposure and effect were good predictors of PAH bioavailability or exposure duration.

The overall hypothesis of this work is that PAH bioavailability and toxicity is overestimated by the default assumption of 100% bioavailability in risk assessment. This global hypothesis has been evaluated through the investigation of three sub-hypotheses: i) repeated exposure to PAHs decreases the bioavailability of the compounds, ii) soil properties affect PAH bioavailability in a predictable manner, and iii) expression of biomarkers of PAH exposure and effect will decrease with decreasing PAH bioavailability but increase with increased exposure duration.

For the first sub-hypothesis, bioavailability of BaP and anthracene were compared on day 1 and day 7 of exposure to PAHs in one of four exposure media. The results of this research are presented in Chapter 3 (THE BIOAVAILABILITY OF POLYCYCLIC AROMATIC HYDROCARBONS FROM DIFFERENT DOSE MEDIA AFTER SINGLE AND SUB-CHRONIC EXPOSURE IN JUVENILE SWINE). The second sub-hypothesis was examined by plotting area under the plasma concentration versus time course curve (AUC) values vs. soil concentration and soil:simulated gastrointestinal fluid partitioning co-efficients. This research is presented in Chapter 4 (IS RECEIVED DOSE FROM INGESTED SOIL INDEPENDENT OF

SOIL PAH CONCENTRATIONS: ANIMAL MODEL RESULTS). Results of testing the third sub-hypothesis are presented in Chapter 5 (DO BIOMARKERS OF EXPOSURE AND EFFECT CORRELATE WITH INTERNAL EXPOSURE TO PAHS IN SWINE?), and examine the effect of dose media, dose level, and exposure length on gene expression, enzyme activity, DNA adduct formation, oxidative stress levels, and micronuclei formation.

Some degree of redundancy in the presentation of this PhD thesis was unavoidable since each research chapter was written as an independent manuscript for publication in peer-reviewed journals. At the time of submission of this PhD thesis, Chapter 3 was previously published in the academic journal Science of the Total Environment, Chapter 4 was previously published in the academic journal Environmental Toxicology and Chemistry and Chapter 5 was previously published in the academic journal Biomarkers.

2 LITERATURE REVIEW

2.1 PAHs in the Environment

2.1.1 Sources and Environmental Distribution

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous compounds of concern for human exposure in the environment. The PAH family includes more than one hundred compounds consisting of two or more fused benzene rings (CCME, 2008). These compounds occur as a major constituent of fossil fuels such as coal and oil, and combustion produces PAHs as a byproduct. Thus, both natural (forest fires, volcanoes) and anthropogenic (energy production, manufacturing) sources of PAHs exist (ATSDR, 1995). Some PAHs are classified as potentially carcinogenic in humans based on animal toxicology studies, and as such, very low regulatory guidelines for environmental media have been developed (CCME, 2008).

PAHs are lipophilic in nature, although lipophilicity does vary between compounds. The log octanol water partitioning coefficients (log Kow) for the 16 US EPA priority PAHs range from 3.4 (naphthalene) to 6.7 (benzo[ghi]perylene) (CCME, 2008). The high lipophilicity of parent PAHs results from the non-polar nature of the compounds, which increases as the compounds have more rings. In general, the smaller compounds, with fewer ring structures, tend to be more water soluble.

Water solubility plays a large role in the fate and transport of compounds in the environment. Highly lipophilic compounds will predominantly partition to soil and sediment, likely due to sorption to organic matter (Meijer et al., 2003, Cabrerizo et al., 2011). Organic matter is considered analogous to lipids in the environment, and as such, lipophilic compounds will preferentially partition to organic matter. Limited environmental transport will occur if compounds preferentially partition to soil and sediment because the advective movement of soil and sediment to other environments is very limited.

PAHs have limited volatility, meaning they will generally be present in the atmosphere in low concentrations. However, due to the magnitude of atmospheric transport, this mechanism is still considered the main medium of transport of PAHs emitted from car exhaust and smokestacks (CCME, 2008). Naphthalene, the smallest PAH with only two rings, has a vapour pressure of 11.3 Pa at room temperature (CCME, 2008). The other 15 US EPA priority PAHs have vapour pressures that range from somewhat volatile ($3.3x10^{-1}$ Pa, acenaphthene) to essentially non-volatile ($1x10^{-8}$ Pa; benzo[ghi]perylene, indeno[123-cd]pyrene, dibenzo[ah]anthracene) (CCME, 2008). Particulate matter in the atmosphere can have sorbed PAH. Humans may experience inhalation exposure to PAHs present on suspended particulate matter. Soot particles have particularly large PAH concentrations as the particles tend to have high concentrations of organic or black carbon (ATSDR, 1995). Like in soil, PAHs sorb to soot particles as the carbon is analogous to lipids. PAHs may be subjected to atmospheric transport while sorbed to soot particles, and thus transported for shorter distances than compounds not bound to particulate matter.

2.1.2 Persistence

Persistence in the environment contributes to the environmental fate of a compound. PAHs are potentially readily degraded by aerobic bacteria in the environment, and as such, can have relatively short environmental half-lives. However, bacterial degradation depends on a number of factors including compound, temperature, organisms present, and the presence of other necessary factors like nutrients, so environmental half-lives can be quite variable. Parent compound half-lives in soil can range from 0.5 days for naphthalene to 5.2 years for pyrene. In ideal conditions, with sufficient microbial biomass and high turnover rates, PAH biodegradation can occur quite rapidly in the aquatic environment (ATSDR, 1995). PAHs with more than four rings tend to resist aerobic biodegradation in water.

Atmospheric PAH degradation occurs by ozone induced oxidation and hydroxylation, and sunlight activates both reactions. Atmospheric degradation occurs more rapidly than in soil and water, with half-lives ranging between 0.4 and 68 hours (CCME, 2008). As atmospheric PAHs tend to sorb to particles, the nature of the particles can affect the persistence of the compounds (Behymer and Hites, 1988). High organic matter content tends to inhibit degradation of atmospheric PAHs (Environment Canada, 1994).

PAHs tend to persist longer in soil than in water, with soil half-lives for parent compounds ranging from approximately 2 days to 58 years (Park et al., 1990, CCME, 2008). Much of this variation results from differences in compounds present, as well as the temperature, microbes, nutrients, pH, and oxygen content in soil. Low molecular weight PAHs are expected to volatilize or biodegrade more rapidly than larger compounds, although PAHs with four or more rings may still have half-lives lasting less than one year (Wild and Jones, 1993). Bioavailability may also influence biodegradation of PAHs in soil as weathering and organic carbon content in the soil may limit the PAH availability to microorganisms (Weissenfels et al., 1992).

2.2 Regulatory Guidelines and Derivations

PAH soil quality guidelines derive from tolerable daily intake values generated through rodent carcinogenesis studies like Culp et al. (1998), as the promary endpoint of concern by government regulatory agencies in human exposure to PAHs is cancer. This study exposed mice to a range of BaP doses in food for 2 years and examined tumor formation throughout the animal. From this research, regulatory agencies can develop a risk specific dose (RSD) for BaP after converting the animal data to a human equivalent dose. RSDs are derived specifically for non-threshold compounds and use a mathematical model to extrapolate the dose response curve to a negligible risk level. The extrapolation is necessary to move from doses that cause a statistically significant increase in tumor formation in laboratory animals to an acceptable

increase in human risk. From this extrapolation, doses representing an increased cancer risk of 10^{-5}, 10^{-6}, or 10^{-7} can be determined from the 95% upper confidence interval of the line. These RSDs are then used to derive soil guidelines that represent increases in cancer risk using default estimations of human soil exposure, absorption from soil, and body weight (Equation 2-1) (CCME, 2008):

$$SQG_{HH} = \frac{RSD \times SF \times BW}{[(AF_G \times SIR) + (AF_L \times IR_S) + (AF_S \times SR)]} + BSC \qquad \text{Equation 2-1}$$

Where:

SQG_{HH} = soil quality guideline for human health

RSD = risk specific dose (mg kg-day-1)

SF = soil allocation factor (unitless)

BW = body weight (kg)

AF_G = relative gastrointestinal absorption factor (bioavailability, unitless, default 100%)

SIR = soil ingestion rate (kg day-1)

AF_L = lung absorption factor (unitless, default 100%)

IR_S = soil inhalation rate (kg day-1)

AF_S = skin absorption factor (unitless, default 34%)

SR = soil dermal contact rate (kg day-1)

BSC = background soil concentration

This equation factors in the three common exposure routes for soil – ingestion, inhalation, and dermal. Risk assessment generally expects oral exposure to provide a significant human exposure to PAHs in soil as the gastrointestinal absorption factor is 100%, especially compared to the default dermal absorption factor of 34%. Oral exposure to soil is also much larger than inhalational exposure to soil-derived particles. The default soil ingestion rate for adults is 20 mg

day^{-1} while toddlers ingest an estimated 80 mg day^{-1}, while adults and toddlers are assumed to inhale 12 µg day^{-1} and 7 µg day^{-1} respectively (CCME, 2008).

Contaminated soil typically contains mixtures of PAHs, which makes toxicity assessment of environmental exposures difficult, especially as the various compounds have different toxic potencies. Toxic equivalency factors are applied to the various compound concentrations to relate the toxicity to BaP, and the results summed to derive a BaP equivalent concentration (CCME, 2008). This is assumed to be representative of the toxicity of the mixture. However, only a limited number of compounds have toxicity equivalency factors available, so their use potentially underestimates the toxicity of a mixture. Equivalency factors also do not consider the presence of other carcinogenic compounds or carcinogenic promoters in the mixture which would affect equivalency factor accuracy (CCME, 2010). Studies assessing the certainty of equivalency factors have found that although equivalency factors accurately represent carcinogenic risk in some cases, in addition to underestimation of risk, they may also overestimate carcinogenic risk for less potent carcinogens (Schneider et al., 2002, CCME, 2010). Furthermore, only parent compounds currently have toxicity equivalency factors, which may exclude the potential toxicity of substituted compounds (Bostrom et al., 2002).

2.3 Human Exposure to PAHs

2.3.1 Incidental Exposure to PAHs in Soil

Soil represents a significant source of PAH exposure in humans. Humans can be exposed to impacted soil through oral, dermal, and inhalation contact, although it is assumed that the most important route of exposure to PAH impacted soil is through the oral route by incidental hand-mouth transfer of soil. Toddlers are considered the most sensitive to PAH exposure in soil as they consume the greatest total mass of soil per day, which translates to a higher dose normalized to body weight as toddlers have a much smaller body weight than adults. Stanek and Calabrese

(1995) assessed the total amount of soil ingested in a day by different age groups by analyzing trace minerals in feces, and determined that children consumed approximately 200 mg soil per day over an 8 day period, which represented the upper 95% confidence interval of the estimate. Single day soil ingestion estimates were less than 15 mg per day for 50% of the study subjects, while the 8 day mean was 45 mg per day or less for 50% of the study subjects (Stanek and Calabrese, 1995). Additionally, these authors assumed that all ingested soil came from the outdoors, which discounts potential indoor sources. Thus, the Canadian Council of Ministers of the Environment (CCME) assumes a default soil consumption value of 20 mg per day for adults and 80 mg per day for toddlers, which accounts for winter seasons when snow cover limits soil accessibility, as well as the uncertainty associated with the derivation of these estimates (CCME, 2006).

Humans typically do not ingest bulk soil in incidental oral exposure scenarios, instead, they ingest smaller size fractions that adhere to hands. The smaller size fractions contain more clay and silt particles which have larger surface area than bulk soils (Ruby and Lowney, 2012). Finer particle size fractions also have enriched organic matter content, which sorbs PAHs. Previous work has demonstrated that the median particle size adhering to hands is between 34 and 40 µm (Yamamoto et al., 2006, Siciliano et al., 2009), and PAHs can be enriched in this size fraction (Siciliano et al., 2010). Although PAH enrichment also occurs in smaller size fractions, the increase in organic carbon present in the smaller size fraction sorbs PAHs and in some cases inhibits their release in gastrointestinal fluid, resulting in decreased bioaccessibility (Siciliano et al., 2010).

Although people generally incidentally ingest soil, some, particularly children, participate in pica behavior. Pica behavior is described as the compulsive ingestion of non-food items,

which can include paper, chalk, or soil. People exhibiting soil pica behavior represent the most sensitive receptors to impacted soil. These people can consume as much as 13 g of soil in a day (Calabrese et al., 1991).

2.3.2 Gastrointestinal Absorption of PAHs

PAH uptake following oral exposure is complicated, with the lipid content of the diet (Stavric and Klassen, 1994), lipid type (Laher et al., 1983, Walker and Ramesh, 2009), the presence of bile (Laher and Barrowman, 1987, Rahman et al., 1986), chylomicrons (Grubbs and Moon, 1973, Laher et al., 1984), and bioaccessibility of PAHs all potentially having an effect on the absorption of PAHs from the gastrointestinal tract. The presence of lipids can increase bioavailability of PAHs (Laher et al., 1984, Stavric and Klassen, 1994), and PAH absorption appears to occur concurrently with lipids (Laurent et al., 2001); however, changing the amount of lipids present does not appear to significantly affect bioavailability (Laher et al., 1984). Reported PAH bioavailability ranges from 5.5% to 100% depending on exposure media, model species, or bioavailability method, with the majority of values reported to exceed 70% (Ramesh et al., 2004).

Movement of PAHs from soil into gastrointestinal fluid is thought to be the first factor that will affect the gastrointestinal absorption of PAHs. Bioaccessibility refers to the fraction of ingested PAHs that enter the gastrointestinal fluid and have the potential to be absorbed, and is often measured with *in vitro* digesters (James et al., 2011, Harris et al., 2013). Bioaccessibility is often thought a conservative bioavailability estimate as what releases into gastrointestinal fluid may not be absorbed. Estimates of bioaccessibility have been validated as appropriate surrogates for bioavailability for lead and arsenic, and a number of lab groups have attempted to validate an *in vitro* model for PAHs (Casteel et al., 1997, Rodriguez et al., 2003, James et al., 2011, Harris et

al., 2013, Duan et al., 2014). Unfortunately, an appropriate bioaccessibility model has yet to be proposed for PAHs (Harris et al., 2013).

Influences of different fat types on bioavailability have also been reported for PAHs (Laher et al., 1983, Walker and Ramesh, 2009). Laher et al. (1983) examined the long and medium chain triglyceride influence on PAH uptake, and found long chain triglycerides had a greater influence on PAH uptake. The authors attributed this to the likelihood that long chain lipid micelles have a larger capacity for lipophilic molecules after lipolysis (Laher et al., 1983). Saturated fat appeared to have an influence on fluoranthene bioavailability at higher doses when compared with unsaturated fats (Walker and Ramesh, 2009). A saturated fat dose vehicle led to a higher bioavailability of fluoranthene at higher doses than a dose vehicle of unsaturated fat (Walker and Ramesh, 2009). The authors attributed this to delays in fluoranthene elimination from the body when ingested with saturated fats, perhaps due to saturated fats entering systemic circulation through absorption into the portal venous system, while unsaturated fats were incorporated into chylomicrons and transported into lymphatic or peripheral circulation (Walker and Ramesh, 2009).

Although lipid content in the diet can affect PAH uptake into intestinal cells, work by Laher et al. (1984) suggests that enterocytes will separate lipids from PAHs following absorption. In this study, they found that chylomicrons appeared in lymph for approximately 7 hours following exposure, while lymph only contained BaP for 2 hours after dosing (Laher et al., 1984). The authors interpreted these results as chylomicrons assisting BaP absorption into systemic circulation initially, but increase in lipid transport into lymph does not equate to increased BaP transport (Laher et al., 1984).

Enterocytes form chylomicrons following lipid transport into gastrointestinal cells, and chylomicrons have been hypothesized to lead to the transport of PAHs in the lymphatic system, effectively bypassing the first pass effect (Grubbs and Moon, 1973, Laher et al., 1984, Busbee et al., 1990). Chylomicrons form in the endoplasmic reticulum, and are then released by enterocytes through exocytosis (Tso and Balint, 1986). Some studies have measured PAHs in lymph following oral exposure, and suggest chylomicrons are the predominant form of PAH transport into blood as a result of the lipophilic nature of PAHs (Grubbs and Moon, 1973, Busbee et al., 1990). However, other studies have shown PAH transport through the lymphatic system accounts for only a small fraction of PAHs absorbed into the organism (Laher et al., 1984, Laher and Barrowman, 1987). These studies used bile and lymph cannulated rats to determine the presence of radiolabeled compound following oral exposure. A more recent study confirmed BaP is predominantly found in bile rather than lymph following oral exposure (Kim et al., 2012).

The presence of bile significantly increases bioavailability of PAHs, particularly larger compounds (Rahman et al., 1986). It is generally accepted that an unstirred water layer in the gastrointestinal tract acts as an absorption barrier to lipophilic compounds (Smithson et al., 1981, Kindel et al., 2010). However, bile salts are an important step in lipophilic compound absorption as bile salts emulsify the lipids and form micelles to allow them to cross the unstirred water layer (Hussain, 2014). Once across the unstirred water layer, passive and active transport convey the micelles into the enterocyte (Hussain, 2014). Larger PAHs are likely affected to a greater extent by bile salts as these compounds have a lower water solubility than smaller PAHs (Rahman et al., 1986).

2.3.3 Distribution of PAHs

Following absorption, PAHs distribute throughout the body via systemic circulation. Biological half-lives of PAHs are considered quite short compared to other environmental contaminants, generally reported as less than 30 hours (Li et al., 2012). This likely results from the rapid metabolism of PAHs once they have entered an organism (discussed in more detail below), and as such, PAHs generally do not bioaccumulate in mammals (ATSDR, 1995). However, although PAHs are eliminated rapidly from the body, they do demonstrate distribution patterns in an organism. Immediately following IV exposure, highly perfused tissues like lung and liver, and to a lesser extent heart and kidney, exhibit elevated PAH levels (Moir et al., 1998). These tissues also see a rapid decrease in PAH levels following the initial exposure with the exception of the lung. These results were attributed to the lung receiving 100% of cardiac output, and is thus the first organ exposed to intravenous exposure (Moir et al., 1998). The lung has metabolic capacity, but it has less metabolic capacity than the liver (Litterst et al., 1975).

Adipose tissue sees a slower, but distinct, distribution phase following IV PAH exposure (Moir et al., 1998). Peak PAH concentrations in adipose tissue were observed between 2 and 5 hours post-exposure, and the peak level appeared to occur later in animals receiving larger doses of benzo[a]pyrene (Moir et al., 1998). PAHs are lipophilic compounds, and as such, would preferentially partition into lipid tissue.

Exposure route appears to affect tissue distribution of PAHs, with first pass metabolism affecting levels of PAHs going to tissues following oral exposure. Withey et al. (1991) exposed rats to pyrene via both oral and IV routes, and observed elevated pyrene levels in fat and liver tissue for both routes. However, pyrene levels in fat were higher in IV exposures when compared to oral exposure, while liver tissue had higher levels in oral exposures compared to IV (Withey et al., 1991). This may be attributed to the liver receiving the initial pyrene dose in oral exposure.

As PAHs are lipophilic entities, the possibility exists that the compounds will partition to cells in whole blood, rather than stay solubilized in plasma. This may affect PAH analysis in blood as analysis is often completed on plasma. If significant partitioning to blood cells occurs, plasma analysis may underestimate circulating PAH concentration. Plasma lipoproteins, in particular, have been hypothesized to bind to hydrocarbons when present in blood (Avigan, 1959). However, *in vitro* plasma partitioning studies conclude that PAH partitioning to lipoproteins likely does not constitute a large portion of PAHs in circulation (Shu and Nichols, 1979). Indeed, a more recent study concluded PAHs enrich in plasma, although this may represent a concentration effect after removing non-plasma components from the blood (Pleil et al., 2010).

2.3.4 PAH Metabolism and Toxic effects

PAHs undergo rapid metabolism in the body, particularly by the cytochrome P450 (P450) family of enzymes. The end goal of metabolic transformation of PAHs is the creation of polar compounds that can then be excreted from the body. In general, enzymes add a more polar group to the PAH, and these groups can range in complexity from a hydroxyl group (Phase I metabolism) to complicated compounds like glutathione (Phase II conjugation). Parent PAHs are toxicologically inert, and only after Phase I metabolism do PAHs become toxic.

Phase II conjugation of PAH metabolites acts primarily as a detoxification reaction in PAH metabolism and excretion. Parent PAHs typically undergo Phase I metabolism to dihydrodiols and phenols and Phase II conjugation occurs following the initial metabolic reaction. Phase II conjugation involves a group of enzymes attaching complex molecules to lipophilic compounds like PAHs prior to excretion in order to increase compound solubility (Ramesh et al., 2004). Phase II conjugates produced in the liver are excreted in the bile and enter the gastrointestinal tract. Once in the gastrointestinal tract, enzymes present in intestinal flora or

the intestine can hydrolyze the metabolites and release parent compound, which can then be re-absorbed (vanSchooten et al., 1997). This phenomenon, termed enterohepatic cycling, can prolong exposure to PAHs and lead to longer apparent compound half-lives (Ramesh et al., 2004). Additionally, the presence of parent compound in the feces indicates that PAH absorption from soil is less than 100%

The most well-known toxic effect of PAHs in humans remains carcinogenesis. PAHs can act both as tumor initiators as well as promoters. PAH tumor initiation is caused by the metabolic activation of the parent compound to a reactive species during Phase I metabolism. Cytochrome P450 (P450) enzymes, particularly CYP1A1, oxidize parent PAHs to increase the water solubility of the compound and likelihood of excretion, and this oxidation can be in the form of epoxide rings (Ramesh et al., 2004). The enzyme epoxide hydrolase breaks these epoxide rings to form two hydroxyl groups. The reactive species of PAHs form when epoxide hydrolase cannot break an epoxide bond and the epoxide reacts with and covalently binds to DNA or proteins in the cell. This generally occurs in epoxides located at bay regions of PAHs, where the epoxide hydrolase cannot react with the epoxide. The most potent metabolite of PAH exposure is thought to be the 7, 8-dihydrodiol-9, 10-epoxide derivative of BaP (Helleburg et al., 2001).

PAH carcinogenesis typically occurs at the site of exposure; that is, lung cancer in inhalation exposures, skin cancer in dermal exposures, and cancer of the gastrointestinal tract in oral exposures. This likely results from the ubiquitous distribution of enzymes in the body attempting to eliminate the compound from the site of exposure, and subsequently causing metabolic activation (ATSDR, 1995). The active metabolites of BaP are very reactive, and as such, will not travel widely throughout the body (Galvan et al., 2005). These reactive metabolites

bind to nearby nucleophiles like DNA which forms adducts, interfering with the function of the molecule.

In addition to DNA adducts, PAHs may also induce carcinogenesis through the production of reactive oxygen species (ROS). PAHs produce ROS through parent compound conversion to quinones by dihydrodiol dehydrogenase (Penning et al., 1996). These quinones can then enter redox cycles which produce ROS, and the cyclical nature of ROS production means that just a few quinones can produce a large amount of ROS (Flowers-Geary et al., 1993). ROS like hydroxyl radicals and superoxide anions can lead to carcinogenesis by attacking DNA (Cavalieri and Rogan, 1995).

Carcinogenesis promotion by PAHs occurs through the activation of the aryl hydrocarbon receptor (AhR). The AhR is typically considered in conjunction with its role in the induction of various P450 enzymes in response to activation. However, other research demonstrates the AhR also plays a role in the upregulation of genes involved in the growth and differentiation of cells (Bostrom et al., 2002). Some PAHs, including BaP, agonize the AhR, meaning that they will activate the AhR, and initiate the downstream responses.

Activation of the AhR may also lead to immunotoxic responses, in general through immunosuppression (Buters et al., 2003). Like with carcinogenesis, immunotoxicity requires metabolically activated PAHs to cause an effect. Immunotoxic effects such as bone marrow damage and decreases in spleen and thymus weights have been observed in organisms exposed to PAHs (Miyata et al., 2001). Bone marrow damage initiation is considered to result from oxidative damage from reactive PAH metabolites, and immunotoxicity occurs as a result of the subsequent depletion of lymphocytes (Galvan et al., 2005). Spleen and thymus are the production site for major participants in an organism's immune response, namely T-cells (thymus) and

immunoglobin M (spleen) (Dean et al., 1985). PAHs have also been demonstrated to suppress the immune system through induction of macrophage apoptosis (van Grevenynghe et al., 2004), suppression of B-cell proliferation (Davis and Burchiel, 1992), and immunoglobin G production (Szczeklik et al., 1994).

In addition to immunotoxic effects, developmental effects, in particular cardiovascular toxicity initiated in early life stages of fish, have been observed in fish embryos exposed to PAHs (Incardona et al., 2005). These effects look similar to those observed with dioxin-like compounds, and as such, are often attributed to AhR mediated activity (Billiard et al., 2006). Cardiovascular effects of AhR mediated toxicity is not clear, but appears related to oxidative stress and growth factor modulation (Goldstone and Stegeman, 2006). The mechanism of action for AhR mediated cardiovascular effects has been derived from dioxin studies, as there appear to be confounding factors in PAH initiation of toxicity. For instance, some PAHs demonstrate cardiac effects similar to those caused by dioxin-like compounds, but the degree of toxicity for PAHs is not correlated to the strength of AhR binding, which is a key factor in dioxin toxicity. As an example, chrysene is a strong inducer of CYP1A1, but has very little cardiac effect (Billiard et al., 2008). Other developmental effects like edema, spinal defects, and hemorrhages in fish have no clear mode of action.

The general toxic effect for PAHs with little to no AhR affinity is considered to be cellular narcosis. Narcosis is characterized by the disruption of the phospholipid bilayer in cells, and occurs when a toxic threshold concentration is reached in an organism. However, more recent research has demonstrated that PAHs do not neatly fit in the narcotic model as PAH mixtures can have synergistic, rather than additive effects as described by the narcotic model (Billiard et al., 2008).

In addition to narcosis, naphthalene can cause hemolytic anemia in humans, and causes respiratory tumors in mice. Hemolytic anemia is a condition that is characterized by lack of oxygen reaching tissues as a result of the destruction of red blood cells. The hemolytic properties of naphthalene are attributed to deficiencies in the glucose-6-phosphate dehydrogenase (G6PD) enzyme (ATSDR, 2005). G6PD catalyzes the conversion of NADP to NADPH, which in turn enables cells to counteract oxidative stress. Red blood cells do not have other methods to combat oxidative stress, and therefore rely on G6PD for defence against insult by oxidative stress (Callellini and Fiorelli, 2008). Thus, G6PD deficiency will lead to greater chance of hemolytic anemia. Like other PAHs, naphthalene is carcinogenic after metabolic activation; however, naphthalene is metabolized by the CYP2F family of enzymes, which are not AhR mediated (Bogen et al., 2008).

Recently, other toxic effects of AhR independent action have been observed for PAHs. For example, cardiovascular effects attributed to AhR independent toxicity of three ringed compounds have been described in developing fish (Incardona et al., 2005). This study exposed wild-type, as well as AhR1, AhR2, and CYP1A knockdown zebrafish embryos to tricyclic PAHs, which are a major constituent of crude oil. These embryos exhibited increased cardiac and morphological changes in the knockdown fish compared with the wild-type controls, which the authors attributed to protective action of the AhR. The mechanism of action for these effects is preliminary, but effects were attributed to blockages of cardiac ion channels. Other research has not been able to reproduce these results; however, this additional research has been completed with 4- and 5-ring PAHs, which have much greater AhR affinity than tricyclic compounds (Billiard et al., 2006). Indeed, Incardona et al. (2005) determined pyrene, a 4-ring PAH

demonstrated classic AhR mediated toxicity, which was suppressed through the knockdown of AhR and CYP1A activity.

2.4 Bioavailability

Using the pharmacokinetic definition, bioavailability refers to the fraction of parent compound which reaches systemic circulation unchanged following an exposure (Chereson, 2002). This definition is preferred as it assumes equilibrium between blood and site of action for a pharmaceutical compound. However, most toxicological exposure studies use a more general definition of bioavailability, namely the fraction of compound the organism internalizes following an oral exposure (Semple et al., 2003). This accounts for compounds like PAHs which cause toxicity through the production of daughter compounds rather than via parent compound, as well as the chance of toxicity occurring in the gastrointestinal tract or liver prior to compounds entering systemic circulation.

If using the more general toxicological definition, PAH bioavailability has been assessed in a number of ways: urinary metabolite formation (Viau et al., 1995), fecal elimination of parent compound (Hecht et al., 1979, Stroo et al., 2000, Juhasz et al., 2014), parent compound and metabolite analysis in blood via radiolabeled compound (Foth et al., 1988, Laurent et al., 2001), and analysis of only parent compounds in the blood (James et al., 2011, Duan et al., 2014). There are advantages and disadvantages to all these methods. Urinary metabolite analysis is a good method in humans due to the non-invasive sample method, and the ability to collect samples frequently. However, analysis is limited to known metabolites, and a knowledge of metabolite ratios compared to parent compound and an understanding of how much metabolite is transferred to the urine, a daunting process as parent PAHs can metabolize into a number of metabolites. Additionally, this method assumes all metabolites are excreted in the urine, which may not be accurate. Thus, urinary metabolites may vastly underestimate bioavailability, and PAH

metabolites in urine are perhaps better used as a biomarker of exposure, rather than an estimate of bioavailability (Bouchard et al., 2002, Viau et al., 2002).

Fecal elimination of parent PAHs also uses a non-invasive method of sample collection, and provides a very conservative bioavailability estimate. Feces are collected for a set time following oral exposure, and total parent compound measured in the feces subtracted from total dose, with the remainder assumed bioavailable (Stroo et al., 2000, Juhasz et al., 2014). Fecal elimination represents a very conservative bioavailability estimate as it does not account for PAHs which may be metabolized by gastrointestinal flora (Stroo et al., 2000), nor does it include compounds converting to inert metabolites which will not cause toxic effects.

Radio-labeled PAHs provide a conservative bioavailability estimate as well, as this method accounts for metabolite formation. In this method, media is spiked with ^{14}C-labeled PAHs and radiometric counts in blood are used to estimate the quantity of absorbed compound (Laurent et al., 2001). This method is preferred by some as it does not differentiate between parent compounds and metabolites. As PAHs metabolically activate, analyzing radio-labeled compounds may more accurately represent what is toxicologically relevant in an organism. However, this method is limited to spiked media, which makes it less comparable to environmental impacted sites as the sites may differ in weathering patterns and PAH composition. Finally, blood sample collection is more invasive than collecting feces and urine.

Parent compound analysis in systemic circulation represents the least conservative method of assessing PAH bioavailability aside from urinary metabolites. Parent compound analysis is not limited to spiked media exposures, and is simpler than including metabolites. Additionally, research suggests that circulating metabolites are no longer reactive, and thus do

not cause systemic toxic effects, and parent compound analysis is sufficient to assess bioavailability (Uno et al., 2004, Galvan et al., 2005).

When analyzing PAH parent compound in systemic circulation, bioavailability is typically calculated from a single exposure to a compound (Hecht et al., 1979, Withey et al., 1991, Moir et al., 1998, Cavret et al., 2003). However, humans are likely to be exposed repeatedly to a mixture of multiple compounds in the environment, including background exposure to PAHs in food (Yebra-Pimentel et al., 2015). The European Food Safety Authority (EFSA) estimated the median dietary exposure to BaP ranged from 235 ng day^{-1} to 389 ng day^{-1} for mean and high dietary consumers, respectively (EFSA 2008). Although this exposure does not often cause a noticeable physiological effect, it still may induce biochemical changes in the body. A common change induced by repeated contaminant exposure is the induction of P450 enzyme activity through the activation of the AhR (Billiard et al., 2006). If enzyme activity increases, more PAHs will be metabolized and excreted. Since PAH bioavailability following oral exposure is influenced by uptake and metabolism in the liver (first pass effect), repeated exposure may increase PAH metabolism and decrease bioavailability if measured as parent compound entering the body.

2.5 Biomarkers

Biomarkers are defined as a cellular or biochemical change or molecular characteristic which we can measure in an organism following a xenobiotic exposure, with the expectation that biomarkers should classify the exposure status of an individual (Watson and Mutti, 2004). These biomarkers typically divide into two categories: biomarkers of exposure, and biomarkers of effect. Biomarkers of exposure are generally defined as reversible changes in cellular components as a result of exposure to a xenobiotic compound, while biomarkers of effect refer to molecular and cellular changes that directly correlate with toxic effects of a xenobiotic exposure.

Common biomarkers of exposure include increased gene expression and enzyme induction, while biomarkers of effect can include micronuclei formation and chromosomal alterations. However, a number compounds may have common biomarkers of effect, so it becomes difficult to specify the compound causing the effects in question. Therefore, assessments of the impact of environmental contaminants prefer biomarkers of exposure as they may be more specific to compound exposures (Silins and Hogberg, 2011). The line between biomarkers of exposure and biomarkers of effect is not distinct, and as such, some overlap exists between these categories. For example, the formation of DNA adducts can be classified as a biomarker of either exposure or effect; DNA adduct repair may occur through nucleotide excision repair, but there also exists a direct link between DNA adduct formation and carcinogenesis.

2.5.1 Biomarkers of Exposure

Urinary metabolite analysis is often described as a PAH biomarker of exposure (Viau et al., 1995, Strickland et al., 1996, Vu-duc and Lafontaine, 1999, Strickland and Kang, 1999). Commonly, 1-hydroxypyrene (1-OHP) is measured in urine to relate internal exposure to PAHs, particularly pyrene, to a received dose (Strickland and Kang, 1999). Urinary metabolites are often used in human biomonitoring studies as sample collection is non-invasive and there is good relationship between received dose and metabolite presence in the urine (Viau et al., 1995).

Activation of the AhR generally drives PAH biomarker of exposure formation, and PAHs can be divided into two groups: those that activate the AhR and cause biological effects through its activation, and those that do not activate the AhR and cause toxicity through different modes of action. Molecular structure generally divides these groups, as PAHs with two or three aromatic rings have been demonstrated to have very weak affinity for the AhR. Conversely, larger molecules demonstrate much higher affinity for the AhR (Bosveld et al., 2002). The

difference in affinity for the AhR was attributed to smaller compounds not having the capacity to bind to the AhR.

Activation of the AhR leads to the upregulation of a number of genes, in particular the CYP1A family. This upregulation occurs when the PAH enters the cell, binds to the AhR, translocates into the nucleus, forms a heterodimer with AhR nuclear traslocator, binds to the xenobiotic response elements of the gene, and induces the production of CYP1A mRNA (Denison and Nagy, 2003). Following production, ribosomes transcribe mRNA into the functional enzyme. It is important to note that increases in gene expression do not always correlate with increases in enzyme activity (Budinsky et al., 2010). Common biomarkers of exposure assessing gene induction and enzyme function include analyzing the fold-change of mRNA of different genes (Messina et al., 2009, Nannelli et al., 2009), or assessing the capability of cells to convert a known enzyme substrate into daughter products (Roos et al., 2002, Zamaratskaia and Zlabek, 2009).

2.5.2 Biomarkers of Effect

Common PAH biomarkers of effect include markers of genomic damage like DNA adducts (Uno et al., 2004, Ramesh and Knuckles, 2006) and micronuclei formation (Zhong et al., 1995, Dertinger et al., 2006, Iarmarcovai et al., 2008), as well as measures of cellular oxidative stress (Penning et al., 1996, Ramesh et al., 2004). A good biomarker of effect for PAHs includes genomic damage as the toxic endpoint of concern for PAHs is carcinogenesis, and DNA adducts are considered precursors to cancer formation. As mentioned previously, the most potent PAH carcinogen remains BPDE, the diol-epoxide metabolite of BaP, and as such, is the most common analyte for DNA adduct quantification following PAH exposure.

Micronuclei form when DNA replication becomes corrupted during the cell cycle as a result of genetic mutations, and is considered a biomarker of genomic instability (Iarmarcovai et

al., 2008). Micronuclei consist of small pieces of DNA not incorporated into the larger nucleus and form their own nuclear envelope. Micronuclei can form in all cell types, but are primarily analyzed in peripheral reticulocytes (immature erythrocytes) as erythrocytes expel their nucleus as part of the maturation process. Thus, if analysis detects genetic material in these cells, micronuclei are present. Studies use reticulocytes as an indicator of acute genomic damage as reticulocytes represent the most recently formed fraction of erythrocytes (Dertinger et al., 2011). This is important in laboratory studies, as it allows for differentiation from damage occurring before exposure. Additionally, micronucleated reticulocytes indicate systemic toxicity, while DNA adduct and oxidative stress analysis may be completed in liver and gastrointestinal tissue. These tissues represent initial exposure to PAHs following oral dosing.

Oxidative stress indicates the formation of ROS in cells following PAH exposure. As mentioned previously, PAHs stimulation of ROS can lead to toxicity (Penning et al., 1996). ROS generation is a more general form of toxicity which may not be specific to PAHs. However, oxidative stress analysis will incorporate toxic effects from all PAHs in a mixture, and are not limited to only BaP. A common method of measuring oxidative stress includes protein carbonyl analysis that assesses the addition of carbonyl groups to cellular proteins (Dalle-Donne et al., 2003). This method of oxidative stress analysis may be preferred as protein carbonyls form relatively quickly following exposure to compounds inducing oxidative stress, and carbonylated proteins remain relatively stable in the cell (Dalle-Donne et al., 2003).

2.6 Connections between Bioavailability and Regulatory Guidelines

Regulatory guidelines for PAHs have been derived from studies exposing animals to benzo[a]pyrene in food. As discussed previously, dose media affects bioavailability of PAHs; therefore, the relationship between soil and food bioavailability (referred to as relative bioavailability, or the bioavailability of PAHs from soil relative to that from food) should be

determined. Although bioavailability varies highly for both media, it is generally thought that PAH soil bioavailability is lower than that from food (Ramesh et al., 2004). Thus, guidelines derived from food exposure studies likely overestimate carcinogenic risk of soil-bound PAHs.

The risk characterization step of risk assessment consists of comparing a chemical exposure estimate to the appropriate guideline to determine the potential risk of the exposure. Risk characterization is quantified by two values: the hazard quotient for threshold compounds and incremental lifetime cancer risk (ILCR) estimate used for non-threshold contaminants (Health Canada, 2007). The hazard quotient is calculated by dividing the estimated exposure by the compound guideline. If this value is less than 0.2, the risk to the exposure is considered negligible. The threshold value is set at 0.2 to account for exposure to the compound from other sources. ILCR calculation involves multiplying the estimated exposure by a cancer slope factor (CSF) to calculate the estimated increase in cancer risk of the exposure (Health Canada, 2007). Both risk characterization methods assume that external doses and absorption following exposure have a linear relationship, which may not be the case (Waschek et al., 1984).

Hazard quotients and ILCR are not constant between exposure groups, and can change depending on a number of factors. The CSF and compound guideline remain the same regardless of exposure group; however, estimated exposure may change. Age group has a large effect on estimated soil exposure as soil ingestion rates are much higher for toddlers compared with other age groups – Health Canada estimates toddlers ingest 80 mg of soil per day as opposed to 20 mg per day for other age groups (Health Canada, 2007). In addition to higher soil ingestion rates, toddlers also have lower body weights than older age groups. Thus, as exposure is normalized to body weight, toddlers receive a higher dose following soil ingestion. In addition to receiving a larger dose than older life stages, juvenile organisms have also been demonstrated to be more

sensitive to toxic insult than later life stages (Atterberry et al., 1997). This likely results from various systems, including metabolic and clearance functions, still undergoing development in juvenile organisms (EMEA, 2005, Hu, 2015).

2.7 Soil Characteristics

Bioavailability of PAHs is widely accepted to vary between different media. In addition, soil properties can influence PAH bioavailability from soils. These soil properties include particle size distribution, organic matter content, and metal concentration in the soil (James et al., 2011, Duan et al., 2014).

Smaller particle size fractions in soil have been demonstrated to affect PAH concentration in soil, as well as oral bioavailability (Siciliano et al., 2010). Decrease in particle size will increase surface area-to-mass ratios of the soil, which may increase adsorption sites for PAHs. However, particle size distribution also affects the organic matter content of the soils and thus influences PAH bioavailability from soil. Smaller particle size fractions have enriched organic matter content compared to bulk soils, which acts as a sorption agent for lipophilic compounds like PAHs (Pernot et al., 2013). Additionally, work by Pernot et al. (2013) suggests that adsorption to organic matter inhibits PAH degradation in soil, rather than surface area of silt particles.

Organic matter may influence PAH bioavailability through limiting bioaccessibility in the gastrointestinal tract as organic matter is considered analogous to octanol in soil (Mackay, 1979). PAHs will preferentially partition to organic matter in the soil due to their lipophilic nature, and as such, become less likely to desorb in gastrointestinal fluids. Soil PAH concentration as well as bioaccessibility and bioavailability correlate with soil organic matter content between similar soils (Carmo et al., 2000, Duan et al., 2014). However, as soil organic matter is not homologous across various soil types, relationships between different soils becomes more difficult (Duan et

al., 2014). Celis et al. (2006) and Pernot et al. (2013) suggest organic matter associated with smaller size fraction has stronger adsorption capacity when compared to bulk soil.

Metals present in the soil as co-contaminants with PAHs may influence bioavailability through affecting the absorption and metabolism of PAHs. Unlike particle size and organic matter content, metals may influence bioavailability through physiological measures like enzyme induction. Cu has been shown to inhibit CYP1A1 activity (Anwar-Mohamed et al., 2009, Korashy and El-Kadi, 2004), which may alter PAH metabolism during the first pass effect. This may affect PAH transfer into systemic circulation, as well as the concentration of circulating parent compound. However, increases in metabolism may also increase the likelihood of toxic metabolite production. Additionally, Cu may increase reactive oxygen species (ROS) and heme oxygenase-1 production, as well as lead to a decrease in cellular glutathione content (Korashy and El-Kadi, 2004, Anwar-Mohamed et al., 2009), and inhibit UDP-glucuronosyltransferase (UGT) activity (Grancharov et al., 2001). Therefore, Cu content in soil may affect the toxicokinetics of PAHs by altering the enzymes responsible for PAH metabolism.

2.8 Animal Model

Toxicological studies of human exposure generally take place in one of four animal species: rodents (Culp et al., 1998, Juhasz et al., 2014), swine (Casteel et al., 1997, Duan et al., 2014), non-human primates (Freeman et al., 1995), and humans (Hecht et al., 1979, Viau et al., 1995). Although humans provide the best model for human exposure, ethics approval remains difficult to obtain for toxicological studies and also to collect samples other than blood, urine, feces, saliva, or hair. Cultured human cell lines are available, but lack the complexity of a complete organism. Non-human primates are the next closest animal model to humans, but it remains difficult to obtain ethics approval to conduct studies in primates. Rodents continue to be the most common animal model for human exposure studies, and are widely used because they

are well characterized, easy to handle, and generally accepted as an appropriate model. However, many differences exist between rodents and humans, including gastrointestinal physiology (Patterson et al., 2008), AhR affinity (Flaveny et al., 2009), and P450 enzyme distribution in tissues (Martignoni et al., 2006) that may make rodents less appropriate for modeling human oral exposure to PAHs.

Swine, on the other hand, may make a more appropriate model for human oral exposure as they have very similar gastrointestinal physiology to humans in both intestinal morphology and cellular structure (Patterson et al., 2008). Swine have the same intestinal divisions as humans, and the intestinal length in swine is in the same proportion to body weight as humans (Patterson et al., 2008). Swine also have similar enterocyte types to humans, and similar intestinal villi structure (Miller and Ullrey, 1987). The gastric secretions of the two species are also similar, in addition to gastric transit times (Cooper et al., 1997). Additionally, swine require nutrients in very similar quantities to humans, which means that swine may absorb nutrients and other compounds to a similar extent as humans (Patterson et al., 2008). Thus, absorption of compounds following oral exposure likely occurs in a similar fashion in swine and in humans.

In addition to anatomical and cellular structure, swine also have very similar enzymatic systems and tissue distribution to humans, in particular P450 enzymes. Swine CYP1A1, 1A2, and 1B1 enzymes, the enzymes most involved with PAH metabolism, share more than 80% amino acid similarity with human counterparts (Puccinelli et al., 2011). However, there are some differences in substrate specificities between human and swine P450 enzymes. CYP1A1 and 1A2 are induced to a lower extent in swine, and showed some substrate differences (Chirulli et al., 2007). CYP1B1 mRNA was detected in swine tissue following induction with β-naphthoflavone, but no 17-β estradiol-4-hydroxylase was observed, a marker of human CYP1B1

activity (Chirulli et al., 2007). Although differences exist between swine and human P450 enzymes, the enzymes remain more closely related than rodent and human analogues (Chirulli et al., 2007). As for tissue distribution, human and swine CYP1A1 resides primarily in intestinal cells when compared to liver, while CYP1A2 occurs in much higher levels in liver for both species (Puccinelli et al., 2011). Finally, CYP1B1 is considered to be primarily extrahepatic in both swine and humans, and is mostly expressed in steroidogenic organs like the spleen (Nannelli et al., 2009).

Human exposure studies generally use one of two species of swine – minipigs and conventional swine. Both species have advantages and disadvantages to their use as models of human exposure. Minipigs are smaller and therefore easier to house and handle than conventional swine. Minipigs are also specifically bred for research, which means they have less genetic diversity and thus less variability in research results than conventional swine (Puccinelli et al., 2011). However, humans also demonstrate wide genetic diversity, such that conventional swine may make a better model for exposure studies. Conventional swine are less expensive and easier to source than minipigs as they are available from commercial hog barns. Humans have more similar P450 enzymes to conventional swine than minipigs, and minipigs demonstrate more variability between genders than conventional swine and humans (Puccinelli et al., 2011). Thus, conventional swine were chosen for this study to assess bioavailability from oral exposure to PAHs.

3 THE BIOAVAILABILITY OF POLYCYCLIC AROMATIC HYDROCARBONS FROM DIFFERENT DOSE MEDIA AFTER SINGLE AND SUB-CHRONIC EXPOSURE IN JUVENILE SWINE

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3.1 Publication Fate and Contribution

A version of this chapter was published in Science of the Total Environment (Peters, R. E., Wickstrom, M. & Siciliano, S. D. 2015. The bioavailability of polycyclic aromatic hydrocarbons from different dose media after single and sub-chronic exposure in juvenile swine. *Science of the Total Environment,* 506**,** 308-314). Ms. Peters was involved in the experimental design, animal handling, and lab work, and completed the bulk of the data collection and analysis, and writing for this chapter. The animal work was completed at the Animal Care Unit at the Western College of Veterinary Medicine, University of Saskatchewan, Canada, under Anumal Use Protocol Number 20080153.

3.2 Abstract

Humans are constantly exposed to contaminants in the environment, which may lead to changes in physiological processes by altering enzyme activities that could affect bioavailability. However, bioavailability estimates are typically made from a single exposure to an animal model, which may lead to overestimating bioavailability. This study uses juvenile swine to model human exposure to benzo[a]pyrene (BaP) and anthracene in certified reference material (CRM), spiked soil, spiked food, or spiked corn oil after one and seven days of dosing. Area under the curve (AUC) was calculated after one and seven days of exposure for both BaP and anthracene for each exposure media. Whereas there were significant differences in AUC between different media, there were no significant changes in AUC after sub-chronic exposure to BaP or anthracene. Average BaP bioavailability for CRM, spiked soil, spiked food and corn oil was 71%, 0.72%, 0.03% and 0.97% respectively. Average anthracene bioavailability was 1.7% and 43% for corn oil and CRM respectively. Anthracene was not detected above background in swine exposed to spiked food and spiked soil. Thus, this study indicates that exposure media impacts bioavailability, but there is no statistical evidence that sub-chronic exposure affects systemic exposure.

3.3 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a family of compounds that are common environmental contaminants (CCME, 2008). PAHs are produced through incomplete combustion of organic compounds and are present in fuel oil, and as such, are released into the environment from fires and fuel processing, and humans can be exposed to them through a number of sources (Garcia-Falcon and Simal-Gandara, 2005, Rey-Salgueiro et al., 2008, Rey-Salgueiro et al., 2009). Although PAHs are ubiquitous, they are commonly found in soil due to their physicochemical properties (Semple et al., 2003). PAHs are highly lipophilic substances, and the

high molecular weight compounds are not particularly volatile, thus they will preferentially stay in soil.

Some PAHs are known or suspected animal carcinogens, and as such, have very conservative environmental guidelines for oral exposure (CCME, 2008). These guidelines are derived with the assumption that the absorption from the guideline media is the same as the exposure media used to derive the guideline; however, PAHs have been shown to have a different bioavailability than that of the original media (Koganti et al., 1998). The definition of bioavailability is the fraction of a compound that reaches systemic circulation following exposure, and bioavailability is calculated by dividing the total amount of compound that reached circulation by the dose without correction for dose media. Bioavailability of parent PAHs from animal dosing studies is commonly reported between 80-100% (Ramesh et al., 2004).

PAH uptake after an oral exposure is complicated, with the lipid content of the diet (Stavric and Klassen, 1994), presence of bile (Laher and Barrowman, 1987, Rahman et al., 1986), and chylomicrons (Grubbs and Moon, 1973, Laher et al., 1984) all potentially having an effect on the absorption of PAHs from the gastrointestinal tract. The presence of lipids have been shown to increase bioavailability of PAHs (Stavric and Klassen, 1994), and PAHs appear to be absorbed concurrently with lipids (Laher et al., 1984, Laurent et al., 2001); however, changing the amount of lipids does not appear to affect bioavailability (Laher et al., 1984). The presence of bile in the intestinal tract has been shown to significantly increase bioavailability of PAHs, particularly for more lipophilic compounds (Rahman et al., 1986). Finally, chylomicrons can lead to the transport of PAHs in the lymphatic system, effectively bypassing the first pass effect (Busbee et al. 1990; Grubbs and Moon 1973; Laher et al. 1984); however, other studies have

shown that PAH transport through the lymphatic system accounts for only a small fraction of what is absorbed into the organism (Laher et al., 1984, Laher and Barrowman, 1987).

When calculated using blood AUC values, bioavailability of PAHs is typically calculated from a single exposure to a compound (Hecht et al., 1979, Withey et al., 1991, Moir et al., 1998, Cavret et al., 2003); however humans are more likely to be exposed repeatedly to a mixture of multiple compounds in the environment, including background exposure to PAHs in food (Yebra-Pimentel et al., 2015). Although these compounds do not often cause a noticeable physiological effect, they still may induce changes in the biochemical processes of the body. A common change induced by repeated contaminant exposure is the induction of enzyme activity. Mono-oxygenase enzymes, such as CYP1A1, CYP1A2, CYP1B1, and the CYP2B, 2C and 3A families are critical to the phase I metabolism and excretion of PAHs and other lipophilic compounds, converting them into more hydrophilic analogs, that can be excreted more readily (Ramesh et al., 2004). If there is an increase in enzyme activity, more of the compound will be metabolized and excreted. Since bioavailability of orally dosed PAHs is influenced by rapid uptake and metabolism in the liver (first pass effect), an increase in enzyme activity in the liver will potentially decrease bioavailability.

The metabolic products of PAHs cause the toxicological effects associated with PAH exposure (Ramesh et al., 2004) but where these metabolites are produced is a critical determinant of their toxicity; in essence, toxicity occurs in the organ where metabolites are formed, not from metabolites in the systematic circulation (Uno et al., 2004, Galvan et al., 2005). Studies conducted with both CYP1A1 knockout mice (therefore have limited free PAH metabolites in systemic circulation) and mice with low affinity AhR characteristics demonstrated that the parent compound is activated at the site of toxic action rather than metabolites formed elsewhere

causing toxicity at the site of toxic action (Uno et al., 2004, Galvan et al., 2005). Metabolites produced in the liver were determined to contribute little to peripheral adduct formation as AhR activation of enzymes predominantly happens in the liver, and mice with low affinity AhR characteristics demonstrated greater peripheral PAH toxicity (Galvan et al., 2005). Similarly, Uno et al. (2004) measured multiple toxic endpoints in CYP1A1 knockout mice exposed to BaP (DNA adducts, body and organ weight, bone marrow cell counts, etc.), and observed significantly higher toxicity in CYP1A1 knockout compared to CYP1A1 wild-type mice. This was attributed to lower circulating parent BaP from CYP1A1 activation in the wild-type mice clearing the compound in the liver (Uno et al., 2004). For these reasons, in this paper we tracked only parent PAH bioavailability.

Although relationships can be made from *in vitro* systems, bioavailability is best measured using an *in vivo* model as a substitute for human exposure. There are four models that are commonly used to predict human exposure; rodents (Withey et al., 1991, Moir et al., 1998, Ramesh et al., 2001), swine (Laurent et al., 2002, Roos et al., 2002), monkeys (Freeman et al., 1995, Akabane et al., 2010), and humans (Hecht et al., 1979, Viau et al., 2002). Although humans and monkeys are the most accurate models for human exposure, monetary and ethical restraints typically exclude their use in bioavailability studies.

Rodents are commonly used as models for human exposure, as they are well defined as a model, rather inexpensive, and easy to handle. However, the gastrointestinal system and nutritional requirements of a rodent are very different from humans. Rodents have different energy expenditures and food intakes than humans in comparison to body size, and the intestinal morphology and gastric microbiota of the rodent also differs from that of humans (Patterson et al., 2008). Therefore, from a physiological perspective, rodents are not the best model for oral

exposure in humans. Swine have emerged as a promising model for human oral exposure to contaminants due to similarity in gastrointestinal anatomy and physiology, down to the cellular level, and as such, have been commonly used as models for a variety of compounds (Roos et al., 2002, Casteel et al., 2006, Budinsky et al., 2008). Additionally, swine have similar nutritional requirements to humans, ensuring that compounds will be absorbed in similar quantities, and at a similar rate (Patterson et al., 2008).

Studies of the effect of sub-chronic exposure on bioavailability are commonly done in association with new drug testing (Andersson et al., 1990, Ferruzzi et al., 2009), and, less commonly, in toxicology studies (Schultz and Shangraw, 2006). No known studies are available for the effect of sub-chronic exposure on PAH bioavailability, although literature is available demonstrating enzymatic increases in response to PAH exposure (Roos et al., 2002, Roos et al., 2004, Harrigan et al., 2006). This study intends to assess the influence of sub-chronic PAH exposure on PAH bioavailability estimates in the swine model, and to characterize the time course of PAH concentrations in swine serum and tissues following oral exposure.

3.4 Materials and Methods

3.4.1 Swine

Female Landrace cross pigs were obtained from the Prairie Swine Centre (Saskatoon, SK), and were housed at the Animal Care Unit of the Western College of Veterinary Medicine (University of Saskatchewan, Saskatoon, SK). Swine were allowed to acclimate for 7 days prior to exposure in the facility and were maintained on water and standard grower ration *ad libitum*. Swine were divided into 2 groups; one to assess effects of sub-chronic exposure on PAH bioavailability from different media (n=24) and one to characterize the time course of PAH uptake into tissues (n=21). Animals were monitored daily during the exposure study by trained animal care staff, and were not observed to suffer ill effects from exposure to PAHs. The study

was reviewed and approved prior to initiation by the University of Saskatchewan Animal Care and Ethics Committee (Animal Use Protocol Number: 20080153).

3.4.2 Sub-chronic Exposure Study

Swine were divided into 4 groups (n=6) and exposed orally to PAHs in one of four media, daily, for 7 days. Exposure media included artificial soil, food (dough ball), corn oil, and a certified reference material (CRM soil). Daily doses of each media were administered in a dough ball composed of feed, oats, flour, and molasses. Certified reference material (CRM 140-100, Resource Technology Corporation Lot 010572) was not modified before being given to swine and each pig was given 5 g of CRM or artificial soil. The CRM soil is a natural clay soil collected from a contaminated site in the United States and contained 15 individual PAHs (Table 3-1. Individual PAH concentration in Certified Reference Material. Standard error is presented in brackets.). Artificial soil was made according to Environment Canada guidelines (2007) and was spiked with benzo[a]pyrene (BaP) and anthracene according to Reid et al. (1998). To simulate exposure to PAHs from food only, swine received dough balls spiked with neat BaP and anthracene. The soil and neat compounds were added to the dough ball with the flour. To simulate bioavailability from a lipophilic media, BaP and anthracene were dissolved in corn oil (Safeway store brand), which was then put into a gel capsule (0.5 ml, gelatin pharmaceutical capsules) and hidden in a dough ball for daily exposure. Swine were dosed with 5 mg kg-bw^{-1} daily of both anthracene and BaP in the artificial soil and food exposure groups. Swine exposed to PAHs in corn oil were given 2.5 mg kg-bw^{-1} daily of both BaP and anthracene. Swine exposed to PAHs in the CRM were given 0.17 kg-bw^{-1} daily of BaP equivalents.

Table 3-1. Individual PAH concentration in Certified Reference Material. Standard error is presented in brackets.

Compound	Concentration (µg/g)
Acenaphthene	4.25 (0.9)
Anthracene	0.28 (0.05)
Benzo[a]anthracene	0.59 (0.05)
Benzo[a]pyrene	0.09 (0.004)
Benzo[b]fluoranthene	1.09 (0.08)
Benzo[k]fluoranthene	0.28 (0.001)
Benzo[ghi]perylene	0.02 (0.01)
Chrysene	0.05 (0.01)
Dibenzo[ah]anthracene	0.62 (0.03)
Fluoranthene	0.12 (0.02)
Fluorene	1.45 (0.3)
Indeno[123-cd]pyrene	0
Naphthalene	0.60 (0.2)
Phenanthrene	0.03 (0.01)
Pyrene	0
Total Benzo[a]pyrene equivalents [a]	0.88 (0.03)

[a] Benzo[a]pyrene equivalents were calculated by applying relative potency factors (Table 3-2) to individual compound concentrations and summing the results.

Table 3-2. Relative potency factors for individual PAH compounds

Compound	Potency factor
Acenaphthene	0
Anthracene	0
Benzo[a]anthracene	0.1
Benzo[a]pyrene	1
Benzo[b]fluoranthene	0.1
Benzo[k]fluoranthene	0.1
Benzo[ghi]perylene	0.01
Chrysene	0.01
Dibenzo[ah]anthracene	1
Fluoranthene	0
Fluorene	0
Indeno[123-cd]pyrene	0.1
Naphthalene	0
Phenanthrene	0
Pyrene	0

Blood samples were collected from the jugular vein of the swine on day 1 and 7 of exposure at 1, 2, 3, 4, 6, 8, 12, and 24 hours post exposure. Serum was separated by centrifugation and stored at 4˚C pending analysis of PAHs.

3.4.3 Tissue Time Course Study

Swine were divided into 7 groups (n=3) and exposed to 5 g of certified reference material (CRM 140-100, Resource Technology Corporation Lot 010572) as described above. Groups of swine were euthanized at 1, 2, 3, 4, 6, 8, and 12 hours post-exposure using a captive bolt gun followed by exsanguination. Tissues collected included samples of stomach, jejunum, ileum, proximal colon, and liver. Blood was collected just prior to euthanization.

3.4.4 PAH Analysis

Serum and tissue extraction and high pressure liquid chromatography coupled with fluorescence detection (HPLC-FD) analysis followed procedures described by James et al. (2011). Briefly, serum samples were extracted by solid phase extraction to remove impurities and then quantified by HPLC-FD. Limits of detection ranged between 0.07 pg and 0.64 pg for the fifteen different compounds contained in the CRM. Tissue samples were freeze-dried and extracted by accelerated solvent extraction prior to analysis by HPLC-FD. The accelerated solvent extraction method used was similar to the soil procedure used in James et al (2011), except that the flush volume was modified from 10% to 100%.

3.4.5 Quality Assurance and Control

Plasma collected at the 0 hour time point was analyzed and used to correct the analytical results from the plasma of the PAH exposed swine. Duplicates, blanks, and spikes were also completed as part of the QAQC process. The average percent deviation of analytical duplicates for swine samples was 18%. Every tenth serum sample was extracted by SPE in duplicate as quality control, every tenth SPE was run with water to act as a blank, and 250µL of a spike

solution (0.1 µg ml^{-1} of each PAH) was added to every tenth sample to measure spike recovery. PAH recovery from the SPE averaged 50%. The HPLC was calibrated with an external standard consisting of 10µg ml^{-1} of each PAH and the calibration was updated daily. On average, percent deviation of analytical system duplicates for swine was 17.5%. The limit of detection for the HPLC analytical system was between 0.07 and 0.64 pg for the various PAHs.

3.4.6 Area Under the Curve and Bioavailability

Serum time course samples were used for area under the curve (AUC) calculations. AUC calculations are used to represent the whole body exposure to a compound following oral dosing. The statistical program R (R Foundation for Statistical Computing, Vienna, Austria) was used to calculate the area under the curve by applying the spline method and calculations were extrapolated to infinity, though there were negligible AUC values for times greater than 48 hours. Further, as serum time course samples were derived from different swine, AUCs are based on total serum load and not serum concentrations. This allows one to correct for differences in blood volume, assumed to be 6.5% of pig weight (Hansard et al., 1953) between samples in a curve.

Bioavailability (BA) was calculated by the following equation (Ehlers and Luthy, 2003):

$$BA = \frac{AUC\ of\ compound\ in\ serum}{known\ daily\ amount\ of\ compound\ given\ to\ pigs}$$ Equation 3-1

Bioavailability was calculated for individual compounds, as well as for BaP equivalents (CCME 2008). BaP equivalents relate the carcinogenic potential of individual PAHs to that of BaP, allowing the concentrations of compounds to be added when present in a mixture. To calculate the AUC and BA of BaP equivalents, AUCs were calculated for each compound, and then summed after applying the appropriate relative potency factor. The relative potency factor for each compound is listed in Table 3-2.

3.4.7 Statistical Analysis

All data was control corrected. Data were assumed to be normally distributed due to small sample sizes. AUC data for both BaP and anthracene were analyzed with repeated measures ANOVA ($\alpha = 0.05$), with length of exposure (day 1 and day 7) and exposure media (spiked corn oil, food, and soil, as well as CRM soil) as the factors. Differences between dose media were further analyzed with a one-way ANOVA ($\alpha = 0.05$), followed by a Tukey's Honestly Significant Difference test as repeated measures ANOVA did not determine significant differences between length of exposure. Differences in AUC between day 1 and day 7 for each media were analyzed with a two-tailed paired t-test, with a Bonferroni correction (corrected $\alpha = 0.0125$).

3.5 Results

The serum time course of BaP equivalents for swine exposed to CRM soil (Figure 3-1) is representative of the time course graphs constructed for BaP and anthracene from swine exposed to PAHs in corn oil, artificial soil and food. There was rapid absorption of PAHs, with peak serum concentrations occurring at 2 hours post-exposure followed by a second peak at eight hours post-exposure, likely due to enterohepatic cycling. The calculated AUC of BaP equivalents for this graph is 2.65 µg, which equates to a bioavailability of 60%.

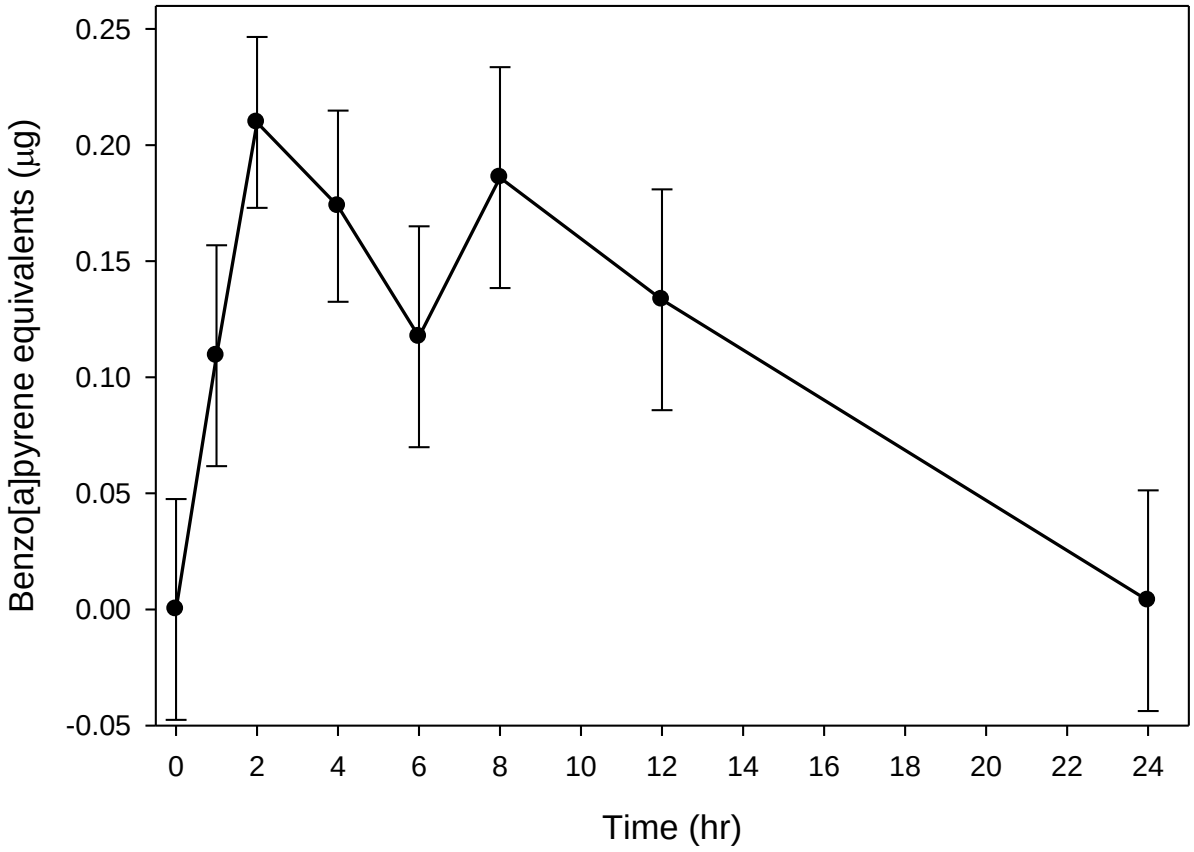

Figure 3-1. Serum time course of total benzo[a]pyrene equivalents in swine following exposure to 5 g of certified reference soil in a dough treat. Each point represents the total amount of benzo[a]pyrene equivalents in the serum. This figure is also representative of other time course graphs in swine exposed to benzo[a]pyrene and anthracene. Error bars represent the standard error of replicate animals at each time point. N=3 at each time point.

Unlike the serum, there is a steady increase in tissue concentration of PAHs which continues well after the serum levels peak (Figure 3-2). PAH concentrations in one tissue is very strongly correlated with levels in all other tissues (Pearson's correlation, $r>0.8$). Ileum and liver tissue have the strongest correlation ($r= 0.991$), with jejunum tissue also correlating strongly with liver ($r= 0.989$) and ileum tissue ($r= 0.984$). Proximal colon tissue concentrations correlate most

weakly with other tissues, with the correlation with ileum tissue the weakest (r= 0.804). This is consistent with the tissues acting as a repository for the compounds following the quick absorption into the blood and transfer via the blood to other parts of the body. Anthracene and BaP plots were very similar to the plot of BaP equivalents, with appreciable amounts of compound still present in the tissues at 12 hours post-exposure (data not shown).

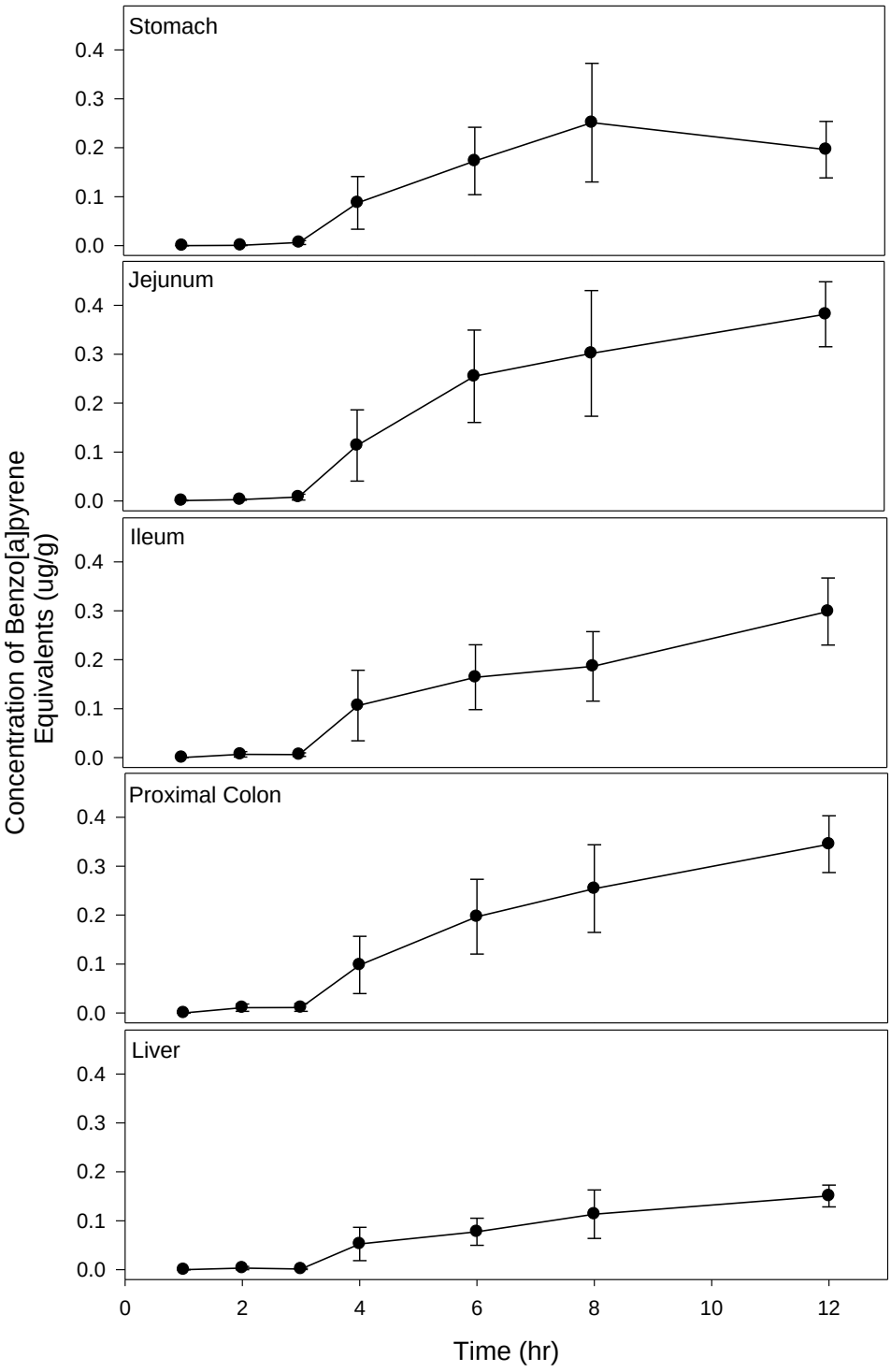

Figure 3-2. Time course of benzo[a]pyrene equivalents in swine tissues following oral exposure to 5 g of certified reference soil in a dough treat. Each point represents the estimate of the mean of swine (n=3) and error bars represent standard error for each time point.

The total internal exposure of swine to anthracene, represented by AUC, showed interaction between dose media and exposure duration (Figure 3-3: $F_{3,41}$= 30.9, $p<0.001$). Sub-chronic exposure had no influence on the calculated AUC for anthracene in any of the media when comparing day 1 to day 7 with a corrected α (paired t-test, $t<1.8$, $p>0.12$). Combined AUC data from day 1 and day 7 for anthracene in swine were compared between the different exposure media and found to be significantly different (one way ANOVA, $F_{3,41}$= 3.1, $p=0.039$). However, following α corrections, there were no significant differences detected between exposure groups (Tukey's HSD, $p>0.075$). BaP internal exposure showed no interaction between dose and exposure time (Figure 3-3, $F_{3,41}$=1.86, $p=0.152$); therefore no further statistics were completed on the data.

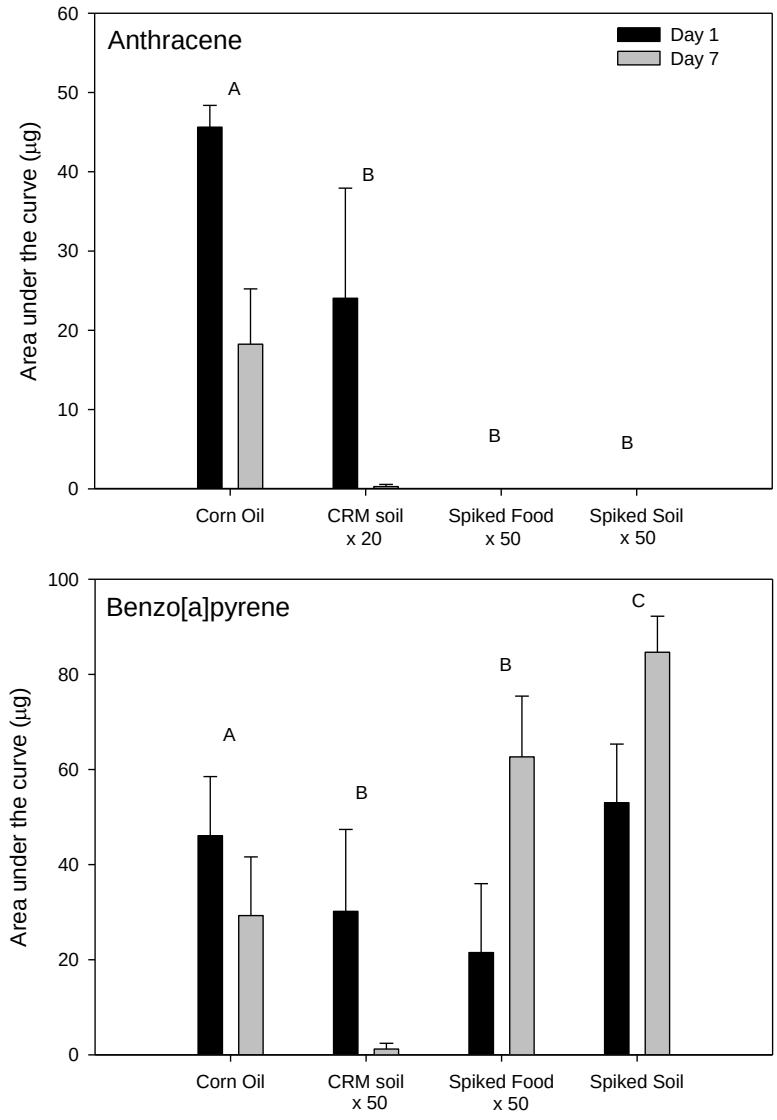

Figure 3-3. Total body exposure estimate calculated by area under the curve of benzo[a]pyrene and anthracene in swine after one and seven days of exposure. Area under the curve measurements were calculated from an eight hour serum time course by the spline method. Swine were exposed to polycyclic aromatic hydrocarbons spiked in food, artificial soil, and corn oil, as well as a certified reference material (CRM). Each point represents the estimate of the mean of swine (n=3) and error bars represent standard error for each time point. Letters denote significant difference between dosing media, as tested with a one-way Analysis of Variance, followed by Tukey's test ($p<0.004$).

Although AUC calculations did not reveal significant differences between time of exposure or exposure media, exposure levels varied for some media, which may affect AUC values. Bioavailability calculations correct for exposure level, and thus make comparisons between exposure media more robust. Bioavailability of both anthracene and BaP in swine showed interaction between length of exposure and exposure media (Repeat measures ANOVA, anthracene: $F_{3,41}$= 3.089, p=0.038; BaP: $F_{3,41}$= 3.513, p=0.023). Further analysis indicated that exposure duration did not have a significant effect on either anthracene or BaP bioavailability in swine (one way ANOVA, anthracene: $F_{1,41}$= 0.655, p=0.423; BaP: $F_{1,41}$= 1.729, p=0.196), so results from day 1 and 7 were combined for further analysis.

Statistical analysis of bioavailabilities revealed significant differences between exposure media for BaP, but not anthracene (one way ANOVA, anthracene: $F_{3,41}$= 2.708, p=0.058; BaP: $F_{3,41}$= 3.245, p=0.032). Post hoc testing indicated that bioavailability from CRM soil is significantly higher than from other media (Fisher's LSD, $0.011<p<0.012$). There were no other differences detected (Fishers LSD, $p>0.95$). Bioavailabilities are presented in Table 3-3.

Table 3-3. Bioavailability of benzo[a]pyrene and anthracene from four exposure media after day 1 and day 7 of exposure (standard error is in brackets)

Compound	Exposure Media	Day 1 Percent Bioavailability	Day 7 Percent Bioavailability	Average Percent Bioavailability
Benzo[a]pyrene	Corn Oil	0.06 (0.02)	0.04 (0.02)	0.05 (0.02)
	CRM Soil	137 (55)	5.5 (5.5)	71 (30)
	Spiked Food	0.0003 (0.0002)	0.0007 (0.0002)	0.0005 (0.0002)
	Spiked Soil	0.04 (0.009)	0.06 (0.004)	0.05 (0.006)
Anthracene	Corn Oil	0.06 (0.003)	0.03 (0.01)	0.04 (0.007)
	CRM Soil	85 (35)	1.0 (1.0)	43 (18)
	Spiked Food	0 (0)	0 (0)	0 (0)
	Spiked Soil	0 (0)	0 (0)	0 (0)

3.6 Discussion

When calculated from a blood time course, bioavailabilities of PAHs are typically only assessed after a single dose (Ramesh et al., 2004). Sub-chronic exposures to PAHs conducted in animals are usually to evaluate the effect on excretion (Jongeneelen et al., 1984), or biomarkers like enzyme activity (Roos et al., 2002, Roos et al., 2004), micronuclei (Uno et al., 2006), and DNA adducts (Ramesh and Knuckles, 2006), as well as to assess carcinogenic potential (Neal and Rigdon, 1967, Culp et al., 1998). However, the induction of CYP enzymes may alter the amount of parent compound reaching systemic circulation after first pass through the liver. In this study of sub-chronic oral dosing in swine, we found no statistical difference in PAH bioavailability after one or seven days of exposure. Seven days is sufficient for full enzyme induction (Litterst and Vanloon, 1974), and thus, it appears that despite the induction of metabolizing enzymes, bioavailability remained similar between exposure days.

Exposure media had a significant effect on bioavailability of BaP, but not anthracene, although anthracene absolute bioavailabilities were very close to being significantly different. In both cases, bioavailabilities of compounds from the CRM were much higher than from other media. Although a dose dependence study with the same CRM soil indicated that a small range of exposures resulted in a linear bioavailability (James et al., 2011), the wide range of bioavailability in this study is may be as a result of the much lower dose the swine received from the CRM soil. This may indicate that large variations in dose have an effect on the bioavailability of PAHs.

With respect to systemic exposure, comparisons are frequently done between soil types (James et al., 2011, Juhasz et al., 2014) and between spiked soil and spiked oil (vanSchooten et al., 1997, Ounnas et al., 2009), as well as with soil amendments added specifically to affect bioavailability (West et al., 2001), demonstrating that oral bioavailability of PAHs is decreased

when present in soil. Here, we find that this does not necessarily occur. Systemic exposure of BaP was highest in spiked soil, while corn oil exposed swine had the highest levels of anthracene.

The high exposure from spiked soil media was unexpected for BaP, especially in relation to the other exposure media, but the results are similar to those seen by van Schooten et al. (1997). That study tested the bioavailability of three PAHs from soil and oil media in rats, and found that AUCs and bioavailability of these compounds when exposed in oil were not consistently higher than when exposed in soil. Both BaP and anthracene had higher bioavailabilities from soil, while pyrene had a higher bioavailability from oil (vanSchooten et al., 1997). Although systemic exposure of BaP, represented by AUC, is much higher in the swine exposed to spiked soil than in swine exposed to spiked corn oil, due to the differences in exposure levels, bioavailability was very similar between the two exposure groups.

Tissue distribution of PAHs in swine is different from serum in that concentrations were still increasing at the end of the time course. The strong correlations between the tissues suggest that the PAHs enter the tissues from systemic circulation, rather than accumulating as they are absorbed through the gastrointestinal tract. An elimination phase in tissue would have been observed if samples were collected at later time points, but the trend observed in this study is similar to that seen in tissues collected by Ramesh et al. (2001) where liver and lung concentrations of BaP peaked at 24 hours post-exposure, and then gradually decreased until the last sample was collected at 72 hours post-exposure (Ramesh et al., 2001). This indicates that tissues are acting as a repository for PAHs following systemic blood circulation and prior to elimination.

Although there were detectable levels of BaP in swine exposed to spiked media (corn oil, food and soil), the bioavailability of BaP from these media was very low (e.g. 0.97%, 0.03% and 0.72%, respectively) as the dose was very high. The same trend was seen in anthracene bioavailability, with bioavailability of anthracene in swine exposed to spiked corn oil being near 1%. There are many other studies exposing rodents to doses of PAHs much higher than was done in this study that do not have such low bioavailability (Bartosek et al., 1984, Ramesh et al., 2001).

The differences in bioavailability may be attributed to the animal model used, method of calculating bioavailability, or compound administration. Swine and rodents have different gastrointestinal physiology, which may affect uptake of compounds. Studies comparing bioavailability estimates in different animal models have been conducted with metals, and they demonstrate decreases in lead bioavailability between rats and swine (Smith et al., 2009) and arsenic bioavailability between rabbits and swine (Ruby et al., 1999). Bioavailability may also be calculated by different methods like fecal elimination, which may underestimate what was absorbed into the organism (Stroo et al., 2000). Furthermore, many studies with high doses have been conducted with dietary exposure, rather than a bolus dose (Rabache et al., 1985, Nemeth and Weyand, 2002, Weyand et al., 2002). This may affect bioavailability exposing the organism to the PAHs over a longer period of time than a bolus dose.

Additionally, food and oil exposure studies conducted in humans typically have bioavailability estimates that exceed 90% (Hecht et al., 1979, Viau et al., 1995). However, these studies use doses that are much lower than the food and soil exposures in this study. These exposures were more comparable to what was exposed from the CRM soil, and much closer to environmentally relevant exposure levels. Also, these cited studies used different methods to

calculate bioavailability; Hecht et al. (1979) analyzed for parent PAHs in feces following oral exposure, and both Viau et al. (1995) and Viau et al. (2002) looked for metabolites in the urine of exposed subjects. These differences make it difficult to compare results between the studies.

The AUC and bioavailability trends in swine for BaP and anthracene were different between both exposure media and time of exposure. Swine exposed to spiked corn oil had the highest AUC of anthracene, but not the highest AUC of BaP, and although extended exposure induced decreases in anthracene AUCs, both increases and decreases were seen with BaP AUCs. These differences may be attributed to the chemical structure and subsequently the systemic treatment of the compound, particularly metabolism. Little information is available on the mammalian metabolic rate of anthracene, although there is more information available for metabolism of BaP (Vadi et al., 1975, Fahl et al., 1978, Prough et al., 1979). However, a study looking at the rate of metabolism rate of a number of PAHs in fungi suggests that there is more than a 15-fold increase in metabolic rate from anthracene to BaP (Pickard et al., 1999). Studies of metabolic rates of other PAHs in rats also suggest that chrysene has a lower metabolic rate than phenanthrene (5-fold difference) (Nordqvist et al., 1981). It is generally accepted that the metabolic rate for smaller compounds like anthracene is quicker than larger compounds like BaP (Pickard et al., 1999). Thus, anthracene would be eliminated more quickly, and affected more strongly by an increase in enzymatic activity, than BaP.

Recently, work has been completed assessing the bioavailability of BaP from different soils in the juvenile swine model (Duan et al., 2014). Bioavailability of BaP was determined to be 22% to 63% from aged soil (Duan et al., 2014). This study is similar to what is completed in this paper; however, there are a few significant differences. Bioavailability of benzo[a]pyrene from spiked soils was estimated using the same animal model and age of pig as this study.

Additionally, AUC estimates in this study as well as Duan et al. (2014) were generated using a blood time course, which is an estimation of systemic exposure to the compounds. However, Duan et al. (2014) only exposed swine to a single dose of a single compound, rather than the 7 days of exposure to a minimum of two compounds completed in this paper.

This study was intended to assess the effects of sub-chronic exposure and different exposure media on bioavailability. Our research indicates that there are significant changes in bioavailability with different media, but not sub-chronic exposure. Studies by Juhasz et al. (2014), Duan et al. (2014), and Ounnas et al. (2009) demonstrate the effect of exposure media on bioavailability, but there are no studies available that test sub-chronic exposure of PAHs. As humans are constantly exposed to these compounds, the effect of sub-chronic exposure is important, and this study demonstrates that bioavailability estimates of PAHs from single exposures are adequate.

Future directions of this research include assessing the bioavailability of a number of different real world soils and determining if soil characteristics like organic matter influence bioavailability. As well, assessing biomarkers of exposure and effect like enzyme induction in liver and ileum and micronuclei in blood will be done to determine if repeated exposure to PAHs affect the expression of these factors.

4 IS RECEIVED DOSE FROM INGESTED SOIL INDEPENDENT OF SOIL PAH CONCENTRATIONS: ANIMAL MODEL RESULTS

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4.1 Publication Fate and Contribution

A version of this chapter has been accepted by the academic journal Environmental Toxicology and Chemistry (January, 2016). Ms. Peters was involved in the experimental design, animal handling, and lab work, and completed the bulk of the data collection and analysis, and writing for this chapter. The animal work was completed at the Animal Care Unit at the Western College of Veterinary Medicine, University of Saskatchewan, Canada, and the Prairie Swine Centre, Saskatoon, Canada, under Anumal Use Protocol Number 20080153.

4.2 Abstract

Polycyclic aromatic hydrocarbon (PAH) bioavailability from ingested soils will vary between soils; however, the nature of this variation is not well characterized. Here, we used the juvenile swine model to link external exposure to internal benzo[a]pyrene (BaP) and anthracene exposure following oral PAH ingestion of 27 different impacted site soils, soots, or spiked artificial soils. Internal exposure of BaP and anthracene, represented by area under the plasma-time curve (AUC), did not correlate to soil concentration in impacted site soils, but did correlate in spiked artificial soil. Point of departure (POD) modeling identified soil PAH concentrations greater than 1,900 mg kg-1 as the point where AUC becomes proportional to external dose. BaP internal exposure below 1,900 mg kg-1 had an upper 95% confidence interval bioavailability estimate of 33% of external exposure. Weak relationships between soil:simulated gastrointestinal fluid PAH partitioning and AUC values indicate that desorption from soil does not play a large role in influencing internal exposure of PAHs. We propose four PAH risk assessment options: (i) assume 100% bioavailability, (ii) assume constant internal exposure below 1,900 mg kg-1, (iii) assume <100%, e.g. 33%, bioavailability below 1,900 mg kg-1, or (iv) model internal exposure through AUC versus soil characteristic relationships. In our opinion, our data best supports option (ii) because we could not detect an increase in AUC with increasing soil concentrations and our best efforts at (iv) do not robustly predict uptake of different PAHs.

4.3 Introduction

Human exposure to polycyclic aromatic hydrocarbons (PAHs) commonly occurs through ingestion of impacted soil. The absorption and bioavailability of PAHs has been studied extensively (see Ramesh et al. 2004 for a thorough review), and it is widely accepted that oral bioavailability of PAHs can differ when present in different media. The observed bioavailability

of PAHs varies between different soil types (Kadry et al., 1995, Stroo et al., 2000, James et al., 2011, Juhasz et al., 2014). These differences arise from contaminant weathering in soil, as well as soil characteristics, which may include soil particle size or chemical partitioning (James et al., 2011, Duan et al., 2014, Juhasz et al., 2014). However, rarely has a wide range of soils been fed to a mammal and PAH bioavailability assessed. Here we fed 19 soils, 4 artificial soils and 4 soot samples to juvenile swine. We used plasma concentrations of parent PAHs to calculate bioavailability and terminal rate constants. Using a large data set is essential to characterizing what occurs when mammals ingest PAHs.

Once ingested, PAHs transfer from the gastrointestinal tract to systemic circulation. Transfer of PAHs into circulation occurs concurrent with lipids (Stavric and Klassen, 1994), and it has been theorized that PAHs are transferred via chylomicron formation within enterocytes and transferred into lymph, which would allow PAHs to bypass the liver and hepatic first pass elimination (Busbee et al., 1990, Hussain et al., 1996). However, a study done in lymph and bile duct cannulated rats determined that about 80% of absorbed PAHs transfer to circulation via hepatic portal transport, rather than lymph (Laher et al., 1984). A more recent study confirms these findings, concluding that approximately twice the PAHs entering into circulation cross through hepatic portal transfer rather than through chylomicron formation and transport through the lymphatic system (Kim et al., 2012). Thus, the majority of ingested PAHs enter the body via the portal vein, to the liver and from there to systemic circulation.

Rapid metabolism of PAHs can confound quantification of PAH uptake following oral exposure. For example, the liver extensively metabolizes PAHs (Ramesh et al., 2004). Analyzing unlabeled metabolites in an organism is a very daunting task because PAHs are a family of compounds, e.g. there are typically at least 9-16 PAHs of interest present in an impacted soil, and

each compound can convert into more than one metabolite (Ramesh et al., 2004). Using ^{14}C labeled compounds eliminates the complicated analysis necessary for unlabeled compounds; however a number of factors make ^{14}C analysis unfavorable for use in PAH bioavailability. First, ^{14}C labeled PAHs may overestimate risk to PAHs, as most absorbed PAHs metabolize to inert metabolites that excrete quickly (Ramesh et al., 2004). Additionally, the use of ^{14}C labeled compounds is limited to spiked soil, and it would be difficult to compare results to those obtained from naturally impacted soils.

Previously, systemic PAH metabolites were thought to be best estimate of PAH bioavailability (Ramesh et al., 2004). These metabolites arise, in a large part, from liver mono-oxygenase enzymes such as CYP1A1, CYP1A2, and CYP1B1. It was initially assumed that toxic metabolites form in the liver and transport in systemic circulation to cause peripheral toxicity. However, animal studies using inbred mouse strains observed that circulating metabolites do not cause bone marrow and spleen toxicity (Legraverend et al., 1983, Uno et al., 2004, Galvan et al., 2005). This is a reasonable observation, as toxic metabolites of PAHs have epoxide groups present on the compound, and as such, are highly reactive and would not travel far in circulation without reacting with epoxide hydrolase or a cellular component. These results should not be taken to imply that the CYP family is unimportant for toxicity, but rather that the first pass effect in which much of the ingested PAHs are metabolized as the portal vein empties into the liver acts as a detoxification reaction. Thus, the assessment of parent PAHs in the systematic circulation may be a better estimate of non-gastrointestinal or hepatic toxicity after oral exposure.

Assessing systemic exposure ignores exposure that occurs in the gastrointestinal tract and liver, which likely have the highest risk to ingested PAHs. Carcinogenic studies in rodents have demonstrated the greatest incidence of tumor formation occurs in the gastrointestinal tract

following oral exposure to PAHs (Neal and Rigdon, 1967, Culp et al. 1998). However, assessing PAH exposure to the gastrointestinal tract and liver is very difficult because of the high metabolic capacity of both intestinal and liver cells (Ramesh et al., 2004). Assessing fecal elimination as a bioavailability estimate following oral exposure has been used to address this, but this method also as drawbacks as gastrointestinal flora can metabolize PAHs without exposure to intestinal cells (Stroo et al., 2000). In addition, enterohepatic circulation will continually reintroduce metabolites and parent compound to the gastrointestinal tract.

Animals, acting as surrogates for humans, are an excellent means to assess internal exposure of PAHs. Swine have become a popular human exposure model, and have been validated as a model for lead and arsenic (Casteel et al., 2006, Juhasz et al., 2008), as well as gaining popularity for organic compounds like PAHs (James et al., 2011, Duan et al., 2014, Peters et al., 2015). Swine are an alternative model to rodents due to the similarities between swine and humans in gastrointestinal physiology and intestinal conformation, as well as the cellular make-up of the organs (Patterson et al., 2008). Biochemically, swine AhR response to agonists like PAHs manifests very similar in magnitude to that of humans (Lesca et al., 1994).

Soils used for assessing bioavailability in mammals usually comes from one of two sources: impacted sites, or spiked soil (Kadry et al., 1995, Stroo et al., 2000, James et al., 2011, Duan et al., 2014, Juhasz et al., 2014, Peters et al., 2015). The advantage of using soil collected from impacted sites is that it represents a realistic exposure medium. However, age and source of the impacts in the soil may be unknown, and there may be impacts other than PAHs present that could affect bioavailability. On the other hand, spiked soils are lab generated, and therefore, source and condition of impacts can be tightly controlled. The disadvantage to using spiked soil in bioavailability studies is it does not always accurately represent real-world conditions, as

weathering duration in the lab does not equal what may be observed in the field, and spiked soil may lack the variability in physical properties that field collected soils may have.

Assessing internal exposure of parent compounds in an animal that ingests contaminated soils allows us to directly compare external to internal exposure of PAHs. With respect to environmental exposure, it is widely assumed that the relationship of dose to plasma concentration will follow a linear trend, where bioavailability is the slope of external to internal exposure. However, toxicologists, especially those concerned with mutagens, have long recognized that the dose-response relationships for toxic effects are typically hockey-stick shaped. A hockey-stick dose-response relationship comprises a linear and a sub-linear component, with the sub-linear component occurring at lower doses. Thus, for example low doses of a mutagen may cause no adverse effect until a break point is reached, at which point adverse effects increase linearly with dose. Commonly, models such as a benchmark dose (BMD), or a threshold dose (Td) model is used for such datasets (Gollapudi et al., 2013). Both Td and BMD calculations utilize the entire dose-response data set and interpolate the data to derive a point of departure where the response begins to differ significantly from the control. In other words, this approach allows one to estimate two slopes in a biphasic relationship. It is exactly this type of relationship, we observed in this study.

4.4 Methods

4.4.1 Soils

Artificial soil was prepared and spiked as in Peters et al. (2015). Soil spiked with BaP and anthracene resulted in swine exposure of 1, 5, 10, and 20 mg kg-bw^{-1} to each compound in 5 g of soil. Soot was provided by the Meyer lab group at the Technical University of Denmark. In short, a composite soot sample collected from several wood-burning stoves in a small Danish town near Roskilde was divided into two treatment groups (treated and untreated) and one group

of soot was treated in contaminant traps, while the other remained untreated. Treated and untreated soot were combined in different ratios to create different PAH concentrations. Soot exposures were designated Soot 1, Soot 2, Soot 3, and Soot 4, and contained 100% treated soot, 50% treated and 50% untreated soot, 17% treated and 83% untreated soot, and 100% untreated soot respectively (V. Gouliarmou, Technical University of Denmark, Roskilde, Denmark, unpublished manuscript). Soils collected from PAH impacted sites in the United Kingdom (n=12), Sweden (n=2), and Canada (n=5) were sieved to less than 250 µm and also fed to swine for a total of 4 artificial soil, 19 impacted site soil, and 4 soot exposures. Soil properties and PAH source are presented in

Table 4-1.

Table 4-1. Soil properties and PAH source for soils collected from PAH impacted sites.

SOIL	LOCATION	PAH SOURCE	SAND (%)	SILT/CLAY (%)	OM (%)
WP1	Sweden	Wood Preservation	42	58	2.4
GW5	Canada	Gas Works	59	41	4.6
BGS 1	United Kingdom	Gas Works	84	16	1.3
BGS 2	United Kingdom	Gas Works	77	23	8.8
BGS 3	United Kingdom	Gas Works	54	46	8.2
BGS 4	United Kingdom	Gas Works	67	33	6.8
BGS 5	United Kingdom	Gas Works	57	43	3.3
BGS 6	United Kingdom	Gas Works	59	41	12
BGS 7	United Kingdom	Gas Works	49	51	7.8
BGS 8	United Kingdom	Gas Works	70	30	13
BGS 9	United Kingdom	Gas Works	38	62	3.9
BGS 10	United Kingdom	Gas Works	90	10	4.8
BGS 11	United Kingdom	Gas Works	39	61	4.9
BGS 12	United Kingdom	Gas Works	63	37	33
COT 1	Canada	Petroleum Impacts	-[1]	-	0.8
COT 2	Canada	Petroleum Impacts	-	-	0.9
COT 3	Canada	Petroleum Impacts	-	-	0.7
COT 4	Canada	Petroleum Impacts	-	-	0.8
COT 5	Canada	Petroleum Impacts	-	-	1.6

[1] Particle size analysis was not completed on COT soils

PAHs in the impacted site soils were extracted by an ultrasonication method. Briefly, 5 ml of 1:6 toluene:methanol solvent mix was added to 1 g of soil. The slurry was sonicated for 2 hours, centrifuged for 15 min at 3000 *g*, passed through a 0.45 µm filter, and stored at -20 ˚C until analysis. Anthracene and BaP concentrations in the soot and soils are presented in Table 4-2.

4.4.2 IV Dose

The intravenous dose was prepared by completing a solvent transfer of a PAH calibration standard containing 16 different PAHs (Supelco PAH Calibration Mix, 10 µg ml^{-1} in acetonitrile, Sigma Aldrich) into glyceryl trioctanoate (Sigma Aldrich). Briefly, four 1 ml calibration standards were combined and the acetonitrile was evaporated to near dryness under a stream of high purity nitrogen gas, after which 10 ml of diethyl ether was added. Approximately half the diethyl ether was evaporated under a stream of high purity nitrogen gas, 1 ml of glyceryl trioctanoate added, and the remaining diethyl ether evaporated.

4.4.3 Swine

Female Landrace cross pigs (7-8 weeks in age, approximately 20 kg) were obtained and housed at the Prairie Swine Centre in Saskatoon, SK. Swine were housed in individual pens and allowed 7 days to acclimate prior to exposure. During the acclimation period, staff trained swine to eat a dough ball consisting of flour, molasses, pig chow, and vanilla. Swine were maintained on standard grower ration at 4% body weight and given water ad libitum. Swine were divided into groups (n=6) and exposed to PAHs by either IV or oral routes, as outlined below. Animals were monitored daily during the exposure study by trained animal care staff, and were not observed to suffer ill effects from exposure to PAHs. This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use (Animal Use Protocol Number: 20080153).

4.4.4 Exposure Study

In order to maximize the data generated by each pig, swine experienced multiple exposures to PAHs in dose media. This was done by exposing swine to a single dose of PAHs, either through oral (i.e. soil or soot) or IV exposure, generating a 48 hour plasma time course, and allowing a 7 day washout period before subsequent exposure. Swine were dosed over a period of 5 weeks, and euthanized at the end of the experiment.

4.4.4.1 Oral Exposure

Swine were given approximately 5 g of soil or 7 g of soot in a dough ball consisting of flour, molasses, pig chow, and vanilla. The soil or soot was added to the dough ball along with the addition of the flour. Swine were allowed to eat the dough ball passively, and generally consumed it in less than a minute.

4.4.4.2 IV Exposure

Swine were moved to a dedicated IV dose area and restrained with a hog snare and handling board. A 1.5 inch, 20 gauge catheter was inserted into an ear vein following topical application of lidocaine to numb the skin. The IV dose media containing PAHs (1 ml) was injected through the catheter, and the catheter flushed with saline. The catheter was removed immediately after the injection was completed, and pressure applied to the injection site until bleeding had stopped. After bleeding ceased, the animals were returned to their individual pens.

4.4.4.3 Blood Collection and Analysis

Whole blood was collected from the jugular vein of four swine per treatment group at 0, 2, 4, 6, 8, 12, and 24 hours post-exposure into heparinized vacutainers. Sample collection was limited to four swine per time point to minimize physical trauma to the animal caused by restraint and blood collection. Blood was stored at 4˚C until plasma separation by centrifugation

(1000 rpm for 15 min) and plasma stored at -20˚C until extraction. Plasma was extracted by solid phase extraction, as in James et al. (2011), and stored at -20˚C until analysis.

4.4.5 High Pressure Liquid Chromatography

Plasma and soil extract was analyzed by high pressure liquid chromatography coupled with fluorescence detection (HPLC-FD) using an Agilent 1260 Infinity system. A 10 µL aliquot of extract was injected on an Agilent PAH Pursuit column (3 µm particle size, 100 mm length, and 4.6 mm inner diameter) guarded by an Agilent MetaGuard 3 µm C18 4.6 mm column. The column was kept at 25˚C during use by a column heater. Run time was set at 30 min and HPLC grade water and acetonitrile (ACN) used as the solvents. The initial solvent gradient was 60:40 ACN:water, with a linear shift to 90:10 ACN:water between 0 min and 20 min. The 90:10 ACN:water gradient was maintained for 5 min, then the gradient was returned to 60:40 ACN:water for 5 min to re-equilibrate the column for the next sample. The Agilent 1260 system was equipped with multisignal acquisition; therefore the excitation wavelength was set at 260 nm, while the 4 fluorescence detectors were set for emission wavelengths of 350 nm, 420 nm, 440 nm, and 500nm respectively.

4.4.6 Quality Assurance and Control

Plasma collected at the 0 hour time point was analyzed and used to correct the analytical results from the plasma of the PAH exposed swine. Duplicates, blanks, and spikes were also completed as part of the QAQC process. The average percent deviation of analytical duplicates for swine samples was 18%. Average spike recovery from plasma during the solid phase extraction process was 70%, and from the ultrasonication extraction process for soil was 94%. The HPLC-FD was calibrated using dilutions of an external standard consisting of 10 µg ml^{-1} of each PAH and the calibration updated daily. Limits of detection for the HPLC-FD were 0.97 ng ml^{-1} for anthracene and 1.74 ng ml^{-1} for BaP. Plasma concentrations were corrected for

partitioning of PAHs into whole blood components, and the average recovery for anthracene and BaP in plasma compared to whole blood were 42% and 43% respectively.

In order to determine if the washout period of 7 days was adequate to allow metabolic processes to return to baseline levels between exposures, one group of swine was exposed to the same soil in week 1 of exposure, as well as week 5, and calculated bioavailabilities were compared. No statistically significant difference was observed between exposure weeks (data not shown).

4.4.7 Pharmacokinetic Parameter Estimations

4.4.7.1 Area Under the Curve

Area under the curve (AUC) calculations were completed on the plasma concentration time course for each compound in individual pigs. AUC calculations are assumed to represent the total body exposure to a compound following an oral or IV dose. AUC was calculated to infinity using the trapezoidal rule in the MESS package (Ekstrom, 2012) in statistical program R (R Team, 2011) using the trapezoidal rule.

4.4.7.2 Absorption and Elimination Rates

Absorption (k_a) and elimination (k_e) rates were calculated for each compound in each soil group as factors of the absorption and elimination slopes in the plasma concentration time course. Flip flop kinetics were observed; therefore, absorption rate was calculated from the slope of the terminal phase of the log plasma concentration time course. The elimination rate constant was calculated from the terminal phase of the IV exposure. Both rates were calculated using PKSolver, an open-source Microsoft Excel add-in (Zhang et al., 2010). Data for individual swine were pooled for each exposure group as blood samples were not collected from each pig at all time points. Thus, standard error was not calculated for absorption and elimination rate constants.

4.4.7.3 Bioavailability

Bioavailability for spiked artificial soil was calculated as the slope of the AUC vs soil concentration relationship. Bioavailability for IV exposure was calculated by dividing the area under the plasma time course by the total exposure (Equation 4-1).

$$BA_{IV} = \frac{AUC_{IV}}{Dose_{IV}}$$ Equation 4-1

Absolute bioavailability of spiked soil was calculated by dividing spiked soil bioavailability by BA_{IV}, as in Equation 4-2.

$$BA_{abs} = \frac{BA_{soil}}{BA_{IV}}$$ Equation 4-2

4.4.8 Point of Departure Calculations

4.4.8.1 Threshold Effect Level

Threshold effect level values were calculated using a piecewise linear model. This model defines a linear relationship for both the low and high part of the dose-response curve, as well as an unknown knot point at the threshold dose. 95% upper and lower confidence intervals were calculated for the threshold by bootstrap analysis. The 95% lower confidence interval is typically reported as the point of departure. This model is available as part of the SiZer package (Sonderegger, 2014) for the statistical software program R (R Team, 2011). The initial slope of the line below the point of departure, along with 95% confidence intervals of the slope, is also calculated with this model.

4.4.8.2 Benchmark Dose

Benchmark doses (BMD) are calculated by fitting models to the dose-response data and using a predetermined response level, commonly 10% from background, to select a BMD. BMD values were determined using the US EPA Benchmark Dose Software (BMDS) Version 2.5, (http://www.epa.gov/ncea/bmds/). This software contains 30 different models that can be used to

calculate a BMD. The exponential model for continuous data was chosen for this data as it provided the best fit. The lower bound 95% confidence limit on the BMD (BMDL) was also calculated, and this value is typically reported as the point of departure. The exponential model does not calculate a sub-linear slope for the data.

4.5 Results and Discussion

Swine anthracene and BaP AUC values following a single exposure to real world soils did not demonstrate a relationship with soil concentration of PAHs (Figure 4-1, anthracene: r^2=0.14, p=0.54; BaP: r^2=0.13, p=0.56). The highest soil concentration of anthracene and BaP (BGS 12) was not included in the regression as it exhibited excessive leverage. Total soil anthracene doses ranged from 0.04 µg to 724 µg and averaged 66 (32) µg, while total BaP doses ranged from 0.01 µg to 1450 µg and averaged 188 (64) µg. As no relationship was found between impacted site soils and internal exposure, average anthracene and BaP AUCs were calculated (standard error (SE) in brackets). Anthracene AUCs ranged from 0.61 µg hr L^{-1} to 14.4 µg hr L^{-1} and averaged 3.6 (0.6) µg hr L^{-1}, while BaP AUCs ranged from 1.0 µg hr L^{-1} to 7.2 µg hr L^{-1} and averaged 2.6 (0.4) µg hr L^{-1}.

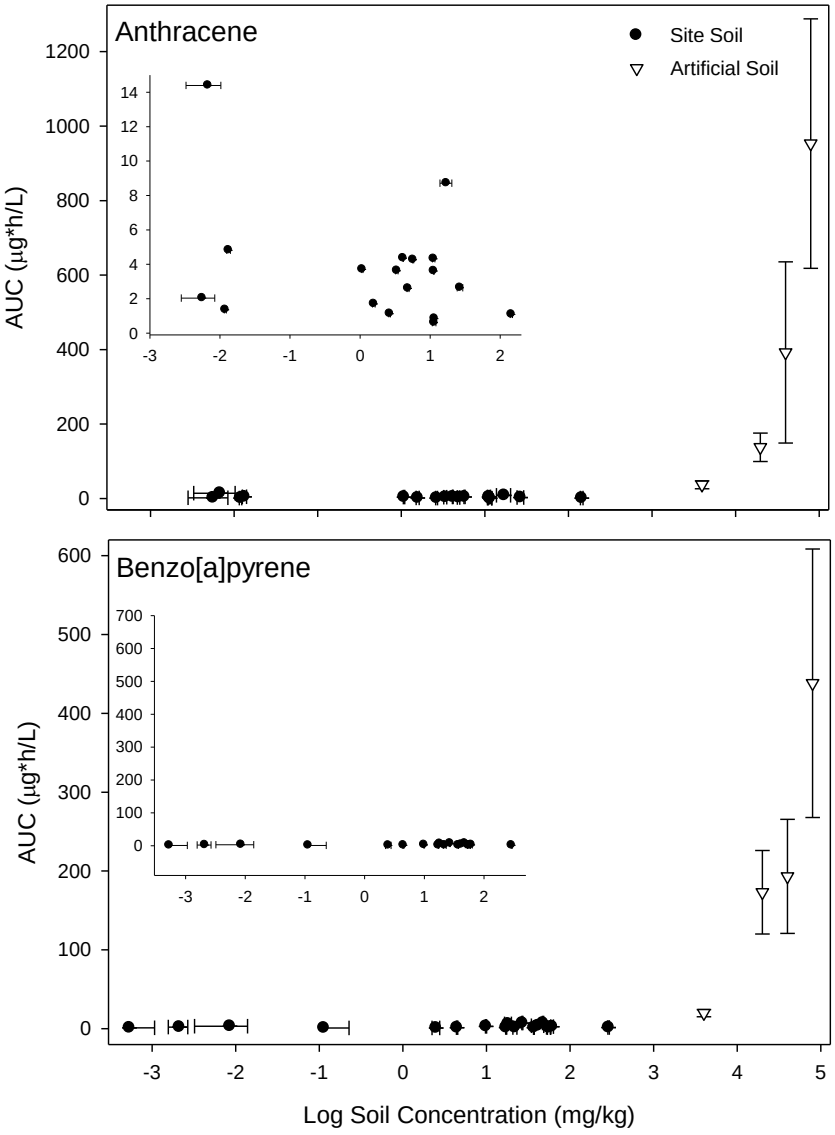

Figure 4-1. The calculated AUC values for anthracene and benzo[a]pyrene in swine after a single exposure to spiked artificial and impacted site soil versus the concentration of anthracene and benzo[a]pyrene in soil. Error bars represent the standard error of the mean for both soil concentration (horizontal, n=3) and AUC (vertical, n=6). Linear regression for impacted impacted site soils (inset) did not demonstrate a relationship between AUC and soil concentration for either anthracene (r^2=0.14, p=0.54) or benzo[a]pyrene (r^2=0.13, p=0.56).

However, linear regression for the spiked soils demonstrated a relationship between AUC and soil concentration for both anthracene (r^2=0.99, p=0.007) and benzo[a]pyrene (r^2=0.95, p=0.02).

Soot is often considered a PAH source in soil and may have different toxicokinetic parameters than soil as soil characteristics may influence chemical desorption and uptake into the organism. BaP and anthracene concentrations in soot corresponded to the range seen in the impacted site soils (Table 4-2), so AUC values were included in the regression analysis. Soot data was not included in the figures, but it corroborates with the values presented for impacted site soil (data not shown).

Table 4-2. Measured soil concentrations of anthracene and benzo[a]pyrene in impacted site soils given to swine. Standard error is in brackets.

Soil	Anthracene (mg kg^{-1})	Benzo[a]pyrene (mg kg^{-1})
WP1	27 (2.4)	18 (1.7)
GW5	1.1 (0.02)	4.5 (0.08)
Soot 1	2	10
Soot 2	5.5	25
Soot 3	7.8	35
Soot 4	9	40
BGS 1	1.6 (0.07)	2.5 (0.3)
BGS 2	11 (0.7)	56 (2.6)
BGS 3	12 (0.6)	55 (2.9)
BGS 4	11 (0.5)	61 (2.4)
BGS 5	5.7 (0.1)	27 (0.3)
BGS 6	4.8 (0.2)	17 (0.2)
BGS 7	3.4 (0.1)	9. 9 (0.2)
BGS 8	4.1 (0.04)	37 (0.4)
BGS 9	2.6 (0.07)	22 (1.2)
BGS 10	17 (3.3)	41 (8.9)
BGS 11	11 (0.1)	48 (0.8)
BGS 12	144 (5.0)	290 (5.8)
COT 1	0.008 (0.003)	0.12 (0.1)
COT 2	0.009 (0.004)	0.014 (0.0005)
COT 3	0 (0)	0 (0)
COT 4	0.013 (0.0008)	0.009 (0.005)
COT 5	0.012 (0.0004)	0.002 (0.0006)

In contrast to real world soils, BaP and anthracene AUCs generated from swine exposed to spiked artificial soil strongly correlated with soil concentration (Figure 4-1). Linear regressions completed for both anthracene and BaP demonstrated AUC has a high dependence on soil concentration (anthracene: r^2=0.99, p=0.007; BaP: r^2=0.95, p=0.02). The strong linear relationship at high doses indicates that absorption in the swine model was not limited by concentration of PAHs in soil. Bioavailability of anthracene and BaP was very low from spiked artificial soil, and was found to be 0.7% and 0.5% respectively. Absolute bioavailability was also determined, and calculated as 1.2% for anthracene and 0.7% for BaP. However, oral and IV exposure routes did not include the same animals, and as such, clearance values may differ which could impact the estimate of absolute bioavailability.

4.5.1 Analysis of the Biphasic External to Internal Exposure Relationship

The shift from no relationship between AUC and dose in impacted site soils to a strong linear relationship between AUC and dose in spiked soils may occur because of biochemical interactions between uptake and soil PAH concentration. PAHs are taken up concurrently with lipids (Stavric and Klassen, 1994), and as such, may be conveyed to systemic circulation via chylomicrons in the gastrointestinal tract. In addition, PAHs could sporadically adsorb to other components in the gastrointestinal lumen, like food, which would reduce oral PAH absorption (Stavric and Klassen, 1994). The dose swine were exposed to in the spiked artificial soil study may have been high enough to overwhelm this adsorption leading to linear PAH absorption, while the impacted site soil had a much higher proportion binding to these other components, which limited extent of absorption.

Point of Departure (POD) modeling of AUC versus soil concentration indicated AUC did not increase until soil concentration values greatly exceeded those typically seen in naturally PAH-impacted soil. PODs calculated using the US EPA Bench Mark Dose Software (BMDS)

were 10,700 mg kg^{-1} and 4,500 mg kg^{-1} for anthracene and BaP respectively. Alternatively, piecewise regression resulted in PODs of 7,500 mg kg^{-1} for anthracene and 1,900 mg kg^{-1} for BaP. This analysis would suggest that below these concentrations, there is a limited link between external dose and internal exposure. However, there are limitations associated with the data generated from spiked artificial soil, namely weathering time. Soil collected from impacted sites had experienced significant weathering time prior to collection, while the spiked artificial soil had only a few weeks of weathering. Additionally, the gap between impacted site and spiked soil concentrations was very large, and as such, may skew the POD models.

4.5.2 Toxicokinetic Parameters of PAHs Ingested with Soil

Like AUCs, anthracene absorption rate constants (k_a) calculated in swine exposed to impacted site or spiked artificial soils do not correlate to soil concentration, while BaP k_a values correlate weakly (Figure 4-2, anthracene: r^2=0.09, p=0.26; BaP: r^2=0.006, p=0.78). Absorption rate constants calculated for spiked artificial soils remained fairly constant, and the range of calculated values did not differ greatly from those calculated for impacted site soils. Average k_a values calculated for combined artificial and impacted site soils were 0.5 (0.2) hr^{-1} and 1.4 (0.4) hr^{-1} for anthracene and BaP respectively.

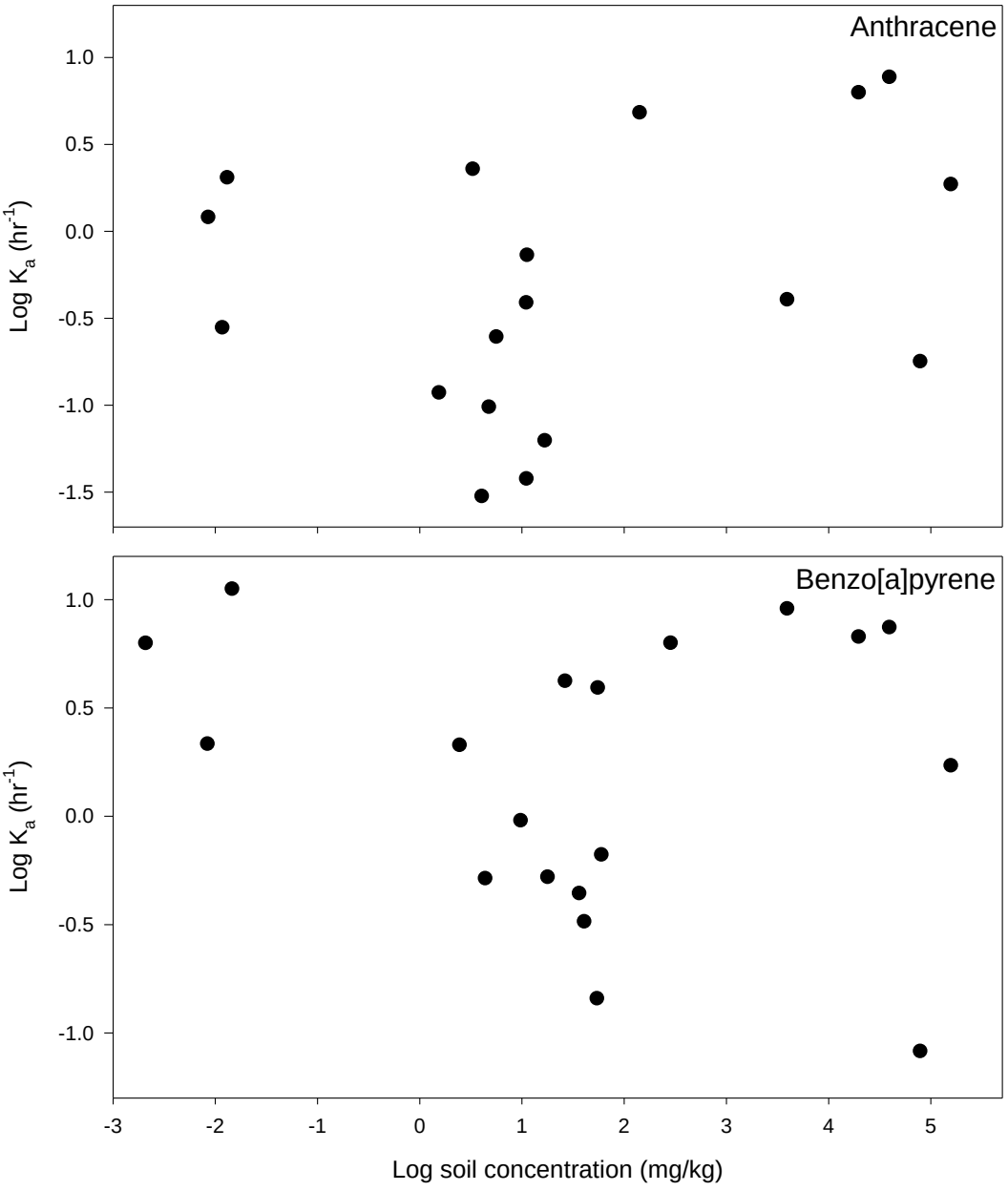

Figure 4-2. Absorption rate constants for anthracene and benzo[a]pyrene in swine following a single exposure to one of 19 impacted site soils or 4 spiked artificial soils versus soil concentration. A linear regression was completed for both benzo[a]pyrene and anthracene, and a significant relationship was not observed for either compound for impacted site soils (anthracene: r^2=0.09, p=0.26; benzo[a]pyrene: r^2=0.006, p=0.78) or spiked soils (anthracene: r^2=0.008, p=0.88; benzo[a]pyrene: r^2=0.24, p=0.41).

Swine k_a values calculated for both BaP and anthracene compare to the range of PAH k_a values available in literature for rodents. Absorption rate constants reported in both rats and mice, for benzo[a]anthracene, pyrene, and phenanthrene, range from 0.69 hr^{-1} to 18.8 hr^{-1} (Modica et al., 1983, Withey et al., 1991, Kadry et al., 1995). The highest reported k_a of 18.8 hr^{-1} was found in rats exposed to 4 mg kg^{-1} pyrene in a study consisting of a range of doses from 2 mg kg^{-1} to 15 mg kg^{-1}, and this value was much larger than the other reported k_a values for other doses from the same study (Withey et al., 1991). If we exclude this value, the highest reported k_a value is 5.0 hr^{-1}, from Withey et al. (1991). Further, Kadry et al. (1995) reported similar k_a values for phenanthrene between exposure media after oral exposure from neat compound, as well as spiked clay and sand (0.69 to 1.4 hr^{-1}). Elimination rate constants calculated following IV exposure for both anthracene and BaP were found to be 5.3 hr^{-1} and 3.7 hr^{-1} respectively.

Calculated k_e values for both anthracene and BaP in orally exposed swine compare to published rodent k_e values for oral exposure; however, the calculated k_e values for swine tended to fall on the high end of the reported range. Published pyrene, benzo[a]anthracene, phenanthrene, and BaP k_e values for both rats and mice range from 0.02 hr^{-1} to 1.3 hr^{-1} (Modica et al., 1983, Withey et al., 1991, Kadry et al., 1995, Ramesh et al., 2001, Uno et al., 2004). Two of these studies contain BaP k_e values, with widely variable results reported: 0.12 hr^{-1} by Ramesh et al. (2001) and 1.3 hr^{-1} by Uno et al. (2004). These two studies were conducted in different species (rats and mice respectively), which may account for the variability in reported k_e values. As with oral exposure, swine k_e values after an IV exposure exceeded published values. Elimination rate constants found in literature for IV exposure of pyrene and BaP range from 0.173 hr^{-1} to 3.5 hr^{-1} (Withey et al., 1991, Bouchard et al., 1998, Lipniak-Gawlik, 1998, Moir et al., 1998). Moir et al. (1998) evaluated the kinetics of BaP in rats over a range of doses, and

reported k_e values ranging from 0.98 hr^{-1} to 2.85 hr^{-1}, the maximum of which is similar to the BaP k_e for swine in this study. Additionally, Lipniak-Gawlik (1998) investigated the influence of other PAHs on pyrene kinetics, and demonstrated that mixtures of PAHs may affect the toxicokinetics of a compound.

4.5.3 Physiological Explanation of Low Dose Responses

Differences in IV and oral exposure elimination kinetics provide the clue to explain why external exposure is not linked to internal exposure at low PAH concentrations. The differences between IV an oral suggest that flip-flop kinetics is occurring. Flip-flop kinetics occur when the absorption rate constant is lower than the elimination rate constant of a compound (Yanez et al., 2011). Work previously published by Withey et al. (1991), and Viau et al. (1999) report that elimination kinetics of pyrene following an IV and oral exposure do not differ significantly. However, both studies used a liquid carrier, a saline/emulphor mix and glucose/emulphor mix respectively, for the oral exposure of pyrene. In this study, the soil matrix may limit the rate of gastrointestinal absorption of PAHs, and therefore induce flip-flop kinetics. In addition to limiting absorption rate, the soil matrix could also limit the extent of PAH absorption by binding PAHs too tightly to be released in the short transit time through the gastrointestinal tract.

Anthracene AUCs weakly correlate to anthracene partitioning from soil in simulated intestinal fluid (r^2=0.18, p=0.13, Figure 4-3). BaP bioavailability (AUC normalized to dose) also correlates weakly with Forest fluid partition co-efficients (James et al., 2016). Both anthracene and BaP demonstrate negative relationships with Forest fluid partitioning co-efficients – as the partitioning co-efficient increases, AUC and bioavailability decrease. Partitioning co-efficients represent the ratio of compound in soil to compound in fluid; therefore, partitioning co-efficients increases signify a greater proportion of compound remaining in soil, rather than fluid. Thus, increases in simulated GI fluid partitioning values indicate a stronger affinity of the compound,

whether anthracene or BaP, to the soil particles, and explains the negative relationship with AUC.

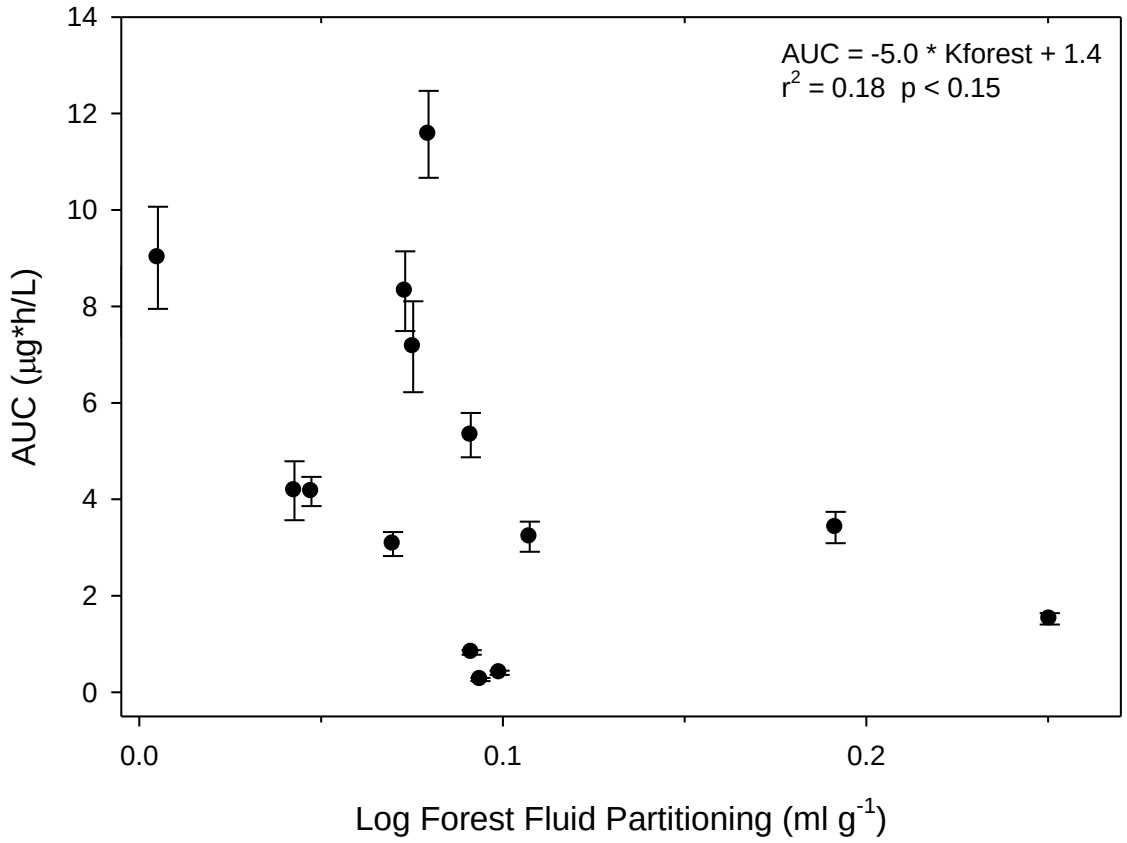

Figure 4-3. Anthracene AUC in swine (n=6) orally exposed to PAHs in impacted site soils (n=14) versus log of simulated intestinal fluid partitioning co-efficient. Linear regression demonstrated a weak relationship between variables (r^2=0.18, p=0.13).

4.5.4 Options for the Risk Assessment of Contaminated Soils

We propose four PAH risk assessment options, as detailed in Table 4-3: (i) assume 100% bioavailability, (ii) assume constant internal exposure below 1,900 mg kg^{-1}, (iii) assume <100%, e.g. 33%, bioavailability below 1,900 mg kg^{-1}, or (iv) model internal exposure through AUC versus soil characteristic relationships. The most conservative, but least accurate, method of risk assessment for PAH impacted sites assumes that 100% of ingested PAHs transfer into an

organism. As demonstrated in this study, as well as others, PAH bioavailability can vary widely depending on dose media, and may lie below 1% in soil (Peters et al., 2015, Ramesh et al., 2004). Therefore, this method may result in extremely elevated risk values for impacted sites.

The second risk assessment option assumes humans absorb a constant amount of PAHs in contaminated soil, irrespective of the contaminant level in these soils. The Incremental Lifetime Cancer Risk (ILCR) is calculated by multiplying the external compound dose to the appropriate cancer slope factor (CSF). Thus, if we assume 100% bioavailability and the average BaP concentration of our soils, the ILCR is $2.3x10^{-5}$ for adults and $3.9x10^{-4}$ for toddlers. Toxicity studies for PAHs base the reference dose value on the external exposure the model organisms received, rather than on what reaches systemic circulation. These studies exposed animals to PAHs in food (Neal and Rigdon, 1967, Culp et al., 1998), and Ramesh et al. (2004) demonstrate that PAH bioavailability from food is near 100%. Thus, if we assume that internal dose is not linked to external dose, then using average BaP AUC value from our study, corrected for assumed adult and toddler soil ingestion rates (CCME, 2006), the ILCR is $6.2x10^{-6}$ for adults and $1.6x10^{-6}$ for toddlers. Although assuming a constant internal dose is very simple, it does not incorporate site-specific variations, and as such, may not accurately represent risk.

The third risk assessment option calculates bioavailability as the slope of the internal-exposure dose curve at environmentally relevant soil concentrations. At these low concentrations, this slope is often termed the sublinear portion because it is not significantly different from zero. Piecewise regression of this sublinear portion indicates that the 95% confidence interval of bioavailability estimates for environmentally relevant soil concentrations range from 2.5% to 33% for BaP and 0% to 36% for anthracene. However, these approaches are highly sensitive to the spiked soil doses, the limitations of which were discussed previously.

Thus, our estimate of 33% soil BaP concentration becoming bioavailable may be too inaccurate to use at a contaminated site.

The fourth, and final, option is to use partitioning to estimate internal exposure of PAHs to humans. For example, in Figure 4-3, there is a weak relationship between partitioning and AUC. This approach can determine internal exposure estimates to site-specific soils but requires site-specific data. Additionally, the observed relationships between partitioning co-efficients and internal exposure in swine were very weak, and as such, may be inaccurate.

Table 4-3. Pros and Cons of Risk Assessment Options for PAHs

Option	Pros	Cons
100% bioavailability	Simple, conservative	May overestimate risk
Constant AUC	Simple, likely realistic	Not site-specific
Constant (<100%) bioavailability	Simple	Highly dependent on spiked soil concentrations, may not be accurate
Soil:fluid partitioning	Site Specific	More complex, weak relationship, more labour intensive

4.6 Conclusions

Analysis of swine anthracene and BaP toxicokinetics demonstrated PAH soil concentration does not change internal exposure of PAHs. This contradicts the common assumption in risk assessment that risk relates linearly to the soil concentration, and therefore external dose, of a compound. There appears to be a point of departure in soil concentrations where internal exposure and external dose become related. Using two different point of departure models indicated AUC and soil dose were only linked at soil concentrations much larger than those typically seen in PAH impacted soils found in the environment. Thus, it may be reasoned humans are exposed to a constant internal dose of PAHs, regardless of external dose. We hypothesize this occurs because of limited absorption coupled with rapid elimination, leading to a reduced amount of circulating compound. As this study measured parent PAHs in systemic

circulation as an indication of internal exposure, decreases in circulating compound would lead to a decreased apparent internal exposure. However, our study design cannot speak to the risk of exposure of the gastrointestinal lining, as PAH exposure to this tissue occurs during the absorption phase, independent of systemic circulation.

5 DO BIOMARKERS OF EXPOSURE AND EFFECT CORRELATE WITH INTERNAL EXPOSURE TO PAHS IN SWINE?

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5.1 Publication Fate and Contribution

A version of this chapter has been published by the academic journal Biomarkers (Peters RE, Wickstrom M, Siciliano SD. 2016. Do biomarkers of exposure and effect correlate with internal exposure to PAHs in swine? Biomarkers, doi: 10.3109/1354750X.2016.1138322). Ms. Peters was involved in the experimental design and animal handling, and completed the bulk of the lab work, data collection and analysis, and writing for this chapter. The animal work was completed at the Animal Care Unit at the Western College of Veterinary Medicine, University of Saskatchewan, Canada, and the Prairie Swine Centre, Saskatoon, Canada, under Anumal Use Protocol Number 20080153.

5.2 Abstract

Biomarkers are often used in toxicology studies to monitor the consequences of organism exposures to complex mixtures, as well as to draw connections between dose and effect. Humans are commonly exposed to polycyclic aromatic hydrocarbons (PAHs), which consist of a family of compounds present as mixtures in the environment. This study exposed swine to PAH mixtures in single and subchronic dose regimens and collected liver and ileum tissue to measure cytochrome P450 (CYP) 1A1, CYP1A2, and CYP1B1 mRNA expression and ethoxyresorufin-O-deethylase (EROD) activity as biomarkers of exposure and 7,8-diol, 9,10-epoxide benzo[a]pyrene (BPDE)-DNA adducts and oxidized proteins as indicators of cellular oxidative stress as tissue biomarkers of effect. Blood was also collected and micronucleated reticulocytes measured as systemic biomarkers of effect. Results indicate that biomarkers of exposure and effect were not affected by dose magnitude, and duration of exposure did not influence mRNA expression or EROD activity. However, exposure duration did produce significant increases in DNA adducts and oxidative stress in liver and ileum tissue. Numbers of micronucleated reticulocytes were not affected by exposure length, suggesting the liver was effective at clearing PAHs to non-toxic metabolites at the doses used in this study. These results suggest biomarkers of exposure may be effective in determining if PAH exposure occurred, while biomarkers of effect in liver and ileum tissue may give more information regarding exposure length and magnitude of dose.

5.3 Introduction

Human environmental exposure studies commonly use biomarkers as uncertainty generally exists with regard to exposures to xenobiotics. Biomarkers are generally defined as a cellular or biochemical change measured in an organism following a xenobiotic exposure, and are often divided into two categories: biomarkers of exposure and biomarkers of effect (Silins

and Hogberg, 2011). Biomarkers of exposure differ from biomarkers of effect as biomarkers of exposure can be repaired and do not have direct links to effects, although there is overlap between categories. Benefits of biomarkers include confirming internal exposure to a compound rather than hypothesizing an external dose, as humans may receive a xenobiotic exposure from any of a number of sources (Silins and Hogberg, 2011). As data from controlled studies in humans is usually lacking, in vivo studies using animal models are often the best source of xenobiotic toxicity data. Rodent models of human exposure are frequently used as rats and mice are easy to handle and well characterized (Galvan et al., 2005, Ramesh and Knuckles, 2006, Juhasz et al., 2014). However, significant anatomical, metabolic, and biochemical differences exist between rodents and humans, such that these animals may not represent the most appropriate models for human exposure (Lu and Li, 2001, Patterson et al., 2008).

Swine are emerging as an alternative animal model for human exposure, especially by the oral route, due to specific advantages over traditional rodent models. Swine have great anatomic similarities to humans, particularly in the gastrointestinal tract, which makes this an excellent model for oral bioavailability studies (Patterson et al., 2008). In addition to anatomic similarities, swine have very similar metabolic and biochemical pathways to humans, making them a good choice for biomarker studies as well (Messina et al., 2009, Nannelli et al., 2009, Puccinelli et al., 2011). Swine aryl hydrocarbon receptor (AhR) binding characteristics are very similar to that of humans, which may lead to comparable enzyme induction between swine and humans when exposed to AhR agonists like polycyclic aromatic hydrocarbons (PAHs) (Lesca et al., 1994). Additionally, conventional swine organs express P450 enzymes to a similar extent as human organs, and swine CYP1A enzymes have >80% similarity to humans (Puccinelli et al., 2011).

Both minipigs and conventional swine have been used in PAH toxicity studies as a model of human exposure (Roos et al., 2002, James et al., 2011, Duan et al., 2014, Peters et al., 2015). Minipigs are bred specifically for laboratory research, and as such, are smaller and easier to handle than conventional swine. Selective breeding of minipigs maintains genetic similarities in the animals, and can reduce variability between individuals (Puccinelli et al., 2011). As with other purpose bred laboratory animals, this increased genetic homogeneity does not represent the human population accurately as humans exhibit wide genetic diversity. Conventional swine typically come from commercial hog operations, and thus demonstrate large genetic diversity. Sources of conventional swine are also easier to find locally and have greater similarities to humans with respect to biochemical factors like enzyme structure than minipigs (Puccinelli et al., 2011).

Soil-bound PAH exposures commonly occur in humans through incidental soil ingestion, and these exposures can induce biomarkers of both exposure and effect. PAHs are potent AhR agonists, and thus induce CYP1A upregulation, among other genes. Typically CYP1A upregulation is assessed by measuring mRNA induction or with enzyme activity assays (Nannelli et al., 2009). mRNA expression represents a very sensitive AhR activation endpoint, with increases in mRNA induced at doses 2 times lower than what is required to induce enzyme activity following exposure to BaP (Shimada et al., 2003). Enzyme activity, as described by and ethoxyresorufin-O-deethylase (EROD) activity acts as an indication of functional protein levels. Many PAHs are metabolically activated to reactive species that can form DNA adducts, which occur when molecules covalently bond with DNA (Ramesh et al., 2004). Benzo[a]pyrene (BaP) is considered the most potent PAH carcinogen, and BaP commonly forms the 7,8-diol, 9,10-epoxide benzo[a]pyrene (BPDE) adduct (Ramesh et al., 2004). As a more general form of

toxicity, PAHs can also form reactive oxygen species, and cause oxidative stress in cells (Ramesh et al., 2004). Additionally, the DNA damage caused by adduct formation or oxidative stress potentially results in production of micronuclei, particularly in reticulocytes, as a function of bone marrow damage (Iarmarcovai et al., 2008).

The organ with the greatest potential for biomarker formation following oral PAH exposure is the liver, as it contains high levels of xenobiotic metabolizing enzymes. It also receives a large dose of absorbed compound as a result of its anatomical location as the recipient of portal blood containing absorbed substances from the gastrointestinal tract. The liver is also considered to be the most prominent factor influencing the bioavailability of foreign compounds in the body (Ramesh et al., 2004). However, the small intestine has also been demonstrated to contain inducible metabolic enzymes, and may play a significant role in influencing metabolism and excretion of PAHs (Kaminsky and Zhang, 2003, Fang and Zhang, 2010). The duodenum is generally considered to be the major site of uptake for most ingested material (Roos et al., 2002), but the ileum may be an uptake site for the absorption of PAHs (Kadry et al., 1995).

Our previous work examined the bioavailability of PAHs in juvenile swine from ingestion of spiked media following single and repeated exposures, and this study assessed biomarkers of PAH exposure and effect in blood and liver and ileum tissue from the same studies. The exposure duration for the repeat exposure study was 14 days as this length of time was assumed to have full induction of biomarkers of exposure and effect. Spiked exposure media (artificial soil, corn oil, food) were amended with concentrations expected to cause biomarker induction, but much higher than environmentally relevant. A certified reference material was also included to provide a more environmentally relevant exposure. Swine were used as a model for human exposure due to their physiological similarities to humans. BaP and anthracene were chosen as

PAHs of interest as BaP represents the prototypical PAH and is considered one of the more potent biomarker inducers. Anthracene was also used to assess the effect of PAH mixtures on biomarker induction as it is a large, non-carcinogenic PAH with a very different structure from BaP. Biomarkers evaluated included mRNA induction of CYP1A1, 1A2, and 1B1; CYP1A activity as determined by EROD assays; BPDE adduct formation; protein carbonyl formation as an indication of oxidative stress; and micronuclei formation in reticulocytes.

5.4 Materials and Methods

5.4.1 Swine

Samples used for biomarker quantification were collected from the swine used in Peters et al. (2015a, 2015b). In short, 8 week old female Landrace cross swine were obtained from the Prairie Swine Centre (Saskatoon, Saskatchewan) and housed in the Animal Care Unit at the Western College of Veterinary Medicine (University of Saskatchewan, Saskatoon, Saskatchewan). Swine were divided into 4 groups; one to assess repeat (subchronic) exposure to PAHs in different media (n=24), one to assess exposure to different concentrations of PAHs in spiked soil (n=24), one to characterize the time course of PAHs in tissues (n=24), and one to act as a control (n=6). Swine were maintained on standard grower ration and water ad libitum. Swine were monitored daily during the exposure study by trained animal care staff and were not observed to suffer ill effects from PAH exposure. This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use (Animal Use Protocol Number: 20080153).

5.4.2 Exposure

Swine used in the following studies were part of larger work which has been published (Peters et al., 2015 and Peters et al., 2016). Swine handling and care is described briefly below.

5.4.2.1 Sub-chronic

Swine were divided into 4 groups (n=6) and exposed daily to BaP and anthracene in spiked corn oil (2.5 mg kg-bw^{-1} both BaP and anthracene), food (5 mg kg-bw^{-1} both BaP and anthracene), or soil (5 mg kg-bw^{-1} both BaP and anthracene), or certified reference material containing a mixture of PAHs (0.17 mg kg-bw^{-1} BaP equivalents) in a dough ball consisting of flour, molasses, pig chow, and vanilla (Peters et al., 2015). BaP equivalents were calculated using potency equivalency factors to relate the potency of various PAHs to BaP as recommended by the Canadian Council of Ministers of the Environment (CCME, 2008). Groups of swine were euthanized following 14 days of exposure using a captive bolt gun followed by exsanguination. Liver and ileum tissues were collected and flash frozen in liquid nitrogen for mRNA, EROD, DNA adduct, and oxidative stress analyses, while blood was collected for micronuclei analysis just prior to euthanization. Tissue samples were stored at -80˚C until analysis. Blood samples were processed for micronuclei analysis immediately following collection.

5.4.2.2 Spiked Soil

Swine were divided into 4 groups (n=6) and exposed to a single dose of both BaP and anthracene in spiked soil at 1, 5, 10, and 20 mg kg-bw^{-1} in a dough ball (Peters et al., 2016). Groups of swine were euthanized 24 hours post exposure, and blood, liver, and ileum samples were collected for the same analyses and processed as described above.

5.4.2.3 Time Course

Swine were divided into 8 groups (n=3) and exposed to a single dose of 0.17 mg kg-bw^{-1} BaP equivalents in certified reference material in a dough ball (Peters et al., 2015). Groups of swine were euthanized at 1, 2, 3, 4, 6, 8, 12, and 24 hours post exposure in the same way as sub-

chronic swine. Liver and ileum tissue samples were collected for mRNA and DNA adduct analyses, flash frozen in liquid nitrogen, and stored at -80 ˚C until analysis.

5.4.2.4 Control Animals

Control swine (n=6) were exposed to dough balls with no additional exposure media daily for 14 days.

5.4.3 Biomarker Analysis

5.4.3.1 mRNA

RNA was extracted from liver and ileum tissues using the RNeasy Plus Mini Kit (Qiagen, Mississauga, Canada) according to the manufacturer's protocol. Total RNA concentration was determined using a Qubit RNA BR Assay Kit (Thermo Fisher Scientific, Burlington, Canada), while purity was assessed with NanoDrop (Thermo Fisher Scientific, Wilmington, USA). Approximately 1 µg RNA was used to synthesize cDNA using the iScript cDNA synthesis kit (Biorad, Philadelphia, USA). Samples were stored at -20˚C until further analysis.

Forward and reverse primers for CYP1A1, CYP1A2, CYP1B1, and glyceraldehyde 3-phosphate dehydrogenase (GADPH) were chosen based on previous work by Nannelli et al. (2009), and p53 forward and reverse primers were chosen based on previous work by Zou et al. (2013) (obtained from Integrated DNA Technologies (Coralville, USA)). Messenger RNA for the genes in question was quantified using qPCR. Samples were prepared for each sample in triplicate by combining 1 µL cDNA, 0.4 µL each of forward and reverse primer for the gene in question, 5.2 µL nuclease free water, and 13 µL Power SYBR Green PCR master mix (Applied Biosystems, Frederick, USA) in a PCR well plate. The rt-PCR program for CYP1A1, CYP1A2, CYP1B1, and GADPH was set similar to Nannelli et al. (2009), and consisted of an initial incubation of 2 min at 50˚C, and a 10 min incubation at 95˚C, followed by 40 cycles consisting

of 15 sec at 95˚C, 30 sec at 60˚C and 45 sec at 72˚C. The rt-PCR program for p53 was set according to Leuchs et al. (2012), and consisted of an initial incubation for 5 min at 95˚C, followed by 40 cycles of 30 sec at 95˚C, 30 sec at 59˚C, and 3.5 min at 72˚C. Fold-change, representing the gene expression difference between control and treated animals, for CYP1A1, CYP1A2, CYP1B1, and p53 was determined using the comparative C_T ($_{\Delta\Delta}C_T$) method using GADPH as the internal control gene (Schmittgen and Livak, 2008).

5.4.3.2 Enzyme Activity

CYP1A activity was measured in microsomes isolated from liver and ileum tissues by ethoxyresorufin-O-deethylase (EROD) analysis as in Budinsky et al. (2008) with minor changes. Briefly, the concentration of resorufin working solution was reduced from 7.5 µM to 0.75 µM, and the concentration of 7-ethoxyresorufin was increase from 23.5 µM to 117.5 µM. Additionally, volumes of bovine serum albumin (BSA) were modified to 0, 5, 12, 24, 48, and 60 µl and volumes of resorufin working solution were modified to 0, 2, 3, 4, 5, and 10 µl. Total well volume was maintained at 200 µl. Standard curves were created by plotting fluorescence versus known levels of resorufin (pmol) and BSA (mg). Final EROD activity was normalized to protein and reaction time, and expressed as pmol resorufin min^{-1} mg $protein^{-1}$.

5.4.3.3 DNA Adducts

DNA was extracted from liver and ileum tissue with the DNeasy Blood & Tissue Kit (Qiagen, Mississauga, Canada) according to the manufacturer's protocol. Total DNA concentration was determined using a Qubit DNA BR Assay Kit (Thermo Fisher Scientific, Burlington, Canada). DNA samples were diluted to 2 µg ml^{-1} in cold 1x PBS and stored at -20˚C until further analysis. DNA adducts were quantified in duplicate with the OxiSelect BPDE DNA Adduct ELISA Kit (Cell Biolabs, Inc, San Diego, USA) according to the manufacturer's

protocol. Concentration of DNA adducts in the final analysis was normalized for tissue weight, and DNA adducts were expressed as ng adducts g tissue^{-1}.

5.4.3.4 Oxidative Stress

Oxidized protein concentrations in liver and ileum tissue has been used as an indication of cellular oxidative stress (Dalle-Donne et al., 2003), and was measured in duplicate with a Protein Carbonyl Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, USA) according to manufacturer protocol. Results are expressed as nmol protein carbonyl ml^{-1}.

5.4.3.5 Micronuclei

Reticulocyte micronuclei concentrations were measured in blood samples using flow cytometry (Litron Laboratories (Rochester, USA)). Blood was collected into heparinized vacutainer tubes and processed according to the manufacturer's protocol prior to shipment to Litron Laboratories for analysis. Results were expressed in percent micronucleated reticulocytes (% MN-RET).

5.4.4 Statistics

One-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference test was used to determine significant changes among dose groups and exposure regimes in all biomarker data sets. Single-tailed t-tests were used to determine significant increases in mRNA expression over control animals. Systat 13 (Systat Software Inc., San Jose, USA) was used for all statistical analyses.

5.5 Results

Induction of P450 genes occurred to a greater extent in repeat exposure groups than in single exposure groups, though the observed changes were not significant (Figure 5-1). Much of the apparent difference between exposure lengths results from the large variability observed

between animals. For example, CYP1A2 fold-change in liver appears to increase approximately 30 times over control, but if the food group is removed, the remaining exposed swine have CYP1A2 expression less than ten-fold that of control animals, very similar to that in single exposure groups. The large variability seen in exposure groups is common in animal models and could be exaggerated by the animal model used in this study. Conventional swine are not bred specifically for research, and as such, are much less genetically homogenous than inbred rat and mouse strains used in laboratory research (Puccinelli et al., 2011). Additionally, variability in subchronic exposure swine may be attributed to the different exposure vehicles influencing internal exposure. Significant CYP1A1 expression increases over control animals was observed in ileum tissue in swine exposed to a single dose of 10 mg kg-bw^{-1} PAHs ($p=0.038$), as well as in swine repeatedly exposed to PAHs in corn oil ($p=0.041$). In the present study CYP1A2 expression increased in groups exposed to single exposures of 5 mg kg-bw^{-1} ($p=0.044$) and 20 mg kg-bw^{-1} ($p=0.033$) relative to controls (Figure 5-1).

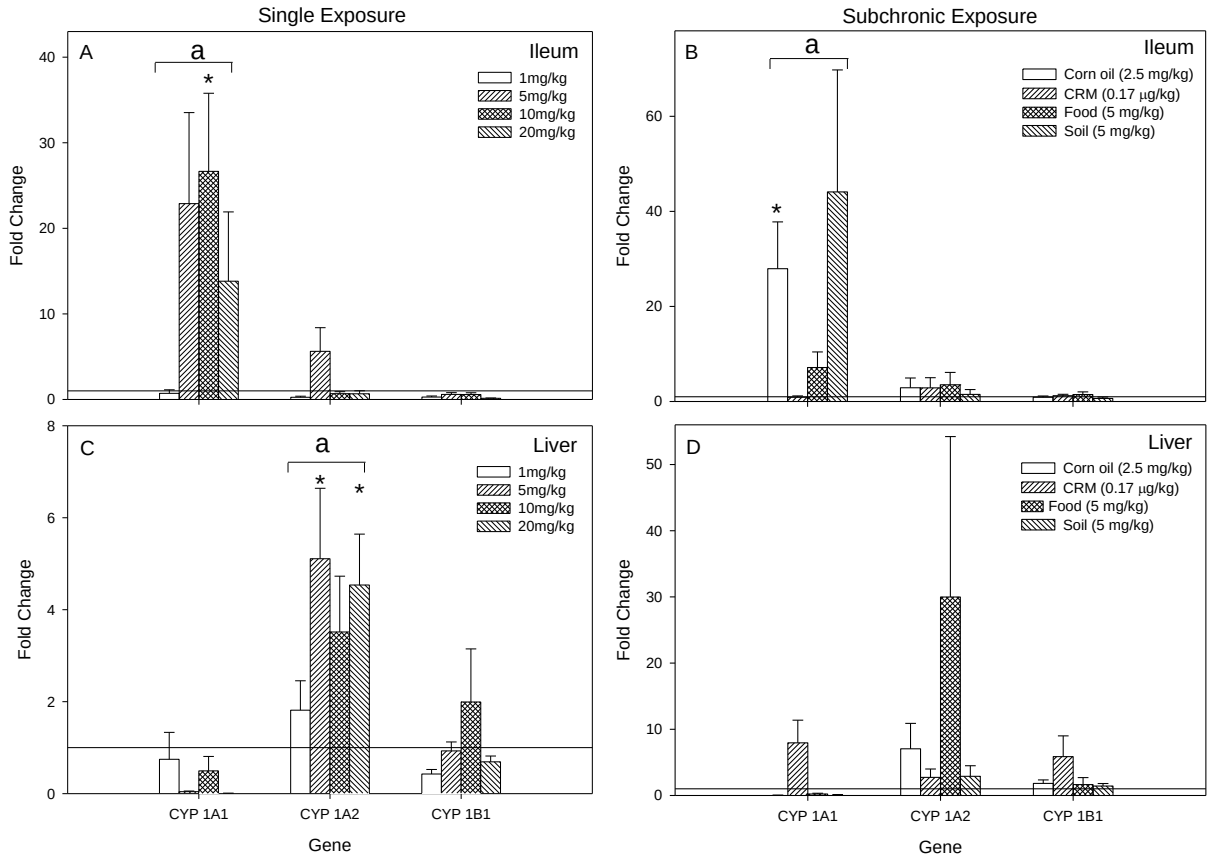

Figure 5-1. Fold change of select cytochrome P450 enzymes in swine liver and ileum tissue (n=6 for each group) following 24 hour exposure to spiked artificial soil (A and C) or 14 day exposure to PAHs in various dose media (B and D). Letters denote significant differences between genes in the tissue ($p<0.001$), while asterisks denote significant increases of the group over control swine ($p<0.045$). The reference line is representative of control animals.

Swine liver demonstrated elevated EROD activity following exposure to PAHs. However, exposure duration did not influence increases (Figure 5-2). Swine exposed to repeated doses of PAHs in food ($p<0.001$) and spiked artificial soil ($p=0.003$), as well as swine exposed to a single dose of 5 mg kg-bw^{-1} in artificial soil ($p=0.001$) demonstrated statistically significant increases in enzyme activity over control animals. However, ileum tissue had very low EROD activity, even in the subchronic study (data not shown).

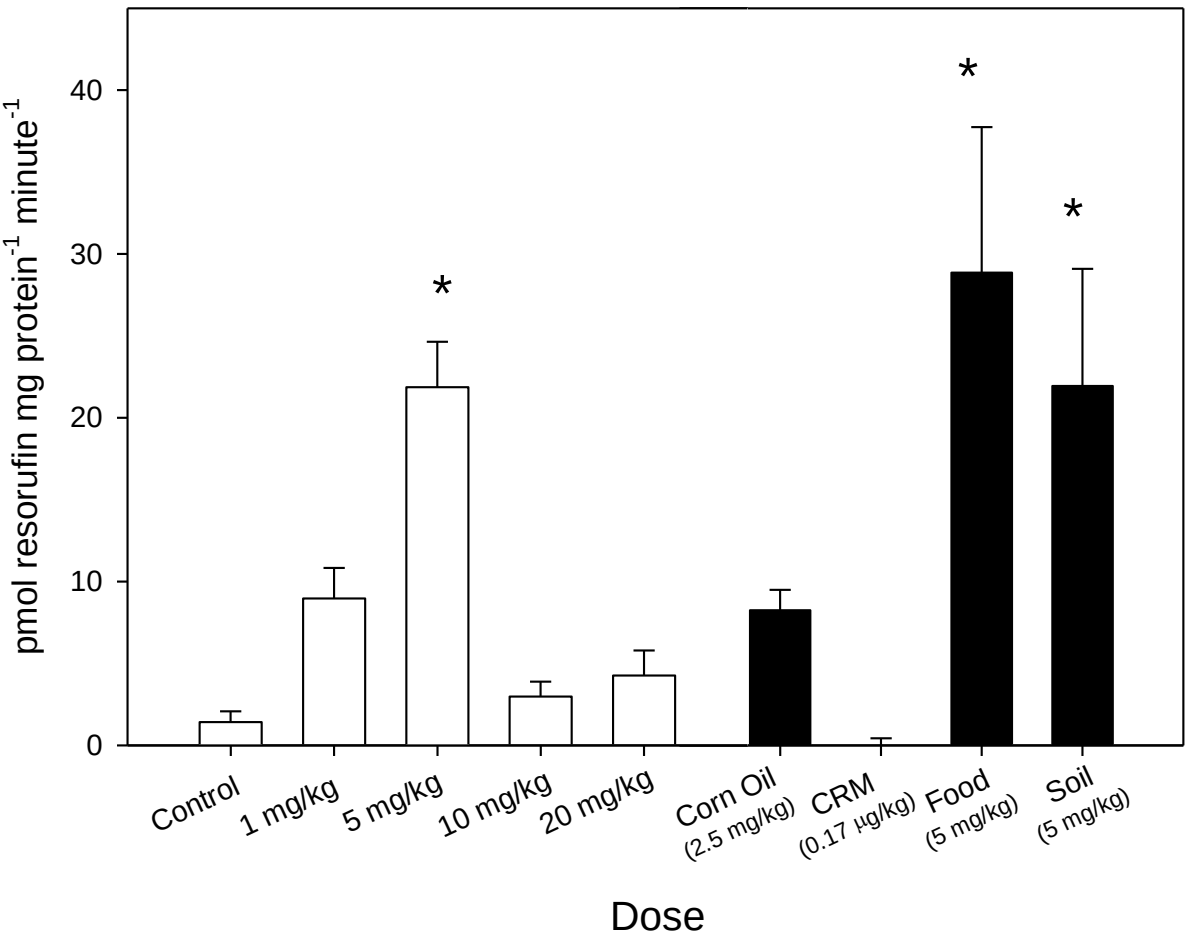

Figure 5-2. EROD activity in swine liver tissue (n=6) as measured by resorufin activity following exposure to PAHs in a variety of dose media. Open bars represent swine exposed to a single dose of benzo[a]pyrene and anthracene in spiked artificial soil at the labeled doses, while black bars represent swine exposed to benzo[a]pyrene and anthracene in the labeled dose media daily for 14 days. Samples for both single and repeat exposure were collected 24 hours following the final dose. Asterisks denote significant differences of groups from control animals ($p<0.009$).

Although exposure duration did not affect the degree of EROD activity in liver, enzyme activity varied with dose of PAHs (Figure 5-2). Significant increases in EROD activity were observed in swine exposed to single (soil) and repeat (soil and food) doses of 5 mg kg-bw^{-1} BaP and anthracene. Swine exposed to 1 mg kg-bw^{-1} in artificial soil have similar elevations of

EROD activity to swine exposed to a comparable dose in corn oil. However, the EROD response was not consistently dose-dependent. Enzyme activity decreased in the higher dose groups (10 and 20 mg kg^{-1}).

Formation and clearance of BPDE DNA adducts occurs very rapidly in liver and ileum tissues following a single oral exposure to PAHs in soil, and do not follow PAH tissue concentration in liver and ileum tissue (Figure 5-3a and Figure 5-3c, tissue concentration from Peters et al. 2015). Tissue BaP equivalent concentration were only reported to 12 hours post exposure. The peak adduct concentration in PAH exposed swine was significantly greater than control animals for both ileum (1 hr post exposure, p=0.005) and liver (4 hr post exposure, p=0.002).

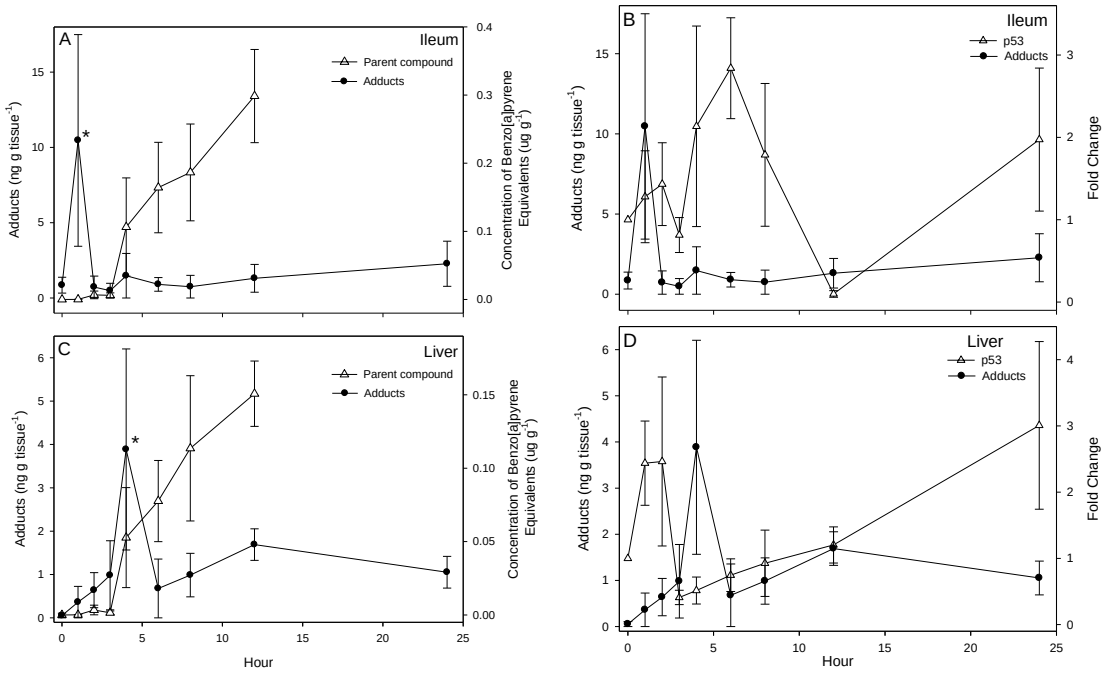

Figure 5-3. Time course of DNA adducts and parent BaP equivalents (A and C) and p53 expression and BPDE adducts in swine liver and ileum tissue (n=3 for each time point) following exposure to PAHs in a certified reference material. Error bars represent standard error and asterisks denote significant difference from control animals (p<0.006).

Analysis of p53 mRNA in swine liver and ileum tissue identified elevated gene expression following single exposure to PAHs, indicating that swine p53 expression responds to PAH exposure (Figure 5-3b and Figure 5-3d). The peak p53 mRNA expression in ileum tissue occurs after peak DNA adducts and corresponds with peak DNA adducts in the liver, suggesting that this peak results from enterohepatic cycling of PAHs. Liver tissue demonstrated a peak in p53 mRNA expression between 1 and 2 hours post-exposure, which appears to correspond with the initial increase in BPDE adducts in liver tissue. In both liver and ileum tissue, p53 expression increases subsequent to increases in DNA adducts, which corresponds with parent PAH concentration in tissues. These results suggest p53 expression in swine tissue is inducible by PAH exposure, which agrees with observations in human tissue (Pei et al., 1999).

Repeated exposure to BaP spiked media increased BPDE adduct concentrations compared to control and single exposure groups (Figure 5-4). Data from day 1 and day 7, as well as liver and ileum, were grouped because no significant differences were observed between dose groups or tissues, and a one way ANOVA detected significant differences between control, single, and repeat exposure scenarios ($p=0.044$). Post-hoc testing did not determine significant differences between groups, which likely resulted from adjusted alpha values for repeat sampling. Liver and ileum tissues from control swine contained detectable concentrations of BPDE adducts, confirming that exposure to BaP is ubiquitous.

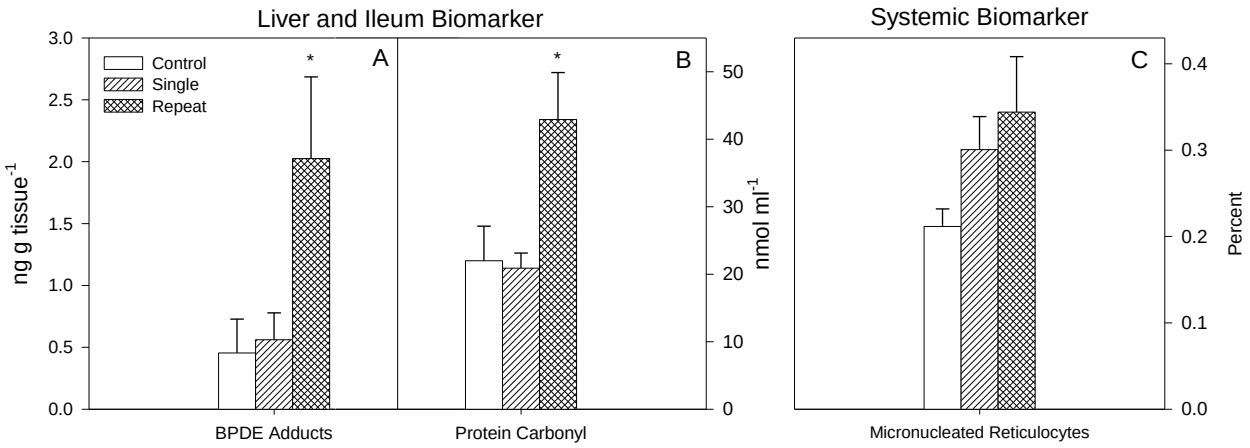

Figure 5-4. Biomarkers of effect in swine in control (n=6), single (n=24), or repeated exposure (n=24) groups to PAHs in a variety of dose media as evidence of tissue (A and B) and systemic (C) effect. DNA adduct concentrations and protein carbonyl levels were quantified in both liver and ileum tissues, while percent micronucleated reticulocytes were measured in blood. Both tissue and dose groups for single and repeat dose studies were combined for BPDE adduct and protein carbonyl graphs as there were no significant differences between groups. Asterisks denote significant differences between dose groups, as determined by one-way ANOVA ($p<0.045$). Dose groups for micronucleated reticulocytes were also combined for single and repeat dose studies as there were no significant differences between groups. Error bars represent standard error for each study.

Like DNA adducts, liver and ileum tissue demonstrated increases in protein carbonyl levels as an indication of cellular oxidative stress following repeated exposure to PAHs (Figure 5-4). A one way ANOVA detected significant difference between groups ($p=0.001$), and subsequent post-hoc testing determined that single and repeat dose groups differed ($p=0.001$); however, the control group did not differ from either dose group ($p>0.145$). Alternatively, swine did not show an increase in percent micronucleated reticulocytes following either single or repeat oral exposure to PAHs (one way ANOVA, $p=0.284$, Figure 5-4). Again, data were grouped for

single and repeat dose scenarios as no significant differences were observed between dose media or tissues.

5.6 Discussion

Swine have been shown as useful models for human exposure for assessing xenobiotic bioavailability (James et al., 2011, Duan et al., 2014, Peters et al., 2015) as well as biomarkers of exposure and effect (Roos et al., 2002, Nannelli et al., 2009, Zamaratskaia and Zlabek, 2009, Puccinelli et al., 2011). Biomarkers of exposure have short half-lives and are not expected to accumulate in tissues, but may more accurately reflect external dose. Biomarkers of effect have longer half-lives which could more accurately represent exposure duration. Our previous work demonstrated that exposure duration did not influence PAH bioavailability (Peters et al., 2015). One of the objectives of the present study was to determine how biomarkers of exposure and effect were influenced by exposure duration and dose level.

Gene induction of CYP1A1, 1A2, and 1B1 enzymes in swine liver and ileum tissue mirrors what is known about the expression of these genes in humans (Puccinelli et al., 2011). Humans tend to have higher CYP1A1 expression in intestinal tissue compared to liver, similar to what we observed in this study. mRNA expression of the gene CYP1A1 was elevated in swine ileum tissue following both single and repeat exposure to PAHs ($p<0.001$), while elevated CYP1A2 was observed in liver tissue following single exposure ($p=<0.001$) (Figure 5-1). Additionally, ileum tissue demonstrates a larger fold-change in enzyme expression than liver tissue in single exposure groups, which likely results from ileum tissue receiving a larger initial PAH exposure during the exposure media transport through the gastrointestinal tract. This difference may also be attributed to the different genes. CYP1A1 is more strongly induced by the AhR, which may account for the differences between genes.

In contrast to what was observed in swine, CYP1A1 mRNA induction commonly occurs in rat liver, and can be observed in human liver following exposure to an inducer (Budinsky et al., 2010). However, induction of CYP1A1 in human liver appears to correlate more with diet than exposure to AhR agonists like PAHs, as the liver of smokers does not always contain elevated CYP1A1 levels (Schweikl et al., 1993, Martignoni et al., 2006). This could indicate that something other than AhR agonism induces CYP1A1 expression in human liver. Additionally, humans and swine have lower AhR expression than rodents, which also potentially results in lower CYP1A1 induction. The CYP1A2 protein structure appears very similar to CYP1A1, and AhR induction is generally considered to act on CYP1A2 expression, but rodent studies demonstrate that CYP1A2 induction can occur in AhR knockout animals, indicating that induction of this enzyme arises from more than the AhR (Sakuma et al., 1999).

Humans frequently have elevated basal CYP1A2 expression in the liver compared to CYP1A1, while CYP1B1 is considered an extrahepatic (outside the liver) enzyme in humans and swine and has been observed in the adrenal gland and cerebral tissues of swine (Puccinelli et al., 2011, Nannelli et al., 2009). Slight elevation of CYP1B1 mRNA was observed in swine liver, which is consistent with previous studies (Messina et al., 2009).

Nannelli et al. (2009) measured induction of these same CYP enzymes in the liver and various brain regions in swine following exposure to a potent AhR agonist, β-naphthoflavone. Swine showed induction of both CYP1A1 and CYP1A2 mRNA in the liver in that study, while CYP1B1 induction was not observed. The difference observed in CYP1A1 induction in liver between Nannelli et al. (2009) and the present study may have resulted from exposure differences as β-naphthoflavone and BaP are both considered relatively strong AhR activators. Nannelli et al. (2009) exposed swine by intraperitoneal injection to 30 mg kg-d^{-1} over a period of

four days. Intraperitoneal exposure mimics oral exposure while bypassing gastrointestinal mucosa. However, much of the dose will be absorbed into the mesenteric blood supply which drains into the hepatic portal vein and subsequently the liver. Thus, bypassing gastrointestinal absorption coupled with a larger exposure could lead to a larger hepatic exposure.

EROD assays are often considered to represent CYP1A1 activity in tissues, and although EROD assays will primarily assess CYP1A1 activity, a number of other enzymes will convert 7-ethoxyresorufin to resorufin. Zamaratskaia and Zlabek (2009) demonstrate biphasic kinetics in EROD assays of swine liver, indicating there are multiple enzymes contributing to resorufin production. One of these enzymes may be CYP1A2, which demonstrated mRNA induction in the liver. Nannelli et al. (2009) also observed biphasic EROD kinetics in liver of swine exposed to β-naphthoflavone, but not in control animals. This suggests the biphasic kinetics were induced by the upregulation of an enzyme in the liver.

The lack of EROD activity in ileum was surprising as we observed significant CYP1A1 gene induction in that tissue. A study conducted in minipigs exposed to PAHs in soil observed significant inductions in EROD activity in the duodenum (Roos et al., 2002). The lack of EROD activity in ileum observed in this study was corroborated by another study examining P450 activity and protein expression in swine hepatocytes and enterocyte cultures (Hansen et al., 2000). Hansen et al. (2000) observed increases in CYP1A1 protein in enterocytes treated with 3-methylcholanthrene and β-naphthoflavone, both potent AhR inducers, but EROD activity was not increased. The authors attributed the discrepancy to the release of an unknown inhibitory factor. In addition, mRNA induction is expressed in fold-change over controls, and protein expression was not quantified in our study. Thus, although induction of CYP1A1 mRNA in ileum occurred, the protein levels may have been too low for measurable increases in EROD

activity. Additionally, Peters et al (2015) demonstrated appreciable parent PAH concentrations in ileum tissue, and the presence of these PAHs could have competed with ethoxyresorufin in the EROD assay, leading to falsely low EROD activity (Petrulis and Bunce, 1999).

The differences in study design between the Roos et al. (2002) and Hansen et al. (2000) and the current study may explain the variability in results. Roos et al. (2002) used Gottingen minipigs as an animal model, while this study and Hansen et al. (2000) used Landrace cross swine. Wide variability in CYP protein expression has been observed between swine species, particularly between conventional swine and minipigs (Puccinelli et al., 2011). Additionally, Roos et al. (2002) investigated effects on the duodenum, while this study focused on the ileum. Enzyme activity is not evenly distributed throughout the length of the small intestine, and the duodenum is generally recognized to have the highest CYP1A activity in the small intestine (Kaminsky and Zhang, 2003). Finally, Roos et al. (2002) exposed the minipigs to impacted soil from industrial sites, while Hansen et al. (2000) and this study used lab generated exposure media. The impacted soil from Roos et al. (2002) may have included compounds that were not identified that blocked the inhibitory factor hypothesized to suppress EROD activity.

Peak DNA adduct formation in liver and ileum in the time course study likely occurs as a result of initial tissue exposure to BaP. Serum BaP equivalent concentrations in swine studied peaked between 2 and 4 hours post-exposure, indicating that the dose had reached systemic circulation, agreeing with the peak of BPDE adducts in ileum occurring at 1 hour post-exposure (Peters et al., 2015). Ileum tissue had higher concentrations of BPDE adducts than liver, perhaps as a result of greater exposure to parent compound, though BPDE adducts result from metabolic activation of parent PAHs. As the ileum has lower metabolic capacity than the liver as demonstrated by mRNA expression and EROD activity, it is curious that BPDE adducts are

higher in the small intestine. Repair mechanisms may act to a greater degree in the liver, resulting in a larger apparent concentration of BPDE adducts in the ileum tissue. Dybdahl et al. (2003) assessed DNA adduct formation in liver and colon in rats following oral exposure to diesel exhaust particulates. The authors observed significantly greater adduct formation in the colon, which was attributed to lower DNA repair capacity in the colon as measured by mRNA expression of OGG1, a nucleotide repair enzyme. Additionally, there may be a dilution effect in the liver as the tissue was not perfused with a physiological buffer to displace blood prior to freezing. The gradual increase in BPDE adducts in liver between 1 and 3 hours post-exposure may indicate the metabolic and repair mechanisms in the liver. The smaller second peak of adducts observed in ileum tissue may be attributed to enterohepatic circulation as it occurs concurrently with the largest adduct peak in liver tissue.

Most time course studies of DNA adduct formation have been conducted over a period of days following exposure, rather than hours (Tombolan et al., 1999, Ericson and Balk, 2000, Briede et al., 2004). Monien et al (2008) examined the rapid formation of liver adducts in rats following exposure to 1-methylpyrene, and observed peak adduct formation 3 hours post-exposure. This observation is consistent with the present study, with slightly earlier adduct formation likely resulting from the use of the intraperitoneal route in the rats, compared to the oral exposure in the swine (Monien et al., 2008).

The lack of correlation between parent tissue concentration and BPDE adduct concentration may result from increases in DNA repair mechanisms in response to cellular challenge. Nucleotide excision repair mechanisms such as global genomic repair is regulated by tumor protein p53 in humans (Hanawalt, 2002). Work in human cell lines demonstrates that BaP does induce p53 regulation of genomic repair, as p53 depleted cells did not repair BPDE adducts,

while cells with normal p53 expression rapidly repaired these adducts (Lloyd and Hanawalt, 2000). Additionally, cell line studies demonstrated that BaP induces p53 upregulation in human cells (Pei et al., 1999). Rodents typically do not have p53 regulated nucleotide excision repair, and as such, may not be the most appropriate animal model for human carcinogenesis (Hanawalt, 2002).

The increases in tissue biomarkers of effect following repeat exposure, coupled with the lack of increase in systemic biomarkers of exposure likely indicate the liver is effective at removing PAHs from systemic circulation at doses used in this study. This agrees with the systemic bioavailability estimates from these studies, which were generally less than 30% (James et al. 2016, Peters et al. 2015). Uptake of PAHs into both the liver and ileum is impacted by the anatomical location of the organs, leading to PAH accumulation in these tissues and increased risk of adverse effects (e.g. carcinogenicity) relative to bone marrow following oral exposure (Culp et al., 1998). Studies addressing bone marrow and other systemic effects typically use larger doses and shorter time periods than carcinogenicity studies, suggesting systemic effects are caused by acute exposures to PAHs, rather than chronic exposures typically experienced by humans (Uno et al., 2004, Galvan et al., 2005).

The increase and evident accumulation of biomarkers of effect in liver and ileum following repeated exposure to PAHs suggests organisms are effective at responding to and detoxifying single exposures, but repeated exposure can lead to a higher probability of effects. The increase of biomarkers in swine subjected to subchronic exposure contrasts with the lack of changes in circulating parent compound following repeat exposure, indicating that while repeated exposure to PAHs did not affect bioavailability of the compounds, it may have led to accumulation of effects (Peters et al., 2015). This trend also differs from that seen in mRNA

expression and enzyme activity, which constitute biomarkers of exposure, where no relationship between exposure duration and biomarkers were observed. This suggests that although mRNA expression and enzyme activity may be good biomarkers of general exposure to PAHs, the magnitude of biomarker response cannot be used to estimate the dose received.

Duration of exposure did not significantly affect biomarkers of exposure like increases in mRNA expression and EROD activity, or micronuclei formation in reticulocytes. However, significant increases were observed in liver and ileum DNA adducts and oxidative stress measures. Additionally, lack of elevation in systemic biomarker response suggests the liver is effective at mitigating systemic effects from an oral exposure. This suggests biomarkers of exposure do not respond to exposure duration while biomarkers of effect may, and the liver and small intestinal tissue, which are involved in the first pass PAH uptake seem at highest risk to oral exposure to PAHs.

6 DISCUSSION AND CONCLUSIONS

Human health risk assessment currently uses a default oral bioavailability estimate of 100% when assessing PAH impacted sites, which may substantially overestimate risk to human health from incidentally ingested soils. Although this conservative approach has the benefit of protecting human health, it results in more conservative cleanup guidelines and may lead to unwarranted, costly cleanup of impacted sites. Assessing site-specific bioavailability of PAHs can be time consuming and costly, as researchers must use animal models to estimate bioavailability values. PAH bioavailability from soil is generally assumed to be lower than bioavailability from exposure media used in toxicity studies, and soil characteristics like organic carbon and particle size are thought to influence the bioavailability of PAHs. Understanding how these factors affect bioavailability should allow more accurate *in vitro* and mathematical exposure modeling. The principal goals of this research project include 1) assessing whether PAH bioavailability and biomarkers of exposure and effect were affected by repeated exposure to PAHs in different media; and 2) determining if PAH bioavailability remained consistent across soil concentrations, and which, if any, soil characteristics affect PAH absorption. Fundamental research questions addressing these goals include: 1) Is PAH bioavailability from soil less than 100%? 2) Does repeated exposure decrease bioavailability? 3) Does repeated exposure increase biomarkers of exposure or effect? 4) Can we determine what soil characteristics influence bioavailability? 5) Can we develop a predictive mathematical model for PAH bioavailability in soil? and 6) Can we develop a PAH bioavailability recommendation for human health risk assessment?

6.1 Principal Findings

PAH bioavailability estimates from impacted site soils and artificially spiked soils are consistently less than 100%, which is contrary to current default bioavailability assumptions used during risk assessment. Estimated bioavailability from a certified reference material was calculated as 60% for BaP equivalents. Absolute bioavailability calculated from the slope of AUC versus soil concentration was 1.2% for anthracene and 0.7% for BaP in spiked soil, while the slope of AUC versus impacted site soil concentration gave a 95% confidence interval bioavailability estimate of 36% and 33% for anthracene and BaP respectively (**Error! Reference source not found.**3). All these bioavailability estimates are much lower than default assumption, indicating that remediation of impacted sites likely occurs to a much greater degree than necessary. Too stringent remediation guidelines may lead companies to one of two approaches to dealing with impacted sites: spending more money than necessary to remediate to prescribed guidelines, or abandoning an impacted site and potentially exposing humans or environmental receptors like plants or soil invertebrates to PAH concentrations above toxicity limits. Neither of these approaches benefit a community or corporation as the money may be better spent elsewhere, or potentially valuable land may sit vacant.

People are typically exposed to PAHs in the environment repeatedly over a period of time. Subchronic exposure to PAHs may induce a number of physiological changes in an organism, in particular induction of metabolic processes (Ramesh et al., 2004). Increased PAH metabolism will lower oral bioavailability as calculated in this research, as we estimate oral bioavailability to be the fraction of the original dose reaching systemic circulation as parent compound. However, increased metabolism may also increase PAH toxicity as PAHs are metabolically activated. PAH oral bioavailability is often estimated from a single dose (Ramesh et al., 2004), but Lipniak-Gawlik (1998) examined the effect of pretreatment of PAHs on

elimination kinetics of pyrene. This study found pyrene had a longer excretion time following PAH pretreatment compared to animals not receiving pretreatment, and differences were attributed to pretreatment PAHs affecting the metabolism of pyrene (Lipniak-Gawlik, 1998). This study differs from the current one in that PAHs used in pretreatment differed from the compound of interest, while the current study pretreated animals with the same compounds as were measured in blood. In the current study, repeated exposure to PAHs does not appear to affect bioavailability of PAHs from different media (Figure 3-3). Thus, our research suggests bioavailability estimates completed after a single exposure will accurately represent internal exposure to PAHs.

Biomarkers of PAH exposure in swine (mRNA expression and EROD activity in liver and ileum tissue) observed in this study likely correlate well with human biochemical reactions to PAH exposure (Figure 5-1, Figure 5-2). Swine are widely recognized as appropriate models for human biomarker studies due to the similarities in CYP enzyme structure and distribution (Puccinelli et al., 2011), as well as similarities in AhR affinity for PAHs (Lesca et al., 1994). This research agrees with published knowledge regarding tissue distribution of CYP enzymes, as CYP1A2 was predominantly found in liver tissue and CYP1A1 was observed primarily in ileum tissue (Figure 5-1). EROD activity was confined to liver tissue, which is contrary to much published literature which found high enzyme activity in the small intestine (Roos et al., 2002, Kaminsky and Zhang, 2003). However, Hanson et al. (2000) observed similar findings in intestinal cell cultures, where CYP1A1 protein content increased following exposure to AhR agonists, but EROD activity remained unchanged. Thus, although PAHs induce gene expression of metabolic enzymes, something appears to inhibit the functionality of the proteins in the small intestine.

Although organ distribution of gene expression and activity in swine agrees with human studies, the magnitude of these markers do not correlate with dose magnitude or exposure duration (Figure 5-1, Figure 5-2), which agrees with the repeat exposure results (Figure 3-3). EROD activity did not appear to change between single and repeat exposure of PAHs, and as EROD activity is often considered analogous to CYP1A1 activity, increases in EROD activity would be expected to decrease PAH systemic bioavailability. Thus, the lack of EROD activity increase agrees with the lack of change in bioavailability estimates.

However, apparent toxicity of PAHs in tissues, as represented by DNA adducts and oxidized protein concentrations, did increase when swine were repeatedly exposed to PAHs in a variety of media. Increases in biomarkers of effect (DNA adducts and oxidative stress measurements) were only observed in liver and ileum tissue. Systemic biomarkers of effect, represented by micronucleated reticulocyte formation were unchanged (Figure 5-4). Liver and ileum tissue are among the group of tissues initially exposed to oral exposure as they are included in the first pass effect, while disruptions in micronuclei formation occur in the bone marrow of an organism. Thus, these results suggest that the first pass effect is effective at reducing the systemic toxicity of a PAH oral exposure.

Gastrointestinal and liver tissue concentrations of parent PAHs increased over the time course until study termination at 24 hours post-exposure, suggesting tissues are subject to PAH exposure for a longer duration than just initial exposure (Figure 3-2). This assumption may also extend to bone marrow, the site of reticulocyte formation. Although tissue concentrations suggest the bulk of PAH exposure occurs after the initial exposure, which would suggest that systemic exposure compares to that of the gastrointestinal tract and liver, the lack of induction in

micronucleated reticulocyte formation indicates that the first pass effect may detoxify PAHs to a greater extent than just removal from circulation would suggest.

PAH concentrations in impacted site soils did not correlate with systemic absorption estimates in swine following oral exposure (Figure 4-1). This likely results from the presence of flip-flop kinetics following oral exposure. The elimination rate constants calculated following IV exposure to PAHs (5.3 hr^{-1} for anthracene and 3.7 hr^{-1} for BaP) were much larger than the elimination rate constant calculated following oral PAH exposure in soil (0.54 hr^{-1} for anthracene and 1.4 hr^{-1} for BaP), indicating absorption of soil-bound PAHs following oral exposure limits elimination. Flip-flop kinetics was not observed in other studies examining both oral and IV exposure; however, these studies used a liquid carrier for oral exposures (Withey et al., 1991, Viau et al., 1999). Soil properties, in particular organic carbon, adsorb PAHs and limit their release in gastrointestinal fluid (Siciliano et al., 2010). Thus, soil may regulate the release of PAHs into gastrointestinal fluid following oral exposure, while the liquid carriers used in other studies will not. Interindividual variation in the physiological system, like intestinal transit times, bile secretion, or enzyme expression can also affect bioavailability, which may explain the lack of relationship between PAH soil concentration or soil characteristics and AUC, as this natural variability could mask absorption differences caused by soil characteristics.

The presence of flip-flop kinetics in swine following oral PAH exposure could be the reason validation of *in vitro* models remains difficult. *In vitro* models have been validated for metals like lead and arsenic (Casteel et al., 1997, Rodriguez et al., 2003) but although multiple attempts to validate *in vitro* models for PAH exposure have occurred, an appropriate model has not been identified (Duan et al., 2014, James et al., 2011). *In vitro* models may not accurately predict PAH absorption following oral exposure, as the *in vitro* models used for PAHs thus far

only mimic the release of compounds into gastrointestinal fluid and do not incorporate absorption, distribution, metabolism, and excretion into estimating internal exposure. As these factors, in particular metabolism, play a major role in influencing PAH parent compound concentration in the blood, *in vitro* models will inaccurately predict internal exposure.

While systemic exposure from impacted site soils did not correlate with PAH concentration in soil, relationship between soil concentration in spiked artificial soil and systemic exposure was observed. A dose-response curve generated after plotting AUC values versus soil concentration for both impacted site and artificial spiked soils suggests a threshold soil concentration exists where PAH uptake becomes related to soil concentration following oral exposure to impacted soil (Figure 4-1). Below this PAH concentration, we expect a human to receive a constant internal exposure to PAHs following oral exposure to contaminated soil, regardless of soil concentration. The lowest threshold, at a soil concentration of 1,900 mg kg^{-1} for BaP, is higher than the maximum impacted site soil concentration used in this study, indicating limitations may exist in the conclusions made regarding the relationship between soil concentration and internal exposure and the application of the threshold dose to impacted site soils. The AUC-soil concentration relationship generated from spiked soil is difficult to compare to results from impacted site soil exposure as the artificial soil media is homologous with only soil concentration changing, while impacted site soils had different soil properties as well as changing PAH concentrations. Additionally, the large gap in soil concentration between impacted site and spiked artificial soil may affect the precision of the threshold dose estimate.

Soil characteristics considered in this research did not appear to correlate with anthracene systemic exposure estimates across a number of impacted site soils (Figure 4-3). This same lack of relationship was observed in the other measured PAHs as well (James et al., 2016). PAH

partitioning coefficients represent the movement of PAHs between media, and PAH release from soil into fluids is thought to be driven by carbon content in the soil (Siciliano et al., 2010). However, PAH partitioning to simulated intestinal fluid may not accurately represent the conditions in the gastrointestinal tract as partitioning is calculated at a pseudo-equilibrium state and soil will pass through the system before reaching equilibrium.

This research suggests that rather than recommending an appropriate bioavailability estimate, assuming a constant exposure from ingestion of impacted soil may be the preferred approach in risk assessment. Flip-flop kinetics of PAHs following oral exposure to impacted soils means that humans would experience equal systemic exposure from PAH impacted soils, regardless of soil concentration, provided PAH concentrations remain below 1,900 mg kg^{-1}. This soil concentration is much higher than the impacted site soils used in this study, although these concentrations may be present in the environment in heavily contaminated sites (USEPA, 1995). For soil PAH concentrations exceeding 1,900 mg kg^{-1}, a bioavailability estimate of 20% for BaP equivalents would provide a conservative estimate of systemic human exposure to PAHs. Additionally, DNA adduct formation, oxidized protein concentrations, and micronucleated reticulocyte formation do not depend on dose, even in subchronic exposure. Rather, the magnitude of these biomarkers depends on duration of exposure. This supports the recommendation of assuming constant internal exposure to PAHs in risk assessment as formation of biomarkers of effect depended more on exposure duration than exposure magnitude.

6.2 Future Directions

Future PAH bioavailability studies should work to assess impacted site soils with PAH concentrations higher than the soils used in this research. Limitations for the dose-response curve generated between AUCs and soil concentrations remain the large gap between low and high doses (approximately 39,500 mg kg^{-1}), as well as the use of spiked artificial soil for the high dose

range. The spiked soils had uniform composition and were not subjected to the degree of weathering as the impacted site soils, so these spiked soils may not have produced comparable results to the impacted site soils. Soils impacted by longstanding historical gas works sites can have PAH concentrations greatly exceeding those in the impacted site soils used in this research, although concentrations may not reach spiked soil concentrations from this research (USEPA, 1995). This could confirm the dose-response relationship observed in this study, as well as provide a more precise threshold estimate.

Additionally, characterizing different types of organic carbon in soil may help to explain variability seen in bioavailability estimates from real world soils. Soils may have both black carbon and amorphous carbon present. Black carbon is produced as a byproduct of incomplete combustion, and as such, can be associated with PAH impacts in soil, while amorphous carbon tends to represent the natural carbon present in the soil. These carbon sources have different properties and varying ratios will change the release rate of PAHs from soil organic carbon. Black carbon is considered to have slow release characteristics, while amorphous carbon releases PAHs more quickly. Knowing the ratio of black carbon to amorphous carbon in soil may assist in accounting for variability observed in calculating PAH bioavailability estimates.

Absolute and relative bioavailability estimates for impacted site soils typically use media spiked with arbitrary concentrations and ratios of PAHs as reference bioavailability estimates. However, there is evidence that varying the PAHs present in media can affect bioavailability (Lipniak-Gawlik, 1998). Thus, additional work assessing the absolute and relative bioavailability of soils using soil extract as the PAH source for IV and food media may address some of the variability observed in the bioavailability estimates generated in this research, and allow for more robust mathematical models predicting bioavailability values to be developed. This would

also allow us to determine how much variability is associated with soil properties as opposed to PAH ratios in soil.

In further development of juvenile swine as a model for human exposure, additional work could be done to assess the swine p53 response to PAHs and the role of swine p53 in nucleotide excision repair. Unlike humans, rodent models, commonly used as human surrogates in genotoxicity studies, do not exhibit p53 regulated nucleotide excision repair (Hanawalt, 2002). This research project demonstrated that the swine p53 gene may be upregulated in response to PAH exposure, but further work to determine if this response is similar in magnitude to that in humans is warranted. Additionally, confirmation that the p53 protein plays a role in the nucleotide excision repair pathway in swine is necessary for the swine model to be used as a human genotoxicity model.

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