Evaluating the Mutagenic Activities of Complex PAH Mixtures in Soil

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Abstract

Risk assessment models for complex mixtures involve (i) non-targeted approaches, based on the risk of a similar mixture, or (ii) targeted approaches, based on the cumulative risk of known mixture components. Ten PAH-contaminated soils were separated into non-polar and polar fractions that were mutagenic in both the Ames/*Salmonella* microsome assay and an *in vitro* mammalian genotoxicity assay employing the FE1 cell line. Targeted and non-targeted models of risk prediction provided overestimations of observed mutagenic activities of the non-polar soil fractions, suggesting non-additive interactions in complex mixtures of PAHs. This suggests that current risk assessment models for complex mixtures may provide conservative estimates of risk for soils contaminated with priority PAHs alone. However, significant underestimations of risk may be obtained if the soils also contain unidentified PAHs, PACs and related compounds, which contribute to the total mutagenic activity of the soil. Estimates of excess lifetime cancer risk resulting from exposure to these soils were assessed using several methods.

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List of Abbreviations

Amp	ampicillin
BjF	benzo[j]fluoranthene
° C	temperature in degrees Celsius
CCME	Canadian Council of Ministers of the Environment
CEPA	Canadian environmental protection act
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
eq.	equivalents
g	gram(s)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IARC	International Agency for Research on Cancer
Kan	kanamycin
kg	kilogram(s)
K _{ow}	octanol/water partition coefficient
M	Molar (moles per litre)
mg	milligram(s)
ml	millilitre(s)
mm	micrometer(s)
mm	millimetre(s)
mM	millimolar

MPa	Megapascal(s)
μg	microgram(s)
μl	microlitre(s)
NaCl	sodium chloride
NADP	nicotinamide adenine dinucleotide phosphate
NAPH	naphthalene
ng	nanogram(s)
PQRA	preliminary quantitative risk assessment
psi	pound(s) per square inch
RDX	cyclotrimethylenetrinitramine
rev	revertant(s)
rpm	rotation(s) per minute
SDS	sodium dodecyl sulfate
SQG _{HH}	soil quality guidelines for human health
Tet	tetracycline
US EPA	United States Environmental Protection Agency
v/v	volume/volume
w/v	weight/volume

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1.0 Introduction

It is estimated that 15,000 - 40,000 contaminated sites exist in Canada [1]. This estimate includes 6207 contaminated sites that are owned by federal departments, agencies and consolidated Crown corporations [2]. Contaminated sites are classified as such because they contain levels of contaminants deemed to be above acceptable levels and therefore, may pose an unacceptable hazard to human health. These sites contain pollutants such as polycyclic aromatic hydrocarbons (PAHs), heavy metals, petroleum hydrocarbons, polychlorinated biphenyls (PCBs), radioactive compounds and explosives. Forty percent of all federal contaminated sites contain elevated levels of PAHs, making PAHs the most abundant class of pollutants [2].

PAHs are known mutagens since they can create DNA adducts. If not repaired, these DNA adducts can result in the replication of an altered DNA sequence (i.e., a mutation). The accumulation of such mutations in oncogenes, tumor-suppressor genes and/or DNA repair genes can result in uncontrolled cell proliferation leading to carcinogenesis [3]. Therefore, mutagenesis is a useful toxicological endpoint to study since it can aid in the detection of carcinogenic agents.

The toxicological, mutagenic and carcinogenic effects of PAHs have been well documented [4-6]. Exposure to PAHs and PAH mixtures is associated with several types of cancers, including breast, esophageal, lung, gastric, colorectal, skin, prostate and cervical cancers [7-9]. PAH exposure can also result in other toxic effects including skin and eye irritation, as well as neuro-, hemato-, cardio-, renal, reproductive, immuno- and developmental toxicities [8]. Therefore, it is not surprising that a great deal of effort has been expended to evaluate the health risks posed by PAH-contaminated sites and there is a growing need to accurately assess the health risks posed by these sites. However, this is

not a simple task. PAH-contaminated sites usually contain complex mixtures of PAHs, related polycyclic aromatic compounds (PACs) and other pollutants, including heavy metals [10]. Moreover, the precise composition of the complex PAH/PAC mixtures is often qualitatively and quantitatively unknown, and may change over time [11, 12].

1.1 PAHs and related PACs

PAHs and PACs are widespread environmental pollutants that are composed of fused benzene rings in linear, angular or clustered arrangements. While the terms ‘PAH’ and ‘PAC’ are often used interchangeably, it is important to make the distinction. Here, the term ‘PAH’ is used to denote polycyclic aromatic hydrocarbons that are composed only of hydrogen and carbon atoms, while the term ‘PAC’ is used to denote related compounds such as oxy-PAHs (i.e., carbonylic PAHs), N-, O- or S-heterocyclics, nitroarenes, and aromatic amines. PAHs and PACs are produced as a result of incomplete combustion reactions associated with natural or anthropogenic processes. Natural sources, while relatively uncommon, include volcanic eruptions and forest fires. Anthropogenic sources include tobacco smoke, diesel emissions, coal tar from manufactured gas plants and coke ovens, and creosote from wood preservation activities [13]. Most PACs and PAHs are generated directly at the source of combustion [14]; however, some PACs, particularly oxy-PAHs, can be formed through the partial degradation of PAHs by bacteria or fungi [15-17], or via Phase I biotransformation reactions in mammals [18].

The knowledge that PAHs pose a threat to human health has prompted government agencies to evaluate the hazards of PAHs for regulatory purposes. Sixteen

PAHs are designated as priority pollutants by the United States Environmental Protection Agency (US EPA) [19]. In this thesis, these sixteen PAHs (Figure 1) will be referred to as priority PAHs. The Canadian Environmental Protection Act (CEPA) lists five PAHs as probable human carcinogens (BaP, BbF, BjF, BkF and I123cdP), and the International Agency for Research on Cancer (IARC) classifies three priority PAHs as probable human carcinogens (BaP, BaA and DahA), and four PAHs as possible human carcinogens (BbF, BkF, I123cdP and BjF) [6]. (Note that Figure 1.1 defines the PAH abbreviations used here.) The U.S. Environmental Protection Agency (US EPA) classifies seven PAHs as B2 carcinogens (i.e., probable human carcinogens). In this thesis, these PAHs (BaA, BaP, BbF, BkF, CHRY, DBahA and I123cdP) will be referred to as B2 PAHs or B2 carcinogens.

Several PACs are mutagenic in bacterial and mammalian cells [20-22], and some PACs, particularly oxy-PAHs can be even more toxic than their parent PAHs [10, 23]. However, few PACs have been classified by the US EPA, CEPA or IARC, and they are therefore not routinely included in risk assessments of PAH-contaminated sites. Representative PACs that have been found at a PAH-contaminated sites are depicted in Figure 1.2.

Figure 1.1 Structures of the 16 PAHs classified as priority pollutants by the US EPA.

Naphthalene
NAPH

ACNP

ACNPY

Fluorene
FLUOR

Anthracene
ANTH

Phenanthrene
PHEN

Pyrene
PYR

Fluoranthene
FLUORA

Benzo[a]anthracene
BaA

Benzo[k]fluoranthene
BkF

Chrysene
CHRY

Benzo[a]pyrene
BaP

Benzo[b]fluoranthene
BbF

Indeno[1,2,3-cd]pyrene
I123cdP

Dibenz[a,h]anthracene
DBaA

Benzo[g,h,i]perylene
BgHiP

Figure 1.2 Structures of selected polycyclic aromatic compounds detected at PAH
contaminated sites.

Benzothiophene

1-indanone

Phenanthridine

2-nitrocarbazole

Dibenzofuran

2-nitrofluorene

11-aminobenzo[a]pyrene

Anthracene-9,10-dione

PAHs at contaminated sites can pose a long-term health hazard because they remain strongly sorbed to organic matter in soil [10]. This is indicated by the low water solubility, high octanol-water partition coefficient (i.e., K_{ow}), and low vapour pressure of PAHs. Table 1.1 summarizes the log K_{ow} of selected PAHs and alkyl-PAHs. The log K_{ow} values for these compounds ranges from 3.3 for low molecular weight PAHs (2 rings) to 6.5 for high molecular weight PAHs (6 rings). Table 1.2 shows the log K_{ow} of some polar PACs. These compounds have notably lower log K_{ow} values than their parent PAHs.

Table 1.1 Hydrophobicity, as log K_{ow} of selected PAHs and alkyl-PAHs.

PAH	Log K _{ow} ^a
Naphthalene*	3.31
2-Methylnaphthalene	3.82
1-Methylnaphthalene	3.82
Biphenyl	4.03
2,6-Dimethylnaphthalene	4.31
Acenaphthylene*	4.02
Acenaphthene*	3.77
2,3,5-Trimethylnaphthalene	4.76
Fluorene*	4.08
Phenanthrene*	4.49
Anthracene*	4.49
1-Methylphenanthrene	4.99
Fluoranthene*	4.95
Pyrene*	4.95
Benzo[<i>a</i>]anthracene*	5.66
Chrysene*	5.66
Benzo[<i>b</i>]fluoranthene*	6.12
Benzo[<i>k</i>]fluoranthene*	6.12
Benzo[<i>e</i>]pyrene	6.12
Benzo[<i>a</i>]pyrene*	6.12
Perylene	6.12
Dibenz[<i>a,h</i>]anthracene*	6.84
Indeno[<i>c,d</i>]pyrene*	6.58
Benzo[<i>g,h,i</i>]perylene*	6.58

* denotes priority PAHs

^a K_{ow} values predicted using clogP (Daylight Chemical Systems Information Inc., Aliso Viejo, CA, USA).

Table 1.2 Hydrophobicity, as log K_{ow} , of selected PACs^a.

PAC	Log K _{ow} ^b	Classification
1-Indanone	1.72	Oxy-PAH
2-Methylanthracenedione	3.48	Oxy-PAH
9-Fluorenone	3.48	Oxy-PAH
Anthracene-9,10-dione	2.14	Oxy-PAH
Benzantracene-7,12-dione	4.65	Oxy-PAH
Benzothiophene	3.17	S-heterocyclic
Carbazole	3.52	N-heterocyclic
Dibenzofuran	4.09	Oxy-PAH
Dibenzothiophene	4.56	S-heterocyclic
Naphthacene-5,12-dione	3.00	Oxy-PAH
7H-benz[<i>d,e</i>]anthracene-7-one	4.65	Oxy-PAH
1-nitropyrene	2.88	Nitroarene
Phenanthridine	3.14	N-heterocyclic
11-aminobenzo[<i>a</i>]pyrene	4.17	Aromatic amine
1-nitrofluorene	2.40	Nitroarene

^a PACs were selected from a previous chemical analysis of soil at a manufactured gas plant site [16].

^b K_{ow} values predicted using clogP (Daylight Chemical Systems Information Inc., Aliso Viejo, CA, USA).

1.2 Sources of PAH contamination

All environmental PAH contamination can be classified as being of petrogenic or pyrogenic origin. As the name implies, petrogenic sources are petroleum-derived sources of PAHs and can include natural oil seeps, as well as waste generated from crude oil and petroleum products [13]. Pyrogenic sources are the most common sources of PAH contamination and arise from the high-temperature combustion of organic material [24]. The most prevalent source of PAH contamination is the anthropogenic production and release of coal tar and creosote from wood preservation processes, coal gasification and coke production [25].

Coal tar is generated as a by-product of coal carbonization that occurs in coke ovens, and during coal gasification at manufactured gas plants. The process of coking involves heating coal at extremely high temperatures in the absence of air to produce metallurgic coke that is used to feed blast furnaces for the production of steel [25, 26]. Coking is a process that is still in use today, and will likely continue in the foreseeable future [26]. At manufactured gas plants, coal is heated at extremely high temperatures in order to isolate the combustible gases [25]. This gas (known as town gas) was widely used for heating during the industrial revolution in Europe and North America. However, the use of town gas for municipal heating has now been replaced with heating by natural gas.

Until the early 20th century, there was no commercial use for coal tar and it was treated as waste and dumped at or near manufactured gas plants and coke ovens. Coal tar contamination at coke ovens and former manufactured gas plants is therefore widespread. However, the commercial value of coal tar was later realized, leading to the production of

creosote for wood preservation. Creosote is generated by the distillation of coal tar at 200 – 400 °C and the subsequent removal of crude naphthalane, phenolic and nitrogen-containing impurities [25]. Creosote is used as a wood preservative for products such as railroad ties, telephone poles and pier pilings [27]. Environmental contamination with creosote is often prevalent at wood-treating facilities and coal-tar processing plants.

Coal tar and creosote are complex mixtures of chemicals, and are known to contain more than 400 known compounds including many PAHs and PACs, although they may actually contain as many as 1000 different compounds [5, 28]. Considering the mutagenic and carcinogenic properties of these PAHs, it is not surprising that coal tar and creosote are also known human carcinogens [29, 30].

Prior to the 1970's, few regulations existed to guide the proper disposal of hazardous waste. Waste products were often dumped directly at the site itself, or were carried off-site for disposal by burial. Accidental spills occurring during the transport of PAH-containing substances are also the cause of some soil contamination. However, awareness of the environmental effects of the improper disposal of hazardous waste in the 1970's prompted the development of regulations guiding the management and remediation of present and future contaminated sites [1].

CCME guidelines

In Canada, the guidelines for the maximum allowable concentrations of PAHs at contaminated sites are defined by the Canadian Council of Ministers of the Environment (CCME). These guidelines establish the maximum allowable concentration of a chemical in soil that will not have a significant effect on the estimated incremental lifetime risk of

cancer. Chemicals that have been evaluated by the CCME are listed on the soil quality guidelines for human health (SQG_{HH}), while chemicals that are currently being evaluated are listed on the interim SQG_{HH} until a guideline becomes available [31]. BaP is the only PAH listed in the Canadian soil quality guidelines, while 9 PAHs are listed in the interim soil quality guidelines (i.e., BaA, BbF, BkF, I123cdP, DBahA, NAPH, PHEN and PYR). Table 1.3 outlines the maximum allowable levels of these PAHs set forth by the CCME in the Canadian soil quality guidelines.

Table 1.3 Maximum allowable PAH concentrations in Canada defined by the CCME.

PAH	CCME guideline ^a (mg/kg soil)
Naphthalene	22
Phenanthrene	50
Pyrene	100
Benzo[<i>a</i>]anthracene	10
Benzo[<i>b</i>]fluoranthene	10
Benzo[<i>k</i>]fluoranthene	10
Benzo[<i>a</i>]pyrene	0.7
Dibenz[<i>a,h</i>]anthracene	10
Indeno[1,2,3- <i>c,d</i>]pyrene	10

^a All levels were obtained from the interim SQG_{HH}, except that of benzo[*a*]pyrene which was obtained from the SQG_{HH} [31].

1.3 PAH and PAC biotransformation reactions

PAHs are indirect-acting mutagens in that they cannot react with DNA until they are metabolically transformed to reactive species that can readily covalently bind to DNA and induce the formation of mutations. To form DNA adducts in mammalian cells, PAHs require cytochrome P-450 (CYP)-mediated oxidation to produce reactive dihydrodiol-epoxides. First, CYP1A1 and/or CYP1A2 catalyze the formation of ring epoxides. Next, epoxide hydrolase catalyzes the formation of a dihydrodiol. The dihydrodiols can then be further metabolized by CYP enzymes to form dihydrodiol esters that can readily bind to DNA to form DNA adducts [32]. Figure 1.3 summarizes this process for BaP, one of the most mutagenic PAHs. This figure shows the formation of the most common BaP metabolite, (+) *trans-anti*-10-(deoxyguanosin-N²-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; however several other stereoisomers can be formed [33]. It should also be noted that not all reactive PAH metabolites will form DNA adducts. Some will be conjugated to glutathione, glucuronic acid or sulfate during Phase II biotransformation reactions that prepare metabolized PAHs for excretion via the biliary system [5, 33]. For a more thorough review of PAH biotransformation reactions, refer to Parkinson [42] or Josephy [43].

Some PACs, namely oxy-PAHs, can readily form DNA adducts without the need for further biotransformation by microsomal enzymes (i.e. they are direct-acting mutagens) [32]. However, most PACs, like the aforementioned PAHs, are also indirect-acting mutagens. Nitroarenes, aromatic amines and N-heterocyclics can be metabolized by oxidative microsomal enzymes in a similar manner to that described above. However, in the absence of microsomal enzymes, nitroarenes can still exert a mutagenic effect

following metabolism by nitroreductases (e.g., bacterial type I and type II nitroreductases) and O-acetyltransferases [22, 34]. Nitroarenes are first converted to arylhydroxylamine intermediates via the reduction of the nitro moiety by nitroreductases (e.g., *Salmonella* classical nitroreductase (Cnr) or *Salmonella* major nitroreductase (Snr)). Acetylation of the arylhydroxylamine by O-acetyltransferase (OAT) followed by deacetylation of the N-acetoxyarylamine intermediate, produces reactive nitrenium and carbenium electrophiles that can react with DNA to form adducts. Aromatic amines and N-heterocyclic compounds can also be transformed by metabolism focused on the nitrogen atom; however, they require initial oxidation by CYP enzymes to generate the arylhydroxylamine. Figure 1.4 demonstrates the biotransformation of 1-nitropyrene and 1-aminopyrene to electrophilic nitrenium and carbenium ions and subsequent DNA adduct formation.

Bacterial nitroreductases have been thoroughly characterized, and are divided into two categories: oxygen insensitive Type I and oxygen sensitive Type II nitroreductases [35]. In mammals, nitroreductase activity is thought to be largely provided by NAD(P)H:quinone oxidoreductase (i.e., DT-diaphorase), xanthine oxidase, and in some cases, aldehyde oxidase. In bacteria acetyltransferase activity is provided by OAT, and in mammalian systems this activity is provided by N-acetyltransferases [36, 37].

Figure 1.3 Biotransformation of benzo[*a*]pyrene to one of its reactive metabolites, (+) benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide, to form the DNA adduct, (+) 10-(deoxyguanosin-N²-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene.

Figure 1.4 Biotransformation of 1-nitropyrene and 1-aminopyrene to produce the DNA adduct, 1-nitropyrene-C⁸-deoxyguanine. Note that in *Salmonella*, reduction reactions are carried out by the classical nitroreductase (Cnr) or the major nitroreductase (Snr). In mammalian systems, reduction can be carried out by several enzymes, including enzymes NAD(P)H:quinone oxidoreductase (i.e., DT-diaphorase), xanthine oxidase and aldehyde oxidase.

1.4 Genetic toxicology of PAHs and PACs

The first indication that exposure to environmental agents is associated with the enhanced incidence of adverse health effects was noted by British surgeon Percival Pott in 1775. He noted a high incidence of scrotal cancer among chimneysweeps in England and concluded that ‘chimney sweeper’s cancer’ might be the result of exposure to chimney soot. Subsequent observations in the late 19th and early 20th century confirmed that exposures to coal tar, chimney soot, as well as coal tar and shale oil distillates did, in fact, induce cancer [38-42]. The carcinogenic nature of these substances is now well documented [30], and it is now known that PAHs and other PACs are the causative agents of cancers induced by coal tar and related substances [30, 41, 43].

The mutagenic properties of physical (e.g. X-rays) and chemical agents (e.g. sulfur mustard) were first documented in the early 20th century [44, 45]. Research carried out in the 1970’s by Bruce Ames and colleagues showed that many mutagens are carcinogens [46, 47], thus establishing a clear empirical and mechanistic link between somatic mutation and cancer. For the past 30 years, the knowledge that environmental agents can constitute a mutagenic and carcinogenic hazard has prompted the concerns of both the general public and the scientific community.

The field of environmental genetic toxicology involves assessment of the genotoxic hazards associated with many occupational and environmental agents. Genotoxic effects can include mutations (i.e., DNA sequence changes) or chromosomal aberrations that result in changes in chromosome structure and/or chromosome number [48]. Structural or numerical chromosomal aberrations can be measured using *in vivo* and *in vitro* methods including the micronucleus assay and the chromosomal aberration

assay [48]. Several *in vivo* and *in vitro* genotoxicity assays have also been developed to measure DNA sequence changes and gene mutations. These are described in further detail below.

The most popular *in vitro* gene mutation assay is the Ames/*Salmonella* mammalian microsome assay, also commonly referred to as the *Salmonella* mutagenicity assay [46, 49, 50]. Similar short-term microbial bioassays include the *trp* reversion mutation assay [51], the *lac*⁺ reversion assay [52], the Mutatox test™ [53] and the BetaGlo® assay [54]. These assays are relatively inexpensive and easy to perform, however they cannot accurately reflect the metabolic, toxicokinetic and DNA repair systems that are present in mammalian systems. Therefore, the assays are generally used as an initial indicator of genotoxicity, and provide guidance for subsequent *in vivo* studies.

Several transgenic rodent mutation systems (reviewed in Lambert et al [48]) have been developed to study gene mutations *in vivo*. Such systems include the Muta™Mouse [55], the BigBlue® mouse and rat [56], the *lacZ* plasmid mouse [57], and the *gpt* delta mouse [58]. These systems can account for toxicokinetic factors and DNA repair processes present in mammals, however they are very costly and time-consuming.

Several mammalian cell lines have also been developed to facilitate *in vitro* mutation studies. Examples of such cell lines include the Muta™Mouse flat epithelial (FE1) cell line [59], the BigBlue® Rat2 line [60] and Chinese hamster ovary (CHO) cells [61]. These cell lines are often used to avoid the time and cost required for *in vivo* studies, while preserving some of the relevant metabolism and toxicokinetic considerations. However, unlike *in vivo* assays, these cell lines cannot account for

processes that may modulate genotoxicity activity at the level of a whole animal (e.g., detoxification and excretion via the bile and urine).

1.4.1 The *Salmonella* mutagenicity assay

The *Salmonella* mutagenicity assay employs a set of histidine-dependent strains (i.e., auxotrophs) of *Salmonella typhimurium* to detect reverse mutations induced by suspected mutagens [50, 62]. The *Salmonella* strains employed contain known mutations at specific *his* loci, and following exposure to a suspected mutagen, reversion frequency is assessed by the ability to grow on minimal medium containing only a small amount of histidine. The small amount of histidine provided is sufficient to allow a few rounds of replication, but is insufficient for sustained growth of histidine auxotrophs [49]. Thus, only bacteria with reverse mutations (i.e., *his*⁺ genotype) form colonies that can be subsequently enumerated.

The *Salmonella* strains used in this assay are engineered to detect either frameshift or base pair substitution mutations. For example, strain TA100 and YG1042 harbour the *hisG46* allele and reversion to *his*⁺ occurs by base-substitution mutations. TA98 and YG1041 harbour the *hisD3052* allele that reverts to *his*⁺ by frameshift mutation [49, 63]. Table 1.4 summarizes the *his* genotypes of the *Salmonella* strains used in this study.

The *Salmonella* strains harbour additional mutations and plasmid-borne genes that increase their susceptibility to chemically induced mutagenesis. The deep rough (*rfa*) mutation results in partial loss of the outer lipopolysaccharide layer, allowing large bulky chemicals to permeate the cell wall. Deletion of the *uvrB* gene results in a compromised nucleotide excision repair system, and an increased sensitivity to mutagenesis. The *uvrB*

deletion also results in partial deletion of the *bio* gene, and the media used in the assay must be supplemented with biotin. The aforementioned strains also carry the multicopy plasmid, pKM101. This plasmid carries the *mucA/B* genes, which encode an error-prone DNA polymerase, PolRI, that bypasses a range of structurally diverse DNA lesions with reduced fidelity, thus enhancing the observed mutagenic potency of many mutagens [64]. Plasmid pKM101 also contains the β -lactamase (i.e., Amp^R) gene, enabling selection by antibiotic resistance [62].

Some *Salmonella* strains have been developed for the purposes of screening particular classes of chemicals. For example, TA102 detects mutations at an ochre (TAA) DNA target contained on the plasmid pAQ1, and is thus useful for detecting chemicals that can induce mutations at A·T sites. This strain also has an intact nucleotide excision repair system, and is thus sensitive to cross-linking agents [62, 65]. YG1041 and YG1042 are derivatives of TA98 and TA100 and can therefore detect frameshift and bases substitution mutations respectively. However, these strains also harbour the multicopy plasmid pYG233, which contains the *OAT* and *cnr* genes, coding for O-acetyltransferase and *Salmonella* classical nitroreductase respectively [63]. Increased cellular activity of OAT and Cnr confer increased sensitivity to nitroarenes, aromatic amines and N-heterocyclic compounds (Refer to Section 1.3 for further details).

Since the *Salmonella* genome does not encode all the proteins necessary to metabolize and activate mutagens, the *Salmonella* assay is often carried out in the presence of an exogenously derived microsomal enzyme mixture commonly referred to as 'S9' [62, 66]. S9 is the 9000×g supernatant fraction of liver homogenate, and the most commonly used S9 mixture is derived from male Sprague-Dawley rats pretreated with

Aroclor 1254 [49]. This material contains the P-450 monooxygenases that are capable of biotransforming and activating mutagens such as PAHs [62].

1.4.2 Flat epithelial (FE1) cell line

Cell lines derived from transgenic rodents have been developed in an effort to more accurately assess the hazards of mutagens, while avoiding lengthy and expensive *in vivo* methods. One such assay utilizes the flat epithelial (FE1) cell line, derived from pulmonary epithelial tissue of the transgenic MutaTMMouse [59]. This cell line is particularly useful for mutagenicity testing because it expresses CYP1A1, an enzyme known to be involved in the biotransformation of PAHs, as well as several glutathione-S-transferase (GST) isozymes, which are involved in the conjugation of metabolized xenobiotics [59]. Moreover, since FE1 cells are derived from MutaTMMouse, they harbour the λ gt10*lacZ* shuttle vector (Figure 1.5), a transgenic DNA target containing the *lacZ* gene (encoding β -galactosidase) flanked by 2 lambda (λ) cohesive ends that facilitates its retrieval and subsequent scoring [67, 68].

Figure 1.5 λ gt10*lacZ* construct in MutaTMMouse FE1 cells (reproduced with permission from Lambert et al [48])

Following exposure to a suspected mutagen, FE1 genomic DNA can be purified, and the *lacZ* shuttle vector recovered by packaging into lambda bacteriophage [69]. The shuttle vectors can then be readily transferred to a *galE*⁻ strain of *Escherichia coli* C (*lacZ*⁻, *galE*⁻, *RecA*⁻, Kan^r, pAA119), and *lacZ*⁻ mutants detected using the selective agent phenyl-β-D-galactoside (PGal) [70]. The *galE*⁻ *E. coli* strain employed contains an inactive UDP-epimerase. When provided with PGal, a substrate for functional β-galactosidase, the strain is unable to convert UDP-galactose into UDP-glucose. Thus, breakdown of PGal in bacteria infected with lambda vectors harbouring wild-type *lacZ* from Muta™Mouse cells will contribute to accumulation of a toxic metabolite (i.e., UDP-galactose) and plaques will not form. However, bacteria infected with mutant copies of the *lacZ* transgenes will survive in the presence of the selective agent (i.e., PGal) and form scorable plaques. Thus, only mutant *lacZ* transgenes will be detected as plaques in the PGal positive selection assay [67]. Figure 1.6 illustrates the selection of *lacZ*⁻ mutants via the PGal positive selection system in *galE*⁻ *E. coli*.

Figure 1.6 The positive selection of *lacZ* mutants via PGal system. Bacteria infected with wild-type *lacZ* transgene copies are able to yield galactose and toxic UDP-galactose, thus inhibiting plaque formation. Bacteria infected with mutant *lacZ* transgenes are unable to produce galactose, and the absence of galactose (and UDP-galactose) permits plaque formation.

1.5 Risk assessment of complex PAH mixtures

Sites that are contaminated by coal tar and creosote contain complex mixtures of mutagens and carcinogens that are persistent pollutants. Therefore the evaluation and management of the health risks associated with these sites is imperative. The ultimate goal of risk assessment for a site contaminated with carcinogenic agents is to determine the excess lifetime cancer risk associated with a given level of exposure at a particular site. These data are then used to prioritize remediation and to determine suitability for agricultural, residential, commercial or industrial use. Risk assessment involves four main steps: (i) hazard identification, (ii) dose-response assessment, (iii) exposure assessment and (iv) risk characterization [71].

Ideally, the risk a contaminated site should be assessed on the basis of all carcinogens in the complex mixture present at the site. However, the identities of all carcinogenic agents in complex matrices, such as coal tar-contaminated soil, are not known, and alternative methods are required to predict the health risk of a contaminated site. These methods typically include a targeted, priority substances approach whereby the risk of a similar mixture is assessed, or the cumulative risk of targeted (i.e. known) chemical constituents in the mixture is assessed [9, 72]. These predictive approaches are commonly used by both US and Canadian regulatory agencies (i.e., US EPA and Health Canada) [73].

Risk assessments are not actual measures of adverse health effects, but are estimates of projected risk, that require a number of assumptions that ultimately influence the risk magnitude. Assumptions include exposure levels for various receptors, the

degree to which contaminants are absorbed by the human gastrointestinal tract, and the assumption that the risks posed by mixture components are cumulative (i.e., additive).

Although the mechanism of action of mutagenic/carcinogenic PAHs is well understood (Section 1.3), current research on the interactions of PAHs in complex mixtures is sparse and sometimes inconsistent. Most studies agree that mutagens (i.e., PAHs) exhibit additive effects in mixtures, however some studies have noted sub-additive effects at higher concentrations or doses [74, 75].

1.6 Hypotheses and objectives of study

Current risk assessment guidelines for complex chemical mixtures state that the risk posed by a complex mixture can be predicted by (i) the risk/hazards of a similar mixture or (ii) the cumulative risk of known mixture components, (e.g., priority PAHs). These guidelines do not consider interactions between mixture components or the risks associated with unidentified mixture components, and require an assumption of risk additivity for known mixture components. It is hypothesized that the mutagenic activity and/or carcinogenic risk of complex PAH mixtures at contaminated sites can be accurately predicted by (i) the mutagenic activity of a synthetic mixture composed entirely of priority PAHs detected at a given site, or (ii) the cumulative mutagenic activity of all priority PAHs detected at a contaminated site. The objectives of this thesis are to:

- (1) Evaluate the mutagenic activities of organic extracts/fractions of soils contaminated with PAHs and PACs from coal tar and/or creosote;
- (2) Evaluate the mutagenic activities of priority PAHs detected in contaminated soils;
- (3) Evaluate the mutagenic activities of synthetic mixtures of priority PAH that mimic the composition of PAH-containing soil fractions;
- (4) Determine if the observed mutagenic activity of the contaminated soil extracts/fractions can be predicted by (i) the cumulative mutagenic activities of all known priority PAHs/PACs or (ii) the mutagenic activity of synthetic priority PAH mixtures.

2.0 Materials and Methods

2.1 Soil samples

The soil samples analyzed in this thesis were obtained from five contaminated sites in Sweden, and were kindly donated by Lars Öberg and Staffan Lundstedt (University of Umeå; Umeå, Sweden). These sites include three former wood impregnation sites, one former gas works site, and one coke oven that is still in operation. Site characteristics are summarized in Table 2.1. These soil sites are currently being investigated by the North Sweden Soil Remediation Centre.

Table 2.1 Description of contaminated sites investigated in this study.

Site	Enterprise	Period	Known pollutants	Soil Description	Vegetation	Sample depth (cm)	Number of Samples
Holmsund, Umeå	Wood impregnation	1943 – 1983	Creosote, CCA ¹ , zinc	Filling material	Sparse	10 – 30	3
Forsmo, Sollefteå	Wood impregnation	1933 – 1950	Creosote, CCA	Material from esker	Sparse	0 – 20	2
Hässleholm	Wood impregnation	1946 – 1965	Creosote, CCA, zinc	Filling material	Sparse	40 – 60	2
Luleå	Coke oven plant	In operation	Creosote, CCA, zinc	Sediment	None	0 – 5	1
Husarviken, Stockholm	Gas works	1893 – 1972	Coal tar, heavy metals, cyanide	Filling material	Sparse	20 – 60	2

¹ CCA: chromated copper arsenate

2.2 Soil extraction and fractionation

2.2.1 Chemicals

All chemicals used for the pretreatment, extraction and fractionation of the soil samples were analytical grade chemicals ($\geq 99\%$) and were obtained from EMD Chemicals (Gibbstown, NJ).

2.2.2 Soil pretreatment

Treatment of collected soils is described in Lundstedt et al [10]. Briefly, samples were sieved to remove larger particles and air-dried at room temperature. The dry soil was ground using a mortar and pestle. All soil samples were stored at -20°C in the dark until use.

2.2.3 Soil extraction

Organic pollutants from all soil samples were extracted using pressurized liquid extraction with the ASE 200 Accelerated Solvent Extractor (Dionex Corp., Sunnyvale, CA). For each extraction, 4 g of soil was placed in a 5 ml extraction cell with an equivalent amount of anhydrous sodium sulfate. Two solvent mixtures were used to extract the organic material from the soil samples: hexane:acetone (1:1 v/v) and methanol:acetic acid (99:1 v/v). Both extraction steps occurred at 2030 psi (14 Mpa) and 150°C with a dynamic extraction time of 7 minutes and two static extractions of 5 minutes each. The two extracts were pooled together and evaporated with ultra-pure nitrogen gas to a final volume of approximately 1 ml, and reconstituted in 1 ml of hexane for clean-up and fractionation.

2.2.4 Sample fractionation

Silica gel columns were used to separate each soil extract into two fractions: non-polar neutral aromatic compounds (i.e. PAHs, alkyl-PAHs and O- and S-heterocyclics) and polar neutral aromatic compounds (i.e. N-heterocyclics, nitroaromatics and oxy-PAHs). The method employed is fully described in Lundstedt et al (2003) [10]. Briefly, glass columns were packed with 25 g of silica gel (10% (w/w) deactivated) and 2 g of anhydrous sodium sulfate. The columns were first rinsed with 80 ml of hexane and the total soil extract was then applied to the column. The columns were eluted with (i) 20 ml of hexane, (ii) 60 ml hexane:dichloromethane (3:1, v/v) and (iii) 120 ml dichloromethane. The first fraction (containing aliphatic hydrocarbons) was discarded, while the latter two fractions were collected separately, evaporated under a stream of nitrogen gas and reconstituted into 500 μ l of DMSO (Sigma Aldrich Canada Ltd, Oakville, ON).

2.3 Chemical analyses

Gas-chromatography/mass spectrometry (GC-MS) chemical analyses of the non-polar neutral soil fractions were carried out by S. Lundstedt in Umeå, Sweden and the methodology employed is described in detail in his doctoral thesis [16]. Briefly, the soil extracts were analyzed using a Fisons gas chromatograph with a 30m DB 5 capillary column (inner diameter 25 μ m, film thickness of 0.25 μ m (J&W Scientific; Folsom, CA)), coupled to an electron impact Fisons MD 800 mass spectrometer (MS) (Fisons Instruments; Manchester, UK). The MS was operated in full scan mode for identification and in single ion monitoring mode for quantification of PAHs. The mass/charge (m/z) ratios of the molecular and selected fragment ions of the 24 PAHs and alkyl-PAHs monitored in this study are listed in Table 2.2.

Table 2.2 PAHs and alkyl-PAHs investigated in this study.

PAH	CAS no.	Molecular weight	<i>m/z</i>
Acenaphthene*	83-32-9	154.2	154, 153
Acenaphthylene*	208-96-8	152.2	152, 151, 76
Anthracene*	120-12-7	178.2	178, 89
Benz[a]anthracene*	56-55-3	228.3	228, 114
Benzo[a]pyrene*	50-32-8	252.3	252, 126
Benzo[e]pyrene	192-97-2	252.3	252, 126
Benzo[b]fluoranthene*	205-99-2	252.3	252, 126
Benzo[g,h,i]perylene*	191-24-2	276.4	276, 138
Benzo[k]fluoranthene*	207-08-09	252.3	252, 126
Biphenyl	92-52-4	154.2	154, 77
Chrysene*	218-01-9	228.3	228, 114
Dibenz[a,h]anthracene*	53-70-3	278.3	278, 139
Fluoranthene*	206-44-0	202.3	202, 101
Fluorene*	86-73-7	166.2	166, 83
Indeno[1,2,3-cd]pyrene*	193-39-5	276.3	276, 138
1-methylnaphthalene	90-12-0	142.2	142, 71
2-methylnaphthalene	91-57-6	142.2	142, 71
2,6-dimethylnaphthalene	584-42-0	156.2	156, 78
2,3,5-trimethylnaphthalene	2245-38-7	170.3	170, 85
Naphthalene*	91-20-3	128.2	128, 64
Perylene	198-55-0	252.3	252, 126
1-methylphenanthrene	832-69-9	192.2	192, 96
Phenanthrene*	85-01-8	178.2	178, 89
Pyrene*	85-01-8	202.1	202, 101

* denotes priority PAHs

2.4 Priority PAHs and mixtures of priority PAHs

All PAHs were obtained from Sigma-Aldrich Canada Ltd (Oakville, ON) and were prepared fresh as required in DMSO. The priority PAHs studied in this thesis are listed in Table 2.2. Synthetic PAH mixtures, comprised solely of the 16 priority PAHs, were created to mimic the composition of each PAH-containing soil fraction. Each PAH mixture was prepared to provide a starting concentration of 500 μg total PAH/ml in DMSO.

2.5 Mutagenic activity of soil fractions

The mutagenic activities of the complex non-polar and polar aromatic soil fractions, obtained by silica gel fractionation, were evaluated using the Ames/*Salmonella* microsome assay and the FE1 MutaTMMouse mutagenicity assay. Note that the Ames/*Salmonella* microsome assay and the FE1 MutaTMMouse mutagenicity assay will be referred to as the *Salmonella* assay and FE1 assay respectively.

The mutagenicity of the non-polar neutral soil fraction (i.e. PAH-containing soil fraction) was predicted by (i) evaluating the mutagenic activity of synthetic priority PAH mixtures (Section 2.4) and (ii) evaluating the mutagenic activity of each priority PAH detected in the soil samples and subsequently employing an assumption of additivity to predict total activity. The predicted mutagenic activity of the non-polar aromatic soil fractions, based on an assumption of additivity of effects induced by the individual priority PAHs, was calculated according to Equation 1.

(1) Total Predicted Mutagenic Activity =

$$\sum_{i=1}^n \text{Observed Activity of Individual PAHs} \times \text{PAH Concentration in Soil}$$

for priority PAHs 1 through n.

Where the observed activity of PAHs is expressed as revertants per μg PAH and the concentration of PAHs in the soil is expressed as μg PAH/mg dry soil.

Predictions on the mutagenic potency of the polar aromatic soil fractions were not carried out, as the identity of the mutagenic compounds that comprise this fraction are unknown. The mutagenic activities of each priority PAH and priority PAH mixture were measured using the *Salmonella* assay and the FE1 assay.

Two-tailed student t-tests ($p \leq 0.05$) were carried out to determine if there was a significant difference between the observed and predicted values for the mutagenic potency of each non-polar neutral soil fraction [76]. Note that the standard errors of the predicted mutagenic activities were calculated as the standard error of prediction when based on additivity of PAHs, and as the standard deviation when based on a synthetic priority PAH mixture [77].

2.6 *Salmonella* Assay

2.6.1 Chemicals, media and media supplements

The protocols for the preparation of all reagents and media used in the *Salmonella* assay and the protocol employed for the standard plate incorporation version of the *Salmonella* assay were based on the Standard Operating Procedures of the Environmental Carcinogenesis Division of the US EPA (Research Triangle Park, NC). These operating procedures were adapted from Mortelmans & Zeiger [62]. All reagents used in the *Salmonella* assay were purchased from Sigma-Aldrich Canada Ltd unless otherwise specified. All water used for the preparation of reagents and media was purified using a Milli-Q Ultrapure water purification system (Millipore Corp., Bedford MA).

Stock solutions of 50X VB salts contained 0.04 M magnesium sulfate, 0.6 M citric acid monohydrate, 2.9 M potassium phosphate (dibasic) and 0.8 M sodium ammonium phosphate [78]. Vogel-Bonner (VB) Salts were filtered with a 0.45 μm Nalgene bottle-top filter (Nalge Nuc, Rochester, NY) and sterilized by autoclaving. Stock solutions of 1.7 M dextrose were also prepared and sterilized by autoclaving. Histidine/biotin stock solutions contained 0.4 M histidine 0.02 mM biotin, and were sterilized by filtration with a 0.2 μm Nalgene bottle-top filter.

Glucose minimal medium was used as the bottom agar in the *Salmonella* assay. Each batch of plates was prepared by autoclaving 2655 ml water and 45 g Difco™ granulated agar (1.5% w/v) (Becton, Dickinson & Company, Le Pont de Claix, France) for 25 minutes at 121°C using a MediaClave media sterilizer (Integra Biosciences; AG, Chur, Switzerland). Once the agar mixture was cooled to 50°C, 200 ml dextrose (2% v/v), 60 ml VB salts (1X), and 30 ml histidine/biotin solution were added to the medium.

The media was dispensed onto sterile petri dishes (26 ml each) using a Tecnomat automatic plate pourer (Fernwald, Germany). Plates were dried overnight at room temperature and then stored at 4°C until use.

Top agar used in the *Salmonella* assay contained 0.6% w/v agar and 86 mM NaCl. Top agar was dispensed into 13 mm x 10 mm glass tubes using a Calibrex 520 bottle-top dispenser (VWR; Mississauga, ON). Prior to each experiment, top agar was autoclaved for 20 minutes and maintained at approximately 50°C in a dry block heater (Dri-Block, Model # DB-3, Techne Ltd; Duxford, Cambridge, England).

2.6.2 S9 metabolic mixture

When exogenous metabolic activation was used it was prepared immediately prior to each experiment. The S9 mixture contained 2% (v/v) microsomal salt solution (0.4 M MgCl₂ and 1.65 M KCl), 5 mM glucose-6-phosphate (G6P), 4 mM nicotinamide adenine dinucleotide (NADP) (Roche Diagnostics; Laval, QC), 50% (v/v) 0.2 M phosphate buffer (pH 7.4), and 5% (v/v) Aroclor-1254 induced rat liver S9 prepared from male Sprague-Dawley rats (protein content: 39.3 to 40.2 mg/ml; Moltox Inc., Boone, NC). Once prepared, the S9 mixture was kept on ice for the duration of the assay.

2.6.3 *Salmonella* strains

Three strains of *S. typhimurium* were used for the *Salmonella* assay: TA98, TA100 and YG1041. TA98 and YG1041 detect frameshift mutations via reversion of the *hisD3052* mutation. TA100 detects base pair substitutions via reversion of the *hisG46* mutation. YG1041 is a derivative of TA98 that contains a multi-copy plasmid harbouring the *OAT* and *cnr* genes, which encode O-acetyltransferase (OAT) and the *Salmonella* classic nitroreductase (Cnr) respectively. The increased activity of OAT and Cnr confer

enhanced sensitivity to nitroarenes and aromatic amines, including N-heterocyclic compounds [63]. The *Salmonella* strains employed here are described in further detail in Table 2.3. TA98 and TA100 were purchased from Moltox Inc (Boone, NC), and YG1041 was provided by Dr. Takehiko Nohmi (National Institute of Health Sciences; Tokyo, Japan).

Prior to using tester strains in the *Salmonella* assay, a preliminary strain check was carried out to ensure the presence and functionality of each essential genetic feature [49, 62]. Growth on nutrient agar plates supplemented with the appropriate antibiotic (i.e., 25 µg/ml ampicillin for TA100 and TA98 and 25 µg/ml ampicillin and kanamycin for YG1041) ensured the presence and maintenance of pKM101 and pYG233 (in the case of YG1041 only). To ensure the presence of the *rfa* mutation, the strains were grown on a nutrient agar plate containing a filter disk treated with 10 µg crystal violet. After a 24-hour growth period, the presence of the *rfa* mutation was confirmed by a zone of inhibition surrounding the filter disk. Histidine dependence was confirmed by a lack of growth following a 72 hour incubation period on minimal medium containing 0.1 mg/ml biotin. Similarly, biotin dependence was confirmed by a lack of growth following a 72-hour incubation period on minimal medium containing 0.1 mg/ml histidine. The *uvrB* deletion (i.e., nucleotide excision repair) gene was confirmed by exposing half of a nutrient agar plate to UV light (30 W) for 8 seconds at a distance of 30 cm. A lack of growth after 24 hours on the UV-exposed half of the plate confirmed the deletion.

Salmonella frozen permanent cultures were prepared by adding 90 µl DMSO to 1 ml of overnight culture in 1.5 ml Eppendorf tubes and storing at -80°C. Overnight cultures of all *Salmonella* strains were grown in 20 ml of Oxoid Nutrient Broth #2 for 16

hours at 37°C and 200 rpm in a shaking water bath (Gyratory Water Bath Shaker, Model # GY6, New Brunswick Scientific; Edison, NJ). Overnight cultures of TA98 and YG1041 were prepared by inoculation with 100 μ l of frozen permanent culture, while overnight cultures of TA100 were prepared by inoculation with a single colony taken from a master plate. If required, overnight cultures were placed at 4°C in the morning until use in the *Salmonella* assay later the same day. Master plates of TA100 were prepared by streaking a frozen culture onto nutrient agar (1.5% agar w/v) and Oxoid nutrient broth (2.5% w/v) supplemented with 25 μ g ml⁻¹ ampicillin. Master plates were incubated for 24 hours at 37°C, and a fresh TA100 master plate was prepared every month.

Strain	Histidine Genotype	Target DNA Sequence	Description	Plasmid-Encoded Genes	Source/Reference
TA98	<i>hisD3052</i>	CGCGCGCG	TA1538(pKM101)	<i>MucA/B, Amp</i>	Maron & Ames (1983)
YG1041	<i>hisD3052</i>	GCGC	TA98(pYG233)	pKM101- <i>MucA/B, Amp</i> pYG233- <i>Cnr, OAT, Tet</i>	Hagiwara et al (1993)
TA100	<i>hisG46</i>	GGG	TA1535(pKM101)	<i>mucA/B, Amp</i>	Maron & Ames (1983)

2.6.4 *Salmonella* mutagenicity assay protocol

All *Salmonella* mutagenicity tests were performed under sterile conditions in a laminar flow hood (Model # BM6-2B-49, Microzone Corporation; Nepean, ON). The standard plate incorporation assay version of the *Salmonella* assay was used to test the mutagenic activity of all soil fractions, priority PAH mixtures, and individual priority PAHs. A brief overview of the method employed is provided below.

A 100 μ l aliquot of the appropriate strain of *S. typhimurium* overnight culture, plus the appropriate amount of the test substance (never exceeding 100 μ l), was added to each tube of top agar. Where required, 500 μ l of S9 metabolic mixture was also added to the top agar. The tube was briefly vortexed, poured evenly onto a minimal media plate and set to dry on a level surface. Once dry, the plates were incubated at 37°C for 72 hours.

A negative (solvent) control and positive control were used during each experiment. 2-aminoanthracene (2-AA) was used as the positive control when S9 metabolic activation was used (0.5 μ g per plate for TA100 and 1 μ g per plate for TA98 and YG1041). Methylmethane sulfonate (MMS) (1 μ g per plate) was used as a positive control for TA100 without metabolic activation and 2-nitrofluorene (2-NF) was used as a positive control for TA98 (2.5 μ g per plate) and YG1041 (0.5 μ g per plate). Each control was tested in triplicate. All positive controls used in the assay were purchased from Moltex Inc. (Boone, NC).

The *Salmonella* assay was used to detect mutations induced by each of the 16 priority PAHs, the synthetic priority PAH mixtures, as well as each of the non-polar neutral and polar aromatic soil fractions (Sections 2.6.5 and 2.6.6). In each case, at least

five concentrations were tested in triplicate. Each plate was counted twice using the ProtoCOL SR automated colony counter (Synbiosis; Frederick, MD) and the mean number of revertants per plate at each concentration was used in all data analyses.

2.6.5 *Salmonella* testing of priority PAHs and PAH mixtures

Each priority PAH and synthetic priority PAH mixture was tested using *Salmonella* strains TA98, TA100 and YG1041 with the addition of metabolic activation at concentrations up to 50 µg total PAH per plate.

2.6.6 *Salmonella* testing of soil fractions

The non-polar neutral and polar aromatic fractions of each soil extract were tested on *Salmonella* strains TA98, TA100 and YG1041 at concentrations up to 50 mg dry soil equivalent per plate. After initial testing, it was noted that the non-polar neutral fractions did not elicit a positive response unless exogenous metabolic activation by S9 was added, and all subsequent *Salmonella* assays on this fraction were performed with the addition of S9. The polar aromatic fractions were tested both with and without the addition of S9.

2.6.7 Statistical analyses

SAS v. 8.02 for Windows was used for statistical analysis of all *Salmonella* assay results [79]. Responses were considered positive if a concentration-dependent increase in the number of revertants was observed and at least a two-fold increase in the number of revertants occurred for two consecutive concentrations. Samples that did not result in a two-fold increase in the number of revertants, but showed a concentration-dependent increase in the number of revertants were considered as marginally positive responses. For each positive (or marginally positive) response, a mutagenic potency (revertants per µg PAH or revertants per mg eq. soil) was calculated. Mutagenic potency is defined as

the slope of the linear portion of the concentration-response curve as calculated using ordinary least squares linear regression analysis [80].

2.7 FE1 MutaTMMouse mutagenicity assay

2.7.1 Chemicals, media and media supplements

All reagents, media and media supplements were obtained from Gibco-Invitrogen (Burlington, ON) unless otherwise indicated.

2.7.2 Flat epithelial (FE1) cell line

A pulmonary epithelial cell line, FE1, derived from the transgenic MutaTMMouse, was used as a mammalian *in vitro* model for evaluating mutagenicity in this thesis [59]. FE1 cells were maintained in DMEM/-F12 (1:1) supplemented with fetal bovine serum (FBS) (2% v/v), penicillin (100 U/ml), streptomycin (100 µg/ml) and murine epidermal growth factor (1 ng/ml) (Roche Diagnostics; Laval, QC). Treatment medium, prepared without the addition of FBS, was used to maintain the cells during all chemical exposures. All incubations occurred at 37°C, 95% humidity and 5% CO₂ in a Sanyo Scientific CO₂ incubator (Model no. MC0-20A1C; Bensenville, IL). All cell culture handling was carried out in a sterile laminar flow hood (Canadian Cabinets Co. Ltd; Model no. BM6-2B; Ottawa, ON).

2.7.3 Mutagen exposures

Approximately 3×10^5 cells were seeded into 100-mm polystyrene culture dishes and grown overnight to approximately 20% confluence. The following morning, the medium on each plate was replaced with 5 ml of treatment medium containing 100 µl of the appropriate dilution of the suspected mutagen (dilutions were freshly prepared in

DMSO). For exposures requiring the addition of exogenous metabolic activation (i.e., S9), the treatment medium also contained 50 μ l Aroclor 1254 induced rat S9 (0.5% v/v) and 105 μ l of S9 cofactor mixture (1% v/v), which contained 1.0 mM Hepes pH 7.2, 1.25 mM $MgCl_2$, 8.25 mM KCl, 1.25 mM G6P and 1.0 mM NADP. The cells were incubated in the treatment medium for 6 hours, washed with phosphate buffered saline (PBS), and incubated for 72 hours in serum-containing medium (Section 2.7.2) for mutation fixation.

FE1 cells were exposed to the 16 priority PAHs as well as 3 non-polar neutral soil fractions (and corresponding priority PAH mixtures) as well as one polar aromatic soil fraction. In each case, at least four concentrations were tested in duplicate. A positive control (0.1 μ g/ml benzo[*a*]pyrene) and negative (solvent) control were run concurrently.

2.7.4 Cell lysis

Following the time period allowed for fixing mutations, the cells were washed in PBS and lysed overnight in a lysis buffer containing 10 mM Tris pH 7.6 (Caledon Laboratories Ltd; Georgetown, ON), 10 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich Canada Ltd), 150 mM NaCl, 1% (w/v) sodium dodecyl sulfate (SDS) and 1mg/ml proteinase K (≥ 20 Units/mg). The lysis buffer was prepared fresh as required and 3 ml of lysis buffer was added to each plate. The following day, each cell lysate was collected in a 15-ml Falcon tube and total genomic DNA was extracted as described below.

2.7.5 DNA extraction

Phenol:chloroform:isoamyl alcohol (25:24:1) was freshly prepared prior to each DNA isolation. Approximately 20 ml of phenol was vigorously mixed with 12 ml (0.6 volumes) of 1 M Tris (pH 8.0) in a 50-ml Falcon tube. The aqueous and organic phases

were then allowed separate for approximately 5 minutes. The aqueous (top) phase was removed and the remaining organic phase was treated with 12 ml of 0.1 M Tris (pH 8.0) as above. The aqueous phase was once again removed, and an equivalent amount of chloroform:isoamyl alcohol (24:1) (Caledon Laboratories Ltd) was added. The tubes were centrifuged at 2500 rpm for 10 minutes in a swinging bucket centrifuge (Sorvall Legend RT, Mandel Scientific Co. Ltd; Guelph, ON). The aqueous phase was removed, and the organic phase was used to extract the DNA from the cell lysates.

An equivalent volume (approximately 3 ml) of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each tube of cell lysate. The tubes were rotated end-on-end using a Caframo rotator (Caframo Ltd; Wiarton, ON) at 20 rpm for 20 minutes. The tubes were then centrifuged at 2500 rpm for 10 minutes (at room temperature) using a swinging bucket centrifuge (Sorvall Legend RT). The top layer (approximately 2.5 ml) was carefully removed with a glass pipette, and transferred to a new 15-ml Falcon tube. An equivalent amount (approximately 2.5 ml) of chloroform:isoamyl alcohol (24:1) was added to each tube and the tubes were rotated and centrifuged as above. The top layer (approximately 2 ml) was removed as before and transferred to a new tube. Twenty-five μ l of 5 mM NaCl (resulting in a final concentration of 200 mM) and 2 volumes of ethanol were added to each tube to precipitate the DNA. DNA was precipitated by manually rolling the tube horizontally on a slanted surface to promote the formation of a DNA bead. The precipitated and coalesced DNA was then spooled onto a sealed Pasteur pipette, briefly rinsed in 70% ethanol, air-dried, and dissolved in 100 μ l of TE buffer (10 mM Tris (pH 7.6), 1 mM EDTA). Isolated DNA was stored at 4°C until further use.

2.7.6 Packaging of transgenic *lacZ* DNA into λ phage

Genomic DNA was packaged into λ phage particles using the Transpack™ lambda packaging system (Stratagene; La Jolla, CA) immediately prior to mutation scoring via the PGal positive selection assay [69]. Briefly, 4 μ l of isolated DNA was transferred to a 1.5-ml Eppendorf tube using a wide-bore pipette tip. A 4.8 μ l aliquot of the ‘patented reagent 1’ of the Transpack system (i.e., red tube) was added to each tube, and thoroughly mixed with the DNA via pipetting. The tubes were spun down for 5 seconds at 10,000 rpm in a benchtop Eppendorf 5417C Centrifuge (Eppendorf Canada; Mississauga, ON) and incubated at 30°C in a water bath for 90 minutes. Following this incubation period, 4.8 μ l of the ‘patented reagent 2’ of the Transpack packaging system (i.e., blue tube) was added to each 1.5-ml Eppendorf tube, and treated as with the red tube. The tubes were once again incubated at 30°C for 90 minutes. Assembled phage particles were then suspended in 500 μ l of SM Buffer (0.1 M NaCl, 0.016 M MgSO₄, 0.05 M Tris (pH 7.6) and 0.01% (w/v) gelatin (J.T. Baker Chemical Co., Phillipsburg, NJ). The assembled phage particles were mixed by rotating at 20 rpm for 30 minutes. Finally, the phage particles with rescued vector sequences were briefly vortexed and spun down with the benchtop Eppendorf centrifuge prior to use in the PGal positive selection assay.

2.7.7 PGal positive selection assay

2.7.7.1 Bacterial strain

A *galE*⁻ strain of *Escherichia coli* (*E. coli* C *lacZ*⁻, *galE*⁻, *RecA*⁻, Kan^r, pAA119) was used in the PGal positive selection assay to score mutations in the *lacZ* transgene copies rescued from FE1 cell genomic DNA [70]. *E. coli* cultures were inoculated from

frozen stocks and propagated overnight in LB broth (2% w/v) supplemented with 0.2% (w/v) maltose (Fisher Scientific Ltd), 50 μ g/ml ampicillin and 20 μ g/ml kanamycin. Frozen permanent cultures of *galE*⁻ *E. coli* C were maintained at -80°C in 15% (v/v) glycerol.

Bottom agar for the PGal positive selection assay was composed of Difco agar (0.75% w/v), Luria-Bertani (LB) broth (0.5% w/v), and 0.1 M NaCl (0.64% w/v), and prepared by autoclaving for 35 minutes at 121°C. Once the agar mixture was cooled to 50°C, the media was dispensed onto sterile petri dishes (8 ml) using a Tecnomat automatic plate pourer. Plates were stored at 4°C until use. Top agar was the same as the bottom agar except for the addition of 10 mM MgSO₄. Top agar was prepared immediately prior to the assay, and was kept molten in a 50°C water bath.

2.7.7.2 *lacZ* positive selective assay protocol

Mutations induced in the *lacZ* transgenes rescued from FE1 cells were detected using the PGal positive selection system [59, 67]. An overnight culture of *E. coli* C *galE*⁻ was diluted 1:100 in LB broth and incubated for approximately 3.5 hours (until OD₆₀₀ ~ 0.1). The culture was centrifuged (Sorvall RC 5C Plus Centrifuge with HS-4 swinging bucket rotor, Mandell Scientific Co. Ltd.) at 2400 rpm for 10 minutes at 15°C. The bacterial pellet was re-suspended in half the original volume of cold LB broth supplemented with 10 mM MgSO₄.

For each concentration tested, 2 ml of culture was used for mutant selection, and 2 ml of culture was used for titre measurement (referred to as selective and titre cultures respectively). The assembled phage particles were added to the selective culture, and adsorbed for 30 minutes at room temperature. A 15 μ l aliquot of the adsorbed selective

culture was then transferred to a titre culture. The titre culture was then mixed with 32 ml of unsupplemented top agar, while the mutant culture was mixed with 32 ml of top agar supplemented with 0.3 % (w/v) phenyl- β -D-galactoside (PGal) (Sigma-Aldrich Canada Ltd). Each top agar tube was distributed evenly among 4 minimal media plates, dried on a level surface, and incubated at 37°C overnight. Mutant plaque forming units (pfu) on the selective plates and total pfu on the titre plates were scored manually the following day.

For each concentration tested, a mutant frequency (MF) was calculated as the ratio of mutant pfu to total pfu. Concentration response curves were constructed by plotting the net MF versus concentration. A mutagenic potency was calculated for each sample as the slope of the linear portion of the concentration response curve. Note that net MF, calculated as the observed MF minus the experiment-specific spontaneous MF was used in calculating all mutagenic potency values. This permits the elimination of day-to-day variations in observed spontaneous mutation frequencies.

2.7.8 FE1 testing of PAHs and PAH mixtures

Preliminary tests were carried out in which FE1 cells were exposed to each priority PAH at a concentration of 10 μ g/ml with and without metabolic activation by S9. Initial testing revealed that no significant difference in mutant frequency occurred with the addition of S9 (determined by Chi-squared tests; in no cases was $p \leq 0.05$), therefore all subsequent exposures occurred without S9. PAHs that induced a significant increase in mutant frequency at 10 μ g/ml were selected for further testing with at least four concentrations.

2.7.9 FE1 testing soil fractions

FE1 cells were exposed to three non-polar neutral soil fractions (Holmsund-1, Holmsund-2 and Husarviken-1) and one polar aromatic fraction (Holmsund-1) with and without the addition of exogenous S9 metabolic activation. Each soil fraction was tested up to a maximum dose of 24 mg soil/ml.

2.8 Cancer risk assessment

The excess lifetime cancer risk associated with exposure to soils from each of the 10 sites was calculated for a typical adult, as well as a construction worker. The techniques employed follow Health Canada's *Guidance on Human Health Preliminary Quantitative Risk Assessment* (PQRA) [81].

First, an exposure factor (EF) was calculated for both adults and construction workers (Equation 2). This exposure factor is a unitless number by which the average daily dose is adjusted based on the frequency and duration of exposure to the soil.

$$(2) \quad EF = \frac{D_1 \times D_2 \times D_3}{LE}$$

Where:

D_1 = Days per week exposed to the site. Adults and construction workers were assumed to be exposed for 5 days per week [81].

D_2 = Weeks per year exposed to the site. Adults were assumed to be exposed for 48 weeks of the year [81]. Construction workers were assumed to be exposed for 16 weeks (i.e. one season) of the year.

D_3 = Total years exposed to the site. Adults and construction workers were assumed to be exposed to the site for a total of 35 years (i.e. the duration of their career).

LE = Life expectancy. The current estimated life expectancy for adults in Canada is 75 years [81]. This life expectancy was assumed to be identical for both typical adults and construction workers.

Second, the average daily dose (ADD) of each B2 PAH inadvertently ingested during the exposure period was calculated. The ADD was calculated using both targeted and non-targeted approaches. The targeted approach calculates an estimated ADD for each B2 PAH present in the complex mixture (Equation 3), while the non-targeted approach calculates an estimated ADD for the complex mixture as a whole, expressed in terms of benzo[*a*]pyrene equivalents (Equation 4). These methods will be referred to as the 'B2 method' and the 'MP (mutagenic potency) method' respectively. The targeted B2 method allows for risk to be calculated based on the concentration of each known carcinogenic PAH in the complex mixture. The MP method assesses the risk of the complex mixture as a whole, accounting for the fact that there are carcinogenic agents in the mixture for which data is not available. Risk is therefore calculated in terms of the equivalent amount of benzo[*a*]pyrene that would be required to yield the mutagenic responses observed here.

$$(3) \quad ADD = \frac{C \times IR \times EF}{BW}$$

Where,

ADD = average daily dose of B2 PAH in the soil (mg PAH/day)

C = Concentration of each PAH in the soil (mg PAH/kg soil).

IR = Soil Ingestion rate (kg soil/day). Adults were assumed to consume 20 mg soil per day while construction workers were assumed to consume 100 mg soil per day during the exposure period [81].

EF = Exposure Factor as calculated in Equation 2.

BW = Body weight. A body weight of 70.7 kg was assumed for both adults and construction workers [81].

$$(4) \quad ADD = \left(\frac{\text{Activity}_{\text{soil}}}{\text{Activity}_{\text{BaP}}} \times IR \times EF \right) \times BW^{-1}$$

Where:

ADD = average daily dose of benzo[a]pyrene equivalents in the soil (mg BaP eq./day).

Activity_{soil} = The sum of the mutagenic activities of the non-polar neutral and polar aromatic soil fractions as measured in the FE1 assay (mutants/dry mg equivalent soil/ml) or the *Salmonella* assay using *Salmonella* strain TA98 (revertants/dry mg equivalent soil).

Activity_{BaP} = the mutagenic activity of benzo[*a*]pyrene (BaP) as measured in FE1 assay (mutants/mg BaP/ml) or the *Salmonella* assay using *Salmonella* strain TA98 (revertants/mg BaP).

IR = Soil Ingestion rate (mg soil/day). Adults were assumed to consume 20 mg soil per day while construction workers were assumed to consume 100 mg soil per day during the exposure period [81].

EF = Exposure Factor as calculated in Equation (2).

BW = Body weight. A body weight of 70.7 kg was assumed for both adults and construction workers [81].

Finally, the total excess lifetime cancer risk of each soil sample was calculated based on the ADD calculated using both the B2 method (Equation 5) and the MP method (Equation 6). Using the B2 method, risk was calculated for each B2 PAH, and an assumption of additivity was subsequently employed to calculate total risk. Using the MP method, the total risk of each soil was calculated using the total estimated exposure to benzo[*a*]pyrene equivalents, therefore alleviating the need for an assumption of additivity.

$$(5) \quad \text{Total Excess Lifetime Cancer Risk} = \sum_{i=1}^n (\text{ADD} \times \text{SF} \times \text{PEF})$$

for B2 PAHs 1 through n

Where,

ADD = Average daily dose ($\mu\text{g PAH/day}$) for each B2 PAH as calculated using B2 method (Equation 3).

SF = Slope factor. The slope factor for BaP is (7.3 per mg soil/kg/day) [82].

PEF = Potency equivalency factor. The PEF for each B2 PAH was used here (Table 2.4)

$$(6) \quad \text{Total Excess Lifetime Cancer Risk} = (\text{ADD} \times \text{SF} \times \text{PEF})$$

Where:

ADD = average daily dose of soil, expressed in terms of BaP equivalents using the BaP potency ratio method (Equation 4).

SF = Slope factor. The slope factor for BaP is (7.3 per mg soil/kg/day) [82].

PEF = Potency equivalency factor. The PEF for BaP was used here (PEF = 1).

Table 2.4 Potency equivalency factors (PEF) for B2 PAHs. All PEF values were obtained from WHO (1998) [83].

B2 PAH	Potency Equivalency Factor*
Benz[<i>a</i>]anthracene	0.1
Chrysene	0.01
Benzo[<i>b</i>]fluoranthene	0.1
Benzo[<i>k</i>]fluoranthene	0.1
Benzo[<i>a</i>]pyrene	1
Dibenz[<i>a,h</i>]anthracene	5
Indeno[<i>1,2,3-cd</i>]pyrene	0.1

3.0 Results

3.1 PAH content of soil samples

The soil samples studied in this thesis are contaminated with varying levels of PAHs derived from contamination with creosote and coal tar. This contamination arose from industrial activities including wood preservation, coal gasification and coking. The levels of 24 PAHs, including 16 PAHs listed as priority PAHs by the US EPA [19], were measured using GC-MS (refer to Figure 1.1 for abbreviations). The total concentration of PAHs in the soil samples ranged from 70 – 9306 μg per g dry soil. The concentrations of the different PAHs varied from each other by up to four orders of magnitude within a given soil sample, and the concentrations of the individual PAHs varied by up to three orders of magnitude across soil samples. For example, the level of FLUOR ranges from 7.6 to 2927 $\mu\text{g/g}$ dry soil and the level of ANTH ranges from 1.4 to 1003 $\mu\text{g/g}$ dry soil. Table 3.1 provides a detailed chemical analysis of each soil sample studied in this thesis.

Four of the ten soils were found to contain each of the 24 measured PAHs. The majority of the PAHs were found in all of the soils: NAPH (and its methylated derivatives), biphenyl and ACNP were the only PAHs that were not detected in all of the samples. The most abundant PAHs in the soil samples were FLUORA, ranging from 11 to 33% of the total, and PYR, ranging from 8 to 27% of the total. Soil samples obtained from wood preservation sites were found to contain high levels of PAHs with four rings (e.g. FLUOR and CHRY), while the gas works site (Husarviken) had high levels of both four- and five-ring PAHs. Meanwhile, the coke oven site (Luleå) had similar concentrations of all sizes of PAHs.

Priority PAHs account for between 85.9% and 95.6% of the total PAH content of each soil sample. The percent composition of B2 carcinogens in the soil samples ranges

from 18.3% to 60.1%. However, no trend could be observed between the proportion of priority PAHs or B2 carcinogens and the type of site from which the soil was obtained.

The relative proportions of certain PAHs in soil can often be used to differentiate between various sources of PAH contamination [24, 84-86]. For example, the ratios of PHEN/ANTH and FLUOR/PYR are temperature-dependent and can be used to differentiate between petrogenic and pyrogenic origins of contamination [84]. A PHEN/ANTH ratio of less than 10 suggests a primarily pyrogenic source, while a ratio of greater than 10 suggests a petrogenic source. Similarly, a FLUOR/PYR ratio of greater than 1 indicates a pyrogenic source and a ratio of less than 1 suggests a predominantly petrogenic source [24]. All samples derived from wood preservation sites (Hsund-1,2,3, Forsmo-1,2 and Hholm-1,2) had quite low PHEN/ANTH ratios, ranging from 0.1 to 0.6, and those derived from coke oven site (Luleå) and gas works sites (Hviken-1,2) had much higher P:A ratios, ranging from 3.4 to 4.9. However, since all PHEN/ANTH ratios were below 10, we can only conclude that the soil samples were all derived from pyrogenic sources. Nine of 10 FLUOR/PYR ratios were greater than one, ranging from 1.3 to 1.8. This also confirms that the PAH contamination in these soils originated primarily from high-temperature combustion sources. One soil extract (Hholm-1) had a FLUOR/PYR ratio less than one ($\text{FLUOR/PYR} = 0.9$), yet it can be safely assumed that this soil is of pyrogenic origin because of its high PHEN/ANTH ratio, as well as the fact that it was collected from the same area as Hholm-2, which is also of pyrogenic origin.

Table 3.1 PAH concentrations detected in contaminated soils (μg PAH/g dry soil).

	Hsund ^a -1	Hsund-2	Hsund-3	Luleå	Forsmo-1	Forsmo-2	Hholm ^b -1	Hholm-2	Hviken ^c -1	Hviken-2
Naphthalene*	9.9	2.5	3.5	13	ND ^d	0.69	6.9	ND	4.1	2.1
2-Methylnaphthalene	5.2	3.4	1.3	9.7	ND	0.26	1.7	ND	2.1	0.78
1-Methylnaphthalene	2.1	0.62	0.28	5.3	ND	0.14	0.48	ND	1.0	0.31
Biphenyl	0.69	0.37	ND	13	ND	ND	0.14	ND	1.1	1.4
2,6-Dimethylnaphthalene	5.3	99	1.1	12	2.4	ND	ND	4.1	0.33	ND
Acenaphthylene*	35	52	2.7	5.7	1.2	2.1	4.3	0.47	0.036	1.4
Acenaphthene*	51	773	44	19	14	4.5	0.77	88	ND	0.22
2,3,5-Trimethylnaphthalene	10	64	5.4	6.2	22	22	ND	8.7	0.13	1.0
Fluorene*	40	447	37	38	28	11	1.4	45	0.042	1.5
Phenanthrene*	46	214	38	73	11	12	11	5.6	6.6	15
Anthracene*	80	1003	58	15	41	78	25	50	1.4	4.4
1-Methylphenanthrene	35	150	36	3.8	53	69	1.9	29	1.4	1.7
Fluoranthene*	1045	2927	502	62	551	910	78	497	7.6	19
Pyrene*	728	1724	358	41	432	746	84	276	5.5	15
Benzo[<i>a</i>]anthracene* [†]	374	620	124	30	136	239	87	129	4.0	11
Chrysene* [†]	397	555	129	30	131	259	100	123	5.0	11
Benzo[<i>b</i>]fluoranthene* [†]	170	214	54	27	53	103	109	50	5.0	10
Benzo[<i>k</i>]fluoranthene* [†]	104	138	34	19	46	79	65	37	4.5	7.4
Benzo[<i>e</i>]pyrene	69	89	26	16	32	71	58	26	4.7	7.0
Benzo[<i>a</i>]pyrene* [†]	78	106	24	21	40	80	76	35	4.1	8.7
Perylene	22	27	6.5	6.3	9.5	18	26	10	1.5	2.6
Dibenz[<i>a,h</i>]anthracene* [†]	21	20	4.2	5.4	4.4	16	13	4.1	1.1	1.9
Indeno[1,2,3- <i>cd</i>]pyrene* [†]	32	47	11	18	18	46	41	16	5.2	9.4
Benzo[<i>g,h,i</i>]perylene*	18	31	8.6	14	13	43	27	11	6.0	6.7
Total PAH Conc.	3377	9306	1509	504	1636	2810	817	1447	70	139
Sum of Priority PAHs	3229	8874	1432	431	1520	2629	729	1367	60	125
Sum of B2 PAHs	1176	1700	380	150	428	822	491	394	29	594

* Denotes priority PAH

[†] Denotes B2 PAH

^a Hsund = Holmsund

^b Hholm = Hässleholm

^c Hviken = Husarviken

^d ND = not detected

3.2 Mutagenicity of soil fractions

Each contaminated soil sample was separated into (i) aliphatic compounds, (ii) non-polar neutral compounds (primarily PAHs), and (iii) polar aromatic compounds. The first fraction, containing the aliphatic compounds, was discarded and excluded from further analysis as it is expected to interfere with GC analyses of PAHs [16]. The mutagenic activities of the latter two fractions (containing non-polar and polar aromatic compounds, respectively) were evaluated using (i) the *Salmonella* mutagenicity assay; and (ii) the FE1 Muta™Mouse mutagenicity assay.

In 2000, the US EPA issued a supplemental guidance document to aid in the risk assessment of chemical mixtures [73]. In this document, the US EPA assumes that the health hazard of a chemical mixture can be defined by the sum of the hazards of the known mixture components, or by the hazard of a similar mixture. In Canada, similar methods are presented in the CEPA priority substances guidance and Health Canada's PQRA report [81]. These methods, which calculate total hazard as the sum of the hazard attributable to the known mixture components, were used in this study to predict the mutagenic activities of the aforementioned complex soil fractions. The predicted mutagenic activities of the PAH-containing soil fractions were first determined by evaluating the mutagenic activities of synthetic priority PAH mixtures prepared on the basis of the chemical compositions shown in Table 3.1. The second method by which the mutagenic activities were predicted involved evaluating the mutagenic activities of each priority PAH detected in the soils and subsequently employing an assumption of additivity to calculate the total predicted mutagenic activity for each soil sample. The mutagenic activities of the priority PAHs and the PAH mixtures were evaluated in the

same manner as the aforementioned soil fractions using (i) the *Salmonella* mutagenicity assay and (ii) the FE1 MutaTMMouse mutagenicity assay.

3.3 *Salmonella* mutagenicity assay

3.3.1 Positive and negative controls

In order to ensure consistent assay response, positive and negative controls were tested during each experiment. The number of spontaneous revertants induced by the solvent (DMSO) for each *Salmonella* strain are presented in Table 3.2 and the number of revertants induced by the positive controls for each strain are presented in Table 3.3.

Table 3.2 Mean number of spontaneous revertants induced by negative
(solvent) controls in the *Salmonella* mutagenicity assay.

Strain	S9	Revertants per plate $\pm$ SEM ^a	N ^b
TA98	-	29.9 $\pm$ 1.6	24
	+	33.7 $\pm$ 1.0	139
TA100	-	116.9 $\pm$ 4.6	18
	+	116.9 $\pm$ 2.4	126
YG1041	-	20.9 $\pm$ 1.7	24
	+	33.3 $\pm$ 1.2	101

^a Standard error of the mean.

^b Number of observations

Table 3.3 Mean number of revertants induced by positive controls in the *Salmonella* mutagenicity assay.

Strain	S9	Chemical	Dose ($\mu\text{g/ml}$)	Revertants per plate $\pm \text{SEM}^a$	N ^b
TA98	-	2-nitrofluorene	2.5	231 $\pm$ 8	12
	+	2-aminoanthracene	1	618 $\pm$ 22	47
TA100	-	Methylmethane sulfonate	1	820 $\pm$ 61	18
	+	2-aminoanthracene	0.5	375 $\pm$ 27	72
YG1041	-	2-nitrofluorene	2.5	312 $\pm$ 7	24
	+	2-aminoanthracene	1	572 $\pm$ 16	72

^a Standard error of the mean.

^b Number of observations.

3.3.2 Observed mutagenicity of the non-polar soil fraction

The mutagenic activities of the non-polar neutral fraction (i.e., PAH-containing fraction) were evaluated using the *Salmonella* mutagenicity assay with and without metabolic activation. However, in all four non-polar soil fractions initially tested, positive responses were not observed unless metabolic activation was used. Therefore, subsequent testing was conducted with metabolic activation. Each soil fraction was tested using three *Salmonella* strains: TA100, TA98 and YG1041. The mutagenic activities (and associated statistics) are presented in Table 3.4.

The mutagenic activities of the non-polar neutral fractions were generally higher for the frameshift strain (i.e., TA98), than for the base pair substitution strain (i.e., TA100). The highest TA100 mutagenic activity was 9.6 revertants/mg eq. soil for Luleå, while the highest TA98 potency was 27.8 revertants/mg eq. soil for Hsund-2. A higher mutagenic activity was observed for the metabolically enhanced frameshift strain (i.e., YG1041) than for its parent strain (i.e., TA98). The YG1041 mutagenic activities ranged from 1.7 revertants/mg eq. soil for Hviken-2 to 35.3 revertants/mg eq. soil for Hsund-1.

Overall, a higher proportion of samples tested positive (or marginally positive) with the frameshift strains (TA98 and YG1041) than with the base pair substitution strain (TA100). Moreover, a higher proportion of fractions tested positive with the metabolically enhanced YG1041, compared TA98. Figure 3.1 illustrates the number of PAH-containing soil (i.e., non-polar neutral) fractions that tested positive or marginally positive with each *Salmonella* strain.

Table 3.4 Mutagenic potencies of non-polar neutral (i.e., PAH-containing) soil fractions determined using the *Salmonella* mutagenicity assay with strains TA100, TA98 and YG1041 plus S9 metabolic activation.

Soil Site	Strain	Mutagenic Activity (revertants/mg eq. soil)	Standard Deviation	r ²	p	Mutation Ratio ^a
Forsmo1	TA100	NM ^b	-	-	-	-
	TA98	1.4	0.3	0.66	<0.0001	2.5
	YG1041	6.6	1.0	0.81	<0.0001	3.5
Forsmo2	TA100	3.2	1.1	0.40	0.012	1.8
	TA98	9.6	2.7	0.61	0.0078	2.5
	YG1041	6.1	1.2	0.75	0.0006	2.5
Hholm1	TA100	3.2	1.1	0.47	0.0144	1.3
	TA98	8.1	0.9	0.93	<0.0001	2.4
	YG1041	11.4	1.2	0.91	<0.0001	5.8
Hholm2	TA100	1.6	0.6	0.4	0.0272	1.1
	TA98	9.6	1.9	0.62	0.0001	1.9
	YG1041	16.7	2.1	0.90	0.0001	4.6
Hsund1	TA100	NM	-	-	-	-
	TA98	7.2	0.9	0.91	<0.0001	2.3
	YG1041	35.3	4.2	0.81	<0.0001	3.1
Hsund2	TA100	NM	-	-	-	-
	TA98	27.8	4.4	0.71	<0.0001	3.6
	YG1041	12.6	2.3	0.66	<0.0001	2.3
Hsund3	TA100	3.6	1.2	0.50	0.0104	1.3
	TA98	2.1	0.5	0.48	0.0015	1.8
	YG1041 ^c	10.7	2.3	0.69	0.0009	2.4
	YG1041 ^c	9.4	1.6	0.68	<0.0001	5.1
Hviken1	TA100	NM	-	-	-	-
	TA98	3.2	0.6	0.90	<0.0001	4.4
	YG1041	2.3	0.1	0.96	<0.0001	7.4
Hviken2	TA100	NM	-	-	-	-
	TA98	5.9	0.6	0.94	<0.0001	4.8
	YG1041	1.8	0.2	0.78	<0.0001	5.9
Luleå	TA100	9.6	2.0	0.69	0.0008	1.9
	TA98	4.0	0.6	0.79	<0.0001	5.3
	YG1041	5.0	1.1	0.68	0.0009	5.4

^a Mutation ratio is defined as the ratio between the mean number of revertants observed at the highest dose used in the calculation of the mutagenic activity and the mean number of spontaneous revertants observed for that day of experiments.

^b Not mutagenic.

^c This sample was tested on two occasions; the geometric mean mutagenic activity is used for all calculations.

Figure 3.1 Percentage of positive and marginally positive responses for the non-polar neutral soil fractions examined using *Salmonella* strains TA100, TA98 and YG1041.

3.3.3 Predicted mutagenicity of non-polar neutral soil fractions

The mutagenic activities associated with exposure to the non-polar neutral fractions studied here were predicted by (i) evaluating the mutagenic activities of reconstituted mixtures composed entirely of priority PAHs detected in the soil samples, and (ii) evaluating the mutagenic activities of all detected priority PAHs individually, and subsequently employing an assumption of additivity to calculate total predicted mutagenic activity. Refer to Section 2.5 for a more thorough overview of the calculation methods employed.

3.3.3.1 Mutagenicity of priority PAHs

Sixteen PAHs, defined by the US EPA as priority pollutants [19], were tested for their mutagenic activities using the *Salmonella* mutagenicity assay with exogenous metabolic activation. PAHs were not tested without the addition of metabolic activation since previous tests in our laboratory indicate that these PAHs do not elicit direct-acting mutagenic activity [87]. Eight of the 16 priority PAHs induced a positive response in at least two of *Salmonella* strains TA100, TA98 and YG1041. Positive responses were observed for BaA, BaP, BbF, BghiP, BkF, CHRY, I123cdP, and DBahA. No significant mutagenic response was observed for ACNPHY, ACNP, ANTH, PYR, PHEN, FLUOR or FLUORA in any of the *Salmonella* strains employed. Table 3.5 summarizes the mutagenic activities (and associated statistics) calculated for the priority PAHs that elicited a positive response.

Regardless of the *Salmonella* strain used, BaP elicited the largest mutagenic response, while DBahA elicited the smallest response. Of the PAHs that tested positive, only CHRY tested negative with TA100, and only DBahA tested negative with TA98.

All of the eight positive PAHs elicited a significant positive response in YG1041. A higher proportion of PAHs elicited positive (relative to marginally positive) responses in the frameshift strains, TA98 and YG1041, versus the base pair substitution strain, TA100. In general, the mutagenic activities of the priority PAHs on the nitroreductase- and O-acetyltransferase-enhanced frameshift strain YG1041 were greater than that for the parent strain TA98. A summary of the PAH responses in each *Salmonella* strain is presented in Figure 3.2.

Table 3.5 Metabolically activated mutagenic potencies of priority PAHs assessed with *Salmonella* mutagenicity assay using strains TA100, TA98 and YG1041.

PAH	Strain	Mutagenic Activity (revertants/ μ g PAH)	Standard Deviation	r^2	p	Mutation Ratio ^a
Benzo[a]anthracene	TA100	15.4	1.1	0.84	<0.0001	2.4
	TA98	4.7	0.6	0.62	<0.0001	3.0
	YG1041	7.1	0.7	0.87	<0.0001	3.5
Benzo[a]pyrene	TA100	57.4	9.4	0.55	<0.0001	2.5
	TA98	245.8	12.9	0.94	<0.0001	8.3
	YG1041	319.6	14.9	0.97	<0.0001	12.1
Benzo[b]fluoranthene	TA100	9.7	1.7	0.48	<0.0001	1.5
	TA98	1.9	0.5	0.29	0.0012	1.7
	YG1041	7.6	1.4	0.70	0.0001	2.5
Benzo[g,h,i]perylene	TA100	8.0	1.1	0.62	<0.0001	1.4
	TA98	7.8	1.2	0.59	<0.0001	2.5
	YG1041	15.8	2.8	0.67	<0.0001	4.3
Benzo[k]fluoranthene	TA100	20.7	2.4	0.72	<0.0001	2.0
	TA98	2.9	0.3	0.65	<0.0001	2.6
	YG1041	21.8	0.8	0.98	<0.0001	4.5
Chrysene	TA100	14.1	2.1	0.65	<0.0001	1.5
	TA98	0.8	0.2	0.44	0.0011	1.5
	YG1041	10.2	2.5	0.56	0.0013	2.2
Indeno[1,2,3-cd]pyrene	TA100	17.0	2.1	0.77	<0.0001	1.8
	TA98	12.8	1.0	0.84	<0.0001	2.8
	YG1041	15.4	2.0	0.79	<0.0001	3.3
Dibenz[a,h]anthracene	TA100 ^b	2.3	0.3	0.6	<0.0001	1.7
	TA100 ^b	5.4	1.5	0.05	0.00031	1.3
	TA98	NM	NM	NM	NM	NM
	YG1041	4.1	0.5	0.83	<0.0001	2.4

^a Mutation ratio is defined as the ratio between the mean number of revertants observed at the highest dose used in the calculation of the mutagenic activity and the mean number of spontaneous revertants observed for that day of experiments.

^b This sample was tested on two occasions; the geometric mean mutagenic potency is used for all calculations.

^c Not mutagenic

Figure 3.2 Percentage of positive and marginally positive responses observed for the sixteen priority PAHs detected by *Salmonella* strains TA100, TA98 and YG1041.

3.3.3.2 Additivity of PAHs to predict mutagenicity of PAH-containing soil fractions

The relative contributions of each PAH to the total mutagenic activity of each PAH-containing soil were calculated (not shown), and summed to predict the total mutagenic activity of each PAH-containing soil fraction (Section 2.5). These predicted mutagenic activities, based on complete additivity of the mutagenic activities of priority PAHs, are presented in Table 3.6.

In most cases, assuming additivity of priority PAHs overestimated the mutagenic activity. In total, 23 of 30 predictions were greater than observed, while 5 of 30 were less than observed. Only 2 of 30 predicted mutagenic activities were approximately the same as their observed activities. In the cases where the mutagenic activity of the non-polar soil fractions was underestimated, the priority PAHs were found to account for 30 to 89% of the activity. Moreover, overestimations were more common for the base pair substitution strain (i.e., TA100), than the frameshift strains (i.e., TA98 and YG1041). Figure 3.3 summarizes the observed and predicted mutagenic activities of the non-polar soil fractions, based on complete additivity of PAHs.

Table 3.6 Predicted mutagenic potencies of non-polar neutral soil fractions as determined
by additivity of priority PAHs.

Soil Site	Strain	Mutagenic Activity (revertants/mg eq. soil)	Standard Error of Prediction ^a	Ratio of Predicted: Observed Mutagenic Potency
Forsmo1	TA100	8.4	241.1	N/A ^b
	TA98	11.3	102.7	7.9
	YG1041	17.3	102.9	2.6
Forsmo2	TA100	14.8	241.0	4.6
	TA98	21.5	102.7	2.2
	YG1041	33.3	102.8	5.5
Hholm1	TA100	9.8	241.1	3.1
	TA98	19.7	102.7	2.4
	YG1041	29.0	102.9	2.5
Hholm2	TA100	7.1	241.2	4.5
	TA98	9.6	102.7	1.0
	YG1041	14.9	102.9	0.9
Hsund1	TA100	19.8	241.0	N/A
	TA98	21.9	102.7	3.0
	YG1041	36.0	102.8	1.0
Hsund2	TA100	28.7	240.8	N/A
	TA98	30.3	102.6	1.1
	YG1041	49.6	102.7	3.9
Hsund3	TA100	6.4	241.2	1.8
	TA98	6.8	102.7	3.3
	YG1041	11.3	102.9	1.1
Hviken1	TA100	0.6	241.3	N/A
	TA98	1.1	102.8	0.3
	YG1041	1.7	102.9	0.7
Hviken2	TA100	1.1	241.3	N/A
	TA98	2.3	102.8	0.4
	YG1041	3.4	102.9	1.9
Luleå	TA100	2.9	241.2	0.3
	TA98	5.5	102.8	1.4
	YG1041	8.2	102.9	1.7

^a Refer to Section 4.5 for discussion of standard error of predictions.

^b Not applicable; ratio could not be calculated because the response observed for the non-polar aromatic fraction was negative.

Figure 3.3 Predicted mutagenic activities of non-polar soil fractions, based on additivity of priority PAHs that were larger, smaller or equal to the observed mutagenic activities.

3.3.3.3 Relationship between PAH levels in soil and mutagenic activity of non-polar neutral soil fractions

Correlation analyses were employed to determine if the priority PAH concentrations were empirically related to the mutagenic activities of the soil fractions. The TA98 mutagenic activities were significantly correlated with the concentrations of 14 PAHs. The YG1041 and TA100 mutagenic activities were significantly correlated with the levels of only two PAHs each; the YG1041 mutagenic activities were correlated with BbF and DBaA concentrations, while the TA100 mutagenic activities were correlated with 1-methylnaphthalene and biphenyl concentrations. It is interesting to note that five PAHs that are not classified as priority PAHs were strongly correlated with the observed *Salmonella* TA98 and TA100 mutagenic activity (i.e., 1-methylnaphthalene, 2-methylphenanthrene, 2,3,5-trimethylnaphthalene, 2,6-dimethylnaphthalene and biphenyl). The Pearson correlation coefficients and associated probability values are summarized in Table 3.7.

Correlation analyses were also carried out to determine if the mutagenic activities observed for the non-polar neutral soil fractions were significantly correlated with (i) the total concentration of measured PAHs, (ii) the total concentration of priority PAHs, or (iii) the total concentration of PAHs classified as B2 carcinogens. The TA98 mutagenic activity was significantly correlated with each one of these, however no significant correlation was observed for TA100 or YG1041 mutagenic activities. The Pearson correlation coefficients and associated probability values are provided in Table 3.8.

Table 3.7 Pearson correlation coefficients showing correlations between PAH concentration and mutagenic activities of non-polar neutral fractions in *Salmonella* strains TA98, YG1041 and TA100^a.

PAH	TA98		YG1041		TA100	
	r^2	p	r^2	p	r^2	p
1-methylnaphthalene	-	-	-	-	0.50	0.0229
1-methylphenanthrene	0.66	0.0043	-	-	-	-
2,3,5-trimethylnaphthalene	0.74	0.0014	-	-	-	-
2,6-dimethylnaphthalene	0.84	0.0002	-	-	-	-
Acenaphthene*	0.62	0.0072	-	-	-	-
Acenaphthylene*	0.85	0.0001	-	-	-	-
Anthracene*	0.84	0.0002	-	-	-	-
Biphenyl	-	-	-	-	0.67	0.004
Benz[<i>a</i>]anthracene*	0.60	0.0081	-	-	-	-
Benzo[<i>b</i>]fluoranthene*	0.47	0.0292	0.41	0.0458	-	-
Benzo[<i>k</i>]fluoranthene*	0.46	0.0315	-	-	-	-
Chrysene*	0.51	0.0211	-	-	-	-
Dibenz[<i>a,h</i>]anthracene*	-	-	0.41	0.0455	-	-
Fluorene*	0.86	<0.0001	-	-	-	-
Fluoranthene*	0.74	0.0014	-	-	-	-
Phenanthrene*	0.74	0.0015	-	-	-	-
Pyrene*	0.65	0.0049	-	-	-	-

* denotes priority PAHs.

^a Only PAHs that showed a significant correlation ($p < 0.05$) in at least one *Salmonella* strain are presented.

Table 3.8 Pearson correlation coefficients showing the correlations between different measures of PAHs and the mutagenic activities of the non-polar neutral soil fractions (TA98 only).

PAH	r^2	p
Total B2 PAHs	0.41	0.0460
Total Priority PAHs	0.65	0.0048
Total PAHs	0.74	0.0013

3.3.3.4 Synthetic mixtures of priority PAHs

The mutagenicity of 10 reconstituted mixtures of priority PAHs were assessed using the *Salmonella* mutagenicity assay. These mixtures are composed entirely of priority PAHs and mimic the relative proportions of each of the 16 priority PAHs detected in the 10 non-polar neutral soil fractions. Preliminary testing of the mixtures did not reveal any direct-acting mutagenicity, therefore all further testing was carried out only with the addition of metabolic activation.

Each PAH mixture elicited a response in all three *Salmonella* strains (i.e., TA100, TA98 and YG1041), however a greater number of samples induced at least a two-fold increase in the number of revertants for the frameshift strains (i.e., TA98 and YG1041). No trend could be observed in terms of the strength of the responses observed across the different *Salmonella* strains. The TA98 activities were greater than the TA100 activities 5 of 10 times. Similarly, the enhanced YG1041 strain elicited responses that were greater than those observed for TA98 5 of 10 times. Table 3.9 presents the mutagenic activities (and associated statistics) for each priority PAH mixture.

Overall, 14 of 30 PAH mixture responses exceeded the corresponding observed mutagenicity of the non-polar neutral soil fractions. Ten of 30 PAH mixture responses were less than the corresponding observed mutagenic, and the mutagenic activity of six PAH mixtures were not significantly different from that of the non-polar neutral soil fractions. Figure 3.4 summarizes the comparisons of the mutagenic activities of the non-polar neutral soil fractions and the synthetic mixtures of priority PAHs.

Table 3.9 Mutagenic potencies of reconstituted priority PAH mixtures assessed using the *Salmonella* mutagenicity assay in the presence of metabolic activation.

Mixture	Strain	Mutagenic Activity (revertants/mg eq. soil)	Standard Deviation	r^2	p	Mutation Ratio ^a
Forsmo1	TA100	3.8	1.1	0.50	0.0031	1.3
	TA98	5.0	0.4	0.91	<0.0001	2.2
	YG1041	5.1	0.6	0.82	<0.0001	3.9
Forsmo2	TA100	8.5	2.1	0.51	0.0009	1.5
	TA98	16.1	1.8	0.88	<0.0001	3.7
	YG1041	21.0	2.9	0.84	<0.0001	3.2
Hholm1	TA100	13.9	0.7	0.97	<0.0001	2.5
	TA98	10.6	1.3	0.87	<0.0001	5.1
	YG1041	14.2	1.0	0.95	<0.0001	6.4
Hholm2	TA100	12.5	1.9	0.82	<0.0001	1.8
	TA98	5.5	0.8	0.88	<0.0001	2.4
	YG1041	10.3	1.2	0.88	<0.0001	3.2
Hsund1	TA100	45.1	7.7	0.71	0.0006	2.6
	TA98	4.6	0.6	0.68	<0.0001	1.6
	YG1041	3.2	0.4	0.80	<0.0001	1.7
Hsund2	TA100	71.1	23.8	0.47	0.0135	1.9
	TA98	19.6	2.7	0.84	<0.0001	1.8
	YG1041	24.3	6.3	0.53	0.002	2.2
Hsund3	TA100	14.6	1.1	0.95	<0.0001	1.8
	TA98	4.9	0.5	0.87	<0.0001	3.5
	YG1041	2.9	0.6	0.62	0.0005	1.9
Hviken1	TA100	1.1	0.1	0.93	<0.0001	2.5
	TA98	0.5	0.0	0.89	<0.0001	6.7
	YG1041	0.4	0.1	0.72	<0.0001	10.2
Hviken2	TA100	0.9	0.1	0.73	<0.0001	2.1
	TA98	0.9	0.1	0.71	<0.0001	6.7
	YG1041	1.0	0.1	0.78	<0.0001	9.5
Luleå	TA100	4.1	0.5	0.82	<0.0001	2.4
	TA98	6.7	0.5	0.93	<0.0001	5.7
	YG1041	4.5	0.4	0.87	<0.0001	7.7

^a Mutation ratio is defined as the ratio between the mean number of revertants observed at the highest dose used in the calculation of the mutagenic activity and the mean number of spontaneous revertants observed for that experiment day.

Figure 3.4 Predicted mutagenic activities of non-polar neutral soil fractions, based on synthetic mixtures of priority PAHs that were larger, smaller or equal to the observed mutagenic activities.

3.3.4 Mutagenicity of polar aromatic soil fractions

Each polar aromatic soil fraction was tested with and without metabolic activation in the *Salmonella* mutagenicity assay. Although the mutagenic responses elicited by polar aromatic soil fractions were significant, they were not as high as those observed for the non-polar neutral fractions (Section 3.3.2). Overall, a higher proportion of polar aromatic samples elicited a positive response when metabolic activation was employed. Regardless of the metabolic capacity of the system, a higher number of polar aromatic fractions tested positive with the frameshift strains (i.e., TA98 and YG1041), than with the base pair substitution strain (i.e., TA100). In fact, when metabolic activation with S9 was not used, none of the polar aromatic fractions elicited a positive response in the base pair substitution strain. Furthermore, regardless of the metabolic capacity of the system, a higher proportion of samples tested positive (versus slightly positive) with the metabolically enhanced YG1041 compared to TA98. The mutagenic activities (and associated statistics) for each polar aromatic soil fraction with and without metabolic activation are presented in Tables 3.10 and 3.11, respectively.

It should be noted that the magnitude of the responses observed without metabolic activation were much less than those observed in the presence of S9 metabolic activation. The enhanced YG1041 strain detected a higher number of positive responses (versus slightly positive responses) than TA98, and had higher mutagenic activities than TA98, regardless of the metabolic capacity of the system. Figure 3.5 summarizes the responses observed for the polar aromatic soil fractions with and without metabolic activation.

Table 3.10 Metabolically activated mutagenic potencies of polar aromatic soil fraction
detected with the *Salmonella* mutagenicity assay using strains TA100, TA98 and
YG1041.

Soil Site	Strain	Mutagenic activity (revertants/mg eq. soil)	Standard Deviation	r ²	p	Mutation Ratio
Forsmo1	TA100	NM ^a	-	-	-	-
	TA98	0.8	0.1	0.64	<0.0001	2.1
	YG1041	5.0	0.8	0.81	<0.0001	2.7
Forsmo2	TA100	3.8	1.0	0.52	0.0024	1.8
	TA98 ^b	7.1	1.6	0.74	0.0028	2.1
	TA98 ^b	12.5	2.3	0.69	0.0001	2.4
	YG1041	10.3	1.3	0.90	<0.0001	2.2
Hholm1	TA100	2.4	0.7	0.55	0.0058	1.2
	TA98	2.5	0.5	0.65	0.0003	2.3
	YG1041	12.0	0.9	0.94	<0.0001	7.7
Hholm2	TA100	NM	-	-	-	-
	TA98	0.8	0.2	0.64	<0.0001	1.8
	YG1041	2.8	0.2	0.94	<0.0001	4.4
Hsund1	TA100	3.8	1.4	0.37	0.0163	1.4
	TA98	3.7	0.3	0.94	<0.0001	3.0
	YG1041	9.3	0.6	0.95	<0.0001	8.6
Hsund2	TA100	NM	-	-	-	-
	TA98	2.8	0.4	0.79	<0.0001	2.3
	YG1041	4.2	0.5	0.86	<0.0001	5.0
Hsund3	TA100	1.9	0.4	0.60	0.0007	1.3
	TA98	0.9	0.1	0.82	<0.0001	2.3
	YG1041	4.1	0.2	0.97	<0.0001	2.9
Hviken1	TA100	2.5	0.5	0.54	0.0001	2.0
	TA98	0.7	0.1	0.69	0.0001	2.5
	YG1041	0.5	0.1	0.67	<0.0001	2.6
Hviken2	TA100	NM	-	-	-	-
	TA98	0.6	0.1	0.72	<0.0001	3.2
	YG1041	0.8	0.1	0.78	<0.0001	3.6
Luleå	TA100	6.0	1.3	0.69	0.0009	1.5
	TA98	1.0	0.1	0.87	<0.0001	3.0
	YG1041	1.8	0.1	0.92	<0.0001	5.2

^a Not mutagenic

^b This sample was tested on two occasions; mean mutagenic potencies will be used for all calculations

Table 3.11 Direct-acting mutagenic potencies of polar aromatic soil fraction measured with the *Salmonella* mutagenicity assay using strains TA98 and YG1041^a.

Soil Site	Strain	Mutagenic activity (rev/mg soil)	Standard Deviation	r ²	p	Mutation Ratio
Forsmo1	TA98	0.6	0.1	0.74	<0.0001	1.7
	YG1041	0.2	0.1	0.29	0.0204	1.5
Forsmo2	TA98	0.2	0.1	0.44	0.0029	1.3
	YG1041	0.7	0.1	0.62	0.0005	1.9
Hholm1	TA98	0.3	0.1	0.30	0.0358	0.6
	YG1041	0.5	0.2	0.39	0.0128	2.0
Hholm2	TA98	0.5	0.2	0.36	0.0178	1.5
	YG1041	0.3	0.1	0.26	0.0321	1.9
Hsund1	TA98	0.2	0.1	0.29	0.0204	1.5
	YG1041	0.3	0.1	0.47	0.0016	1.7
Hsund2	TA98	NM ^b	-	-	-	-
	YG1041	0.3	0.1	0.54	0.0005	1.7
Hsund3	TA98	0.4	0.1	0.75	<0.0001	1.6
	YG1041	0.4	0.1	0.53	0.0007	1.8
Hviken1	TA98	0.3	0.1	0.38	0.0066	1.4
	YG1041	1.0	0.2	0.76	<0.0001	2.4
Hviken2	TA98	0.3	0.1	0.58	0.0002	1.5
	YG1041	0.7	0.1	0.88	<0.0001	2.6
Luleå	TA98	0.2	0.1	0.25	0.0353	1.4
	YG1041	1.2	0.3	0.54	0.0062	1.8

^a No polar aromatic soil fractions tested positive with TA100

^b NM = Not mutagenic

3.4 Mutagenicity assessment using the Muta™Mouse FE1 assay

3.4.1 Positive and negative controls

Positive and negative controls were routinely tested to ensure proper functioning of the assay. BaP (0.1 $\mu\text{g/ml}$) was used as the positive control for the assay and the solvent (DMSO) was used as the negative control. Mean spontaneous mutant frequencies induced by DMSO and BaP-induced mutant frequencies are summarized in Table 3.12.

Table 3.12 Spontaneous and positive control mutant frequencies for Muta™Mouse FE1
cells^a

Agent	Min	Max	Mean Mutant Frequency $\pm$ SEM^b ($\times 10^{-5}$)
DMSO	16	75	37.0 ± 1.5
Benzo[<i>a</i>]pyrene	291	999	645 ± 44

^a N = 75

^b Standard error of the mean

3.4.2 Mutagenic potency detection limit for the MutaTMMouse FE1 assay

To account for day-to-day variation in the spontaneous mutant frequency the smallest meaningful increases in *lacZ* mutant frequency were calculated separately for soil fraction analyses, analyses of priority PAH mixtures and analyses of individual priority PAHs. These potency ‘detection limits’ were calculated as the slope of the line joining the observed 5th to 95th percentiles of the negative (DMSO) control across the range of soil (or PAH) concentrations tested. The detection limit calculated for the soil samples is 0.14×10^{-5} mutants/mg eq. soil/ml and that for the PAHs and PAH mixtures is 3.3×10^{-5} mutant frequency/ μ g PAH/ml.

3.4.3 MutaTMMouse FE1 mutagenicity in the presence of exogenous S9 activation

During the initial stages of testing, FE1 cells were exposed to the appropriate test substance with and without metabolic activation by exogenous S9. All exposures carried out with the addition of the exogenous metabolic activation system did not elicit a positive response. This phenomenon was observed for BaP (Figure 3.6), the Holmsund-1 PAH mixture, the Holmsund-2 non-polar neutral and polar aromatic soil fractions, and has been previously observed in our laboratory. This result is not unexpected since FE1 cells have an inherent metabolic capacity, and therefore do not require the addition of exogenous S9 [59]. Further addition of metabolic activation enzymes may inhibit the mutagenic activity of PAHs (See Section 4.2). All further testing was conducted without exogenous S9 metabolic activation.

Figure 3.6 *lacZ* mutant frequency induced by benzo[*a*]pyrene in the Muta™Mouse FE1 assay with and without metabolic activation by exogenous S9.

3.4.4 Mutagenicity of the non-polar neutral soil fraction

Three non-polar neutral soil fractions were tested for their ability to induce mutations in the *lacZ* transgene using the Muta™Mouse FE1 cell assay (i.e., Hsund-1, Hsund-2 and Hviken-1). FE1 cells were exposed to a range of doses of each fraction and a mutagenic activity was calculated as the slope of the linear portion of the dose response curve. The mutagenic activities observed for these three soil fractions are outlined in Table 3.13.

Each of the three samples induced a significant positive response; however, a difference of four orders of magnitude was observed between the samples. The highest mutagenic activity was observed for Hsund-2, while the lowest mutagenic activity was observed for Hviken-1. This range of activities is not unexpected given the large range in detected PAH levels.

Table 3.13 Mutagenic activities observed for non-polar neutral soil fractions with the FE1 assay.

Soil Site	Mutagenic Activity (net mutants ($\times 10^{-5}$)/mg eq./ml)	Standard Error	r^2	p
Hsund-1	16.0	3.0	0.65	0.0007
Hsund-2	522.7	35.3	0.77	<0.0001
Hviken-1	0.7	0.1	0.72	<0.0001

3.4.5 Predicted mutagenicity of non-polar neutral soil fractions

As described previously for the *Salmonella* mutagenicity assay (Section 3.3.3), the mutagenic activities observed with the FE1 assay were predicted using two methods. First, the mutagenic activity of each priority PAH was assessed with the FE1 assay, and the relative contribution of each priority PAH to the mutagenicity of each soil sample were calculated and summed to predict a total mutagenic activity for each of the three soils. Second, the mutagenic activity of each of the three soils was predicted using a reconstituted priority PAH mixture.

3.4.5.1 Mutagenicity of individual priority PAHs

FE1 cells were exposed to each PAH at an initial concentration of 10 µg/ml. If a significant positive response was not observed at this concentration (determined by Chi-squared test; in no cases was $p < 0.05$), no further testing was carried out as the response was considered to fall below the detection limit (see Section 3.4.2). However, if a significant response was observed at 10 µg/ml, FE1 cells were exposed to a range of concentrations and a mutagenic potency value was calculated for the PAH.

Five of 16 priority PAHs induced a significant increase in mutant *lacZ* frequency on the FE1 assay: BaP, BbF, BkF, CHRY and DBahA. The responses of these PAHs are summarized in Table 3.14. The remaining 11 priority PAHs did not elicit a significant response at 10 µg PAH/ml, and were not tested further (ACNP, ACNPHY, ANTH, BaA, BghiP, I123cdP, FLUORA, FLUOR, NAPH, PHEN and PYR).

Table 3.14 Mutagenic activities of priority PAHs detected examined using the FE1 assay.

PAH	Mutagenic Activity (net mutants ($\times 10^{-5}$)/ μg PAH/ml)	Standard error	r^2	p
Benzo[<i>a</i>]pyrene	3989.0	247.2	0.80	<0.001
Benzo[<i>b</i>]fluoranthene	63.8	5.6	0.76	<0.001
Benzo[<i>k</i>]fluoranthene	6.1	0.4	0.88	<0.001
Chrysene	3.9	0.4	0.77	<0.001
Dibenz[<i>a,h</i>]anthracene	362.1	38.5	0.57	<0.001

3.4.5.2 Additivity of PAHs to predict mutagenic activity of the PAH-containing soil fraction

The contributions of each PAH to the total mutagenic activity of each of the three non-polar neutral soil fractions were calculated and summed to predict total mutagenic activity (Section 2.5). These predicted mutagenic activities, based on complete additivity of the mutagenic activities of priority PAHs, are presented in Table 3.15. For Hsund-1 and Hviken-1, the PAHs overestimated the mutagenic activity by more than 2000-fold. However, for Hsund-2, the PAHs accounted for only 90% of the total observed mutagenic activity with the FE1 assay.

Table 3.15 Predicted FE1 mutagenic activities based on complete additivity of priority
PAHs.

Soil Site	Mutagenic Activity (net mutants ($\times 10^{-5}$)/mg eq./ml)	Standard Error	Ratio of Predicted/Observed Activity
Hsund-1	331.3	315.4	20.7
Hsund-2	446.1	315.1	0.9
Hviken-1	17.1	317.3	22.2

3.4.5.3 FE1 Mutagenicity of priority PAH mixtures

Three reconstituted priority PAH mixtures, corresponding to the non-polar neutral fractions of Hsund-1, Hsund-2 and Hviken-1, were tested for their ability to induce mutations in the *lacZ* transgene of FE1 cells. Each of the three PAH mixtures induced a significant response in the FE1 assay. The observed mutagenic activities of these mixtures are presented in Table 3.16. The mutagenic activities ranged 5 orders of magnitude, however, unlike the responses observed for the PAH-containing soil fractions, where Hsund-2 induced the largest response and Hviken-1 induced the smallest response, Hviken-1 induced the largest response, and Hsund2 induced the smallest response.

In 2 of 3 cases (i.e., Hsund-1 and Hviken-1), the synthetic priority PAH mixtures overestimated the observed mutagenic activity of the non-polar soil fractions. For Hviken-1, the activity was overestimated by more than 46,000 times. In the case of Hsund-2, the priority PAH mixture underestimated the observed mutagenic activity of the soil.

Table 3.16 Mutagenic activities of synthetic priority PAH mixtures determined using the
FE1 assay.

Mixture	Mutagenic Activity (net mutants ($\times 10^{-5}$)/mg eq. soil/ml)	Standard Error	r^2	p	Ratio of Predicted: Observed Activity
Hsund-1	42.2	2.9	0.84	<0.0001	2.634
Hsund-2	4.4	0.3	0.93	<0.0001	0.008
Hviken-1	35598.2	2264.4	0.93	<0.0001	46231

3.4.6 FE1 Mutagenic potency of polar aromatic soil fractions

The mutagenicity of the polar aromatic fraction from Hsund-2 was also tested on the FE1 assay. This fraction elicited a relatively low mutagenic potency of $3.89 \pm 0.35 \times 10^{-5}$ mutants/mg eq. soil/ml ($r^2 = 0.86$, $p < 0.0001$). This mutagenic activity is significantly elevated relative to spontaneous, however it is low relative to the other samples studied. Figure 3.7 depicts the response of the polar aromatic fraction of Hsund-2.

Figure 3.7 FE1 mutagenic activity of the polar aromatic soil fraction of Holmsund-2.

3.5 Cancer risk assessment

The total excess lifetime cancer risk associated with exposure to each of the contaminated soils studied here was calculated for a typical adult and for a construction worker. These two exposure scenarios required calculation of dose in mg BaP equivalents/kg body weight/day. Moreover, two separate methods were employed to calculate the dose of BaP equivalents. The method labeled “B2 method” used the concentration of each B2 PAH and published PEFs (potency equivalency factors) [83] to calculate the daily dose of BaP equivalents. The method labeled “MP method” (i.e., mutagenic potency method) used *Salmonella*- or MutaTMMouse FE1-derived mutagenicity data and a slope ratio method to derive an equivalent concentration, and ultimately dose of BaP equivalents (see Section 2.8). All assumptions used in the risk assessment calculations are outlined in Section 2.8 and are summarized below in Table 3.17.

Table 3.17 Assumptions for assessment of excess lifetime cancer risk due to non-dietary ingestion of contaminated soils by a construction worker and a typical adult.

Parameter	Assumption
Soil Contamination	The PAH levels in the contaminated soils are constant across time and space
Exposure	Exposures occur only at the contaminated sites
	Exposures occur only during a regular working day for the duration of the career of an adult and construction worker
	The soil ingestion rate for adults and construction workers is constant across time, space and subject
Bioavailability	The PAHs present in the contaminated soils are adsorbed and bioavailable to the same extent as in food (i.e. oral slope factors for non-dietary and dietary ingestion are the same)

Regardless of the method used to calculate the daily dose, the excess lifetime cancer risk associated with exposure to these contaminated soils is greater for a construction worker than for an adult. In general, soils with higher levels of B2 PAHs had higher estimates of excess lifetime cancer risk. Furthermore, risk estimates calculating using the MP-Salmonella method are always greater than those calculated using the B2 method. Risk estimates calculated using the MP-FE1 method are significantly lower than the risk estimates calculated using the MP-Salmonella or B2 methods. The excess lifetime cancer risks for a construction worker and an adult are presented in Figure 3.8 and 3.9 respectively.

Figure 3.8 Excess lifetime cancer risk associated with the non-dietary ingestion of contaminated soils by a construction worker. Each of the ten soils is plotted with respect to the total concentration of B2 PAHs present in the sample (ranging from 28.9 to 1700 μg B2 PAHs/g soil).

Figure 3.9 Excess lifetime cancer risk associated with the non-dietary ingestion of contaminated soils for a typical adult. Each of the ten soils is plotted with respect to the total concentration of B2 PAHs present in the sample (ranging from 28.9 to 1700 μg B2 PAHs/g soil).

4.0 Discussion

4.1 PAH contamination of soils

The levels of twenty-four PAHs were measured in ten coal tar- and creosote-contaminated sites. All 16 priority PAHs were detected in all of the soil samples, with the exception of naphthalene and acenaphthene, which were only detected in 80% and 90% of the soils, respectively. The other PAHs detected (see Table 3.1) include biphenyl and alkylated derivatives of several priority PAHs. The total PAH concentration ranged from 70 to 9306 $\mu\text{g/g}$ dry soil (geometric mean = 1023 $\mu\text{g/g}$ dry soil). These may be considered elevated concentrations, but they are not unexpected given that the samples were collected from industrial areas [88].

PAHs have previously been found in creosote-contaminated soils [27, 89, 90], which is not unexpected since PAHs comprise approximately 85% of all identified compounds in creosote [91]. Previous studies have also detected PAHs in coal tar-contaminated soils found at manufactured gas plant and coke oven sites [26, 92-95]. Contamination arising from the production of coal tar (including creosote) also contain N-, S- and O-heterocyclics and oxy-PAHs [10, 27, 91, 96], however, these compounds were not measured in the soils studied here.

Maximum allowable PAH levels

In Canada, the Canadian soil quality guidelines for human health (SQG_{HH}) define the maximum allowable PAH concentrations for soil at industrial sites [97]. These guidelines are used as a tool to determine if the levels of contaminants in soil could pose a threat to humans. The levels of BaP in all soils studied here exceed the recommended level of 0.7 mg/kg in the SQG_{HH} by at least one order of magnitude. The geometric mean

BaP concentration for the soils studied here is 32.4 mg/kg. The levels of all other PAHs listed in the guidelines, except naphthalene, are exceeded for most of the soils examined. Therefore, under CCME guidelines, from a targeted chemical perspective, every soil studied here would be categorized as unacceptably hazardous to humans.

Sources of PAH contamination

The ratios of PAHs detected in these soil samples (i.e., PHEN/ANTH and FLUOR/PYR) indicate that all the soils studied here are of pyrogenic origin (see Section 3.1). This is not unexpected since all the soils were sampled from industrial sites that employ(ed) high-temperature combustion processes. The production of coal tar at manufactured gas plants and coke ovens occurs at temperatures exceeding 1000°C [16], and the distillation of creosote from coal tar occurs at temperatures ranging from 200 – 400°C [25, 98]. Interestingly, the FLUOR/PYR ratios were notably higher for Luleå, Hviken-1 and Hviken-2, compared to all other soils. These PAH ratios clearly separate the wood preservation sites from the others (i.e., the manufactured gas plant and coke-oven site). However, accurately differentiating between different pyrogenic sources, such as a gas works site and a coke oven, is difficult, and cannot be accomplished with the data shown here. More accurate forensic analyses for classification of pyrogenic contamination requires knowledge about a broad spectrum of PAHs, PAH derivatives such as alkylated PAHs, as well as aliphatic compounds [25].

4.2 *Salmonella* mutagenic activity of organic fractions from contaminated soils

All of the non-polar neutral soil extracts studied here required exogenous S9 metabolic activation to elicit a mutagenic effect, indicating that this fraction contains only indirect-acting mutagens that require oxidation by S9 microsomal enzymes. This indirect activity is likely due to the PAHs and alkyl-PAHs detected in the soils (Table 3.1).

Numerous studies have shown that PAHs are not directly mutagenic in *Salmonella*, rather they are indirect acting mutagens (also known as pro-mutagens), and they must be converted to electrophilic intermediates by cytochrome P450 enzymes in order to exhibit mutagenic activity [5, 87, 99]. This fraction may also contain O- and S-heterocyclics [10, 87], yet the mutagenic profiles of these types of compounds is rarely available.

Moreover, further chemical characterization would be required to validate their presence in the samples studied here.

The polar aromatic soil fractions induced mutations in the *Salmonella* assay with and without metabolic activation, however the strongest responses were observed in the presence of metabolic activation. The direct-acting mutagenicity (i.e., without S9) is likely due to the presence of oxy-PAHs and nitroarenes that are capable of forming DNA adducts in *Salmonella* in the absence of S9 microsomal enzymes [20, 100, 101].

Conversely, the S9-activated mutagenicity is likely due to aromatic amines and N-heterocyclic compounds that must be converted to hydroxylamines by microsomal enzymes in order to elicit a mutagenic effect [102].

Nitroarenes and aromatic amines (including N-heterocyclic compounds) are pervasive environmental pollutants that are mutagenic in several mutagenicity assays [20, 103, 104]. Their presence in the soil samples studied here is supported by the response

elicited on *Salmonella* strain YG1041. Over-expression of *cnr* and *OAT* in YG1041 confers enhanced sensitivity to nitroarenes and aromatic amines [63, 105]. Their presence in the samples examined would not be surprising as previous studies have detected such compounds in coal tar-contaminated soils [16] and creosote-contaminated soils [89]. The chemical fractionation scheme employed in this study to segregate the more polar compounds in the polar aromatic fractions, however the high response on YG1041 with S9 for the non-polar neutral fraction suggests that some polar nitrogen-containing compounds may have eluted in the non-polar soil fraction. Indeed, nitroarenes have been found to co-elute with PAHs during fractionation procedures such as high performance liquid chromatography (HPLC) [106]. Nevertheless, co-elution of nitroarenes in the non-polar neutral fraction is not likely to be the case here because the non-polar soil fractions lack a direct-acting mutagenicity, and nitroarenes do not require S9 activation to exert a mutagenic effect in YG1041 (i.e., they can readily be activated to arylhydroxylamines by *cnr* and to N-acetoxyesters by *OAT*) [20, 63, 107]. Alternatively, the non-polar neutral fractions may contain indirect-acting aromatic amines and N-heterocyclic compounds that require activation by S9 as well as *OAT* (i.e., oxidation by S9 to produce arylhydroxylamines and *OAT* to produce unstable N-acetoxyesters) [108]. The high response on YG1041 to the non-polar neutral fractions may also be due to the presence of O- and S-heterocyclics. These compounds elute in the non-polar fraction [10, 87] and, while the metabolism of these compounds is not fully understood, it is possible that their activity may be enhanced by *OAT* and/or *cnr*. A more thorough chemical analysis is warranted to investigate the presence of aromatic amines and O-, N- and S- heterocyclic compounds in the non-polar neutral fractions.

The mutagens present in both the polar and non-polar fractions showed a tendency to induce frameshift mutations rather than base pair substitutions, as indicated by the higher proportion of positive responses on TA98 and YG1041 compared to TA100. In fact, the polar aromatic fraction did not exhibit any base pair substitution activity in the absence of metabolic activation, suggesting that there are no direct-acting mutagens capable of inducing base pair substitution mutations in this fraction. These observations are not surprising as high frameshift activity has been previously been observed at creosote-contaminated sites [109, 110], and other PAH-contaminated sites [111]. One published study has demonstrated that oxy-PAHs are strong base substitution mutagens [22], however in this study, only marginally positive responses were elicited by the polar aromatic fractions on TA100 + S9, and no activity was detected on TA100 without S9. This may suggest that the concentrations tested were not high enough to elicit a significant positive response. A recent review of mutagens in contaminated soil by White and Claxton confirms that most of the mutagenic activity observed at contaminated sites is primarily S9-dependent frameshift activity, although it should be noted that most of the reviewed studies only used frameshift strains to measure soil mutagenic activity [1]. Furthermore, nitroarenes, N-heterocyclics and oxy-PAHs have been found to produce mainly frameshift mutations [20, 100].

The *Salmonella* mutagenic activities observed for the PAH-contaminated soils examined here are in the same range as those observed for contaminated soils at other industrial sites (per mg soil) [1]. The aforementioned review by White and Claxton noted that organic extracts from industrial soils yield mutagenic potency values on TA98 with S9 activation that range from very low values (i.e., less than 0.01 revertants per mg) to

extreme values that exceed several hundred revertants per mg. However, the White and Claxton review also notes that the extremely potent extracts were largely derived from heavily contaminated Superfund sites and/or sites contaminated with highly mutagenic nitroarene explosives (e.g., trinitrotoluene, RDX). Geometric and arithmetic mean values for the 587 mutagenic potency values (i.e., TA98 with S9 for organic soil extracts) reviewed by White and Claxton are 0.95 ± 0.2 and 21.7 ± 3.8 revertants per mg, respectively. Thus, the TA98 mutagenic potency values (with S9) shown here (i.e., between 1.4 and 27.8 rev per mg, see Table 3.4) are similar to those presented in the published literature. However, it should be emphasized that detailed chemical characterization of the soils discussed in White and Claxton is rarely available, and therefore, it is not possible to determine if the PAH concentrations in the contaminated soils are similar to those examined in this study. Nevertheless, some the sites reviewed by White and Claxton are known to have high levels of identified PAHs (e.g., more than 1000 mg per kg total PAHs), and these samples elicited mutagenic responses on TA98 with S9 activation that are similar to those observed here. In addition, recent analysis of additional samples from the Husarviken site near Stockholm revealed similar mutagenic activity values for similar levels of PAH contamination [87].

4.3 FE1 mutagenic activity of organic fractions from contaminated soils

The FE1 assay was used as an *in vitro* mammalian model to validate the results obtained with the *Salmonella* assay. Direct-acting mutagenicity was observed for the non-polar neutral and polar aromatic soil fractions, and the addition of S9 inhibited the mutagenic response. Inhibition by S9 preparations has previously been observed with

this cell line [59], and may result from a reduction in contact between the cell and the test article (i.e., reduced bioavailability of the mutagenic agent). The microsomal proteins may provide a physical barrier that effectively reduces exposure of FE1 cells, thus resulting in a lower effective concentration of PAHs and an inability to induce mutations in the *lacZ* transgene. Furthermore, FE1 cells express GST and CYP1A1, have an endogenous metabolic capacity, and do not require the addition of S9 to convert PAHs to reactive mutagens [59].

All three of the tested non-polar neutral soil fractions (i.e., Hsund-1, Hsund-2 and Hholm-1) elicited positive responses on the FE1 assay, thus validating the presence of mutagens in these fractions. This activity is likely due to the presence of PAHs and alkyl PAHs, compounds that are known to be mutagenic in other cell lines, as well as the *in vivo* version of the MutaTMMouse assay [48, 112]. However, O- and S-heterocyclic compounds may also contribute to the mutagenicity of these fractions.

One polar aromatic fraction was tested with this assay (Hsund-2), and this sample also elicited a mutagenic response. However the observed response was lower than that obtained for the corresponding non-polar neutral fraction; a similar trend to that already observed in the *Salmonella* assay. As previously noted, the mutagenicity of this polar soil fraction is likely due to the presence of nitroarenes, aromatic amines, N-heterocyclic compounds and oxy-PAHs, since these compounds have been shown to elute in this fraction, and many are known mutagens [20, 22, 87, 100]. In fact, some of these compounds (i.e., nitroarenes and N-heterocyclic compounds) are known to be mutagenic in similar transgenic rodent assays, including the *in vivo* version of the MutaTMMouse system [48, 113]. However, it should be noted that there are profound mechanistic

differences between the metabolism of nitroarenes in mammals and bacteria, and the process of nitro-compound reduction in mammals is relatively poorly understood [35]. As such, mammalian and bacterial mutagenicity assays may produce apparently incompatible results for nitroarene-contaminated soils. In mammals, reduction of the nitroarenes to an arylhydroxylamine is thought to be carried out by NAD(P)H:quinone oxidoreductase (i.e., DT-diaphorase), xanthine oxidase, and aldehyde oxidase [32]. In Gram-negative bacteria (e.g., *Salmonella*) reduction is carried out by *snr* and *cnr*. Moreover, acetyltransferase activity is carried out by N-acetyltransferases in mammals, as opposed to *O*-acetyltransferases in bacteria [36].

4.4 Relationship between soil mutagenic activity and PAH concentrations

Eight of the 16 priority PAHs induced positive responses on at least two *Salmonella* strains (i.e., BaA, BaP, BbF, BghiF, BkF, CHRY, I123cdcP and DBahA), and these results are in agreement with previous studies on PAH mutagenicity in *Salmonella* [6, 114]. Statistical analyses revealed that the mutagenic activities of the non-polar neutral soil fractions were significantly correlated with the levels of 17 PAHs, including 14 priority PAHs (Table 3.7). The overwhelming majority of these relationships were observed for TA98 activity. White and Claxton also noted an empirical relationship between TA98 mutagenic activity (with S9) and the concentrations of several PAHs in soil [1], and similar results were also recently obtained for samples of settled house dust [114].

It is interesting to note that the concentration of BaP was not significantly correlated with the mutagenic activities of the soils in any *Salmonella* strain. This was

unexpected since BaP is generally considered to be the PAH that constitutes the greatest mutagenic and carcinogenic hazard to a contaminated matrix (e.g., soil). For example, oral slope factors used to calculate the excess cancer risk associated with exposures to soils contaminated with priority PAHs are all scaled to those generated from BaP carcinogenicity experiments [4, 82, 115].

The mutagenicity of all priority PAHs was also evaluated with the FE1 assay, however only five compounds yielded positive responses (i.e., BaP, BbF, BkF, CHRY and DBahA). The response observed for BaP are comparable to those already published [59], however, this thesis constitutes the first test of any other PAHs with this assay. Mutagenic and/or carcinogenic responses have been observed for these five PAHs in other *in vitro* and *in vivo* mammalian systems [6, 116].

4.5 Predicted mutagenic activity of soils

The mutagenic activities of the soils were predicted using two methods described in the US EPA's *Supplementary Guidance Document for Conducting Health Risk Assessments for Chemical Mixtures* [73]. Under CEPA, Canadian regulatory authorities employ similar, yet less thoroughly defined methods [117]. In the absence of mixture (geno)toxicity data, the US EPA recommends that the (geno)toxicity of a sufficiently similar mixture should be evaluated. For the soils studied here, the genotoxicity of a reconstituted (i.e., synthetic) PAH mixture, composed entirely of detected priority PAHs was the 'sufficiently similar mixture' examined. In the absence of a sufficiently similar mixture, the EPA recommends that the (geno)toxicity of each hazardous component of the mixture be evaluated separately, and the mixture (geno)toxicity be assumed to be

equal to the sum of the contributions from the mixture components [72]. In this study, the mutagenic activities of the detected priority PAHs were assessed, the activities were assumed to be additive, and the contributions from each priority PAH were summed in order to predict the total mutagenic activity of a given soil (see Section 2.5). It should be noted that the predicted mutagenic activities are only comparable to the substances that elute in the non-polar neutral soil fractions, since the polar aromatic soil fractions were not chemically characterized.

The majority (~70%) of predicted mutagenic activities, whether based on complete additivity of the individual PAH constituents, or on the activity of a synthetic priority PAH mixture, were greater than the observed mutagenic activities of the non-polar neutral soil fractions in both the *Salmonella* and FE1 assays (Section 3.3.3 and Section 3.4.5). Predictions based on additivity of the priority PAHs were up to 8-fold higher when measured using the *Salmonella* assay, and up to 22-fold higher when measured using the FE1 assay. Predictions based on synthetic priority PAH mixtures were up to 10-fold higher when measured using the *Salmonella* assay, and up to 4×10^4 higher when measured using the FE1 assay. Nevertheless, 25% of all predictions proved to be underestimations of the observed non-polar neutral mutagenic activities, and 8% of the predictions were reasonably accurate representations of the observed activities of the non-polar neutral fractions.

The aforementioned results suggest that the mutagenic activities of PAHs are rarely additive. This apparent lack of support for the additivity paradigm was somewhat surprising since published support for the additivity paradigm is fairly extensive [74, 75, 118, 119], and the assumption of additivity is routinely employed by regulatory agencies

to calculate the total risk posed by chemical mixtures [12, 73]. Several explanations for these non-additive effects can be hypothesized upon closer examination of the mixtures themselves, and the mechanisms through which they exert their mutagenic effect.

Overestimations of actual activity may be caused by antagonistic interactions between PAHs in mixtures. Antagonism between PAHs has been documented, and one study noted the inhibition of benzo[*a*]pyrene metabolism by benzo[*e*]pyrene [120]. Another study noted the inhibition of nitroarene mutagenicity by some oxy-PAHs [121]. Inhibition of the enzymes responsible for the metabolism of PAHs may be the cause of the sub-additive effects observed in this and other studies [122, 123]. However, the most likely cause of sub-additive effects is saturation of the metabolic enzymes required for the conversion of PAHs to reactive metabolites capable of causing DNA damage [74]. This contention is supported by the fact that predictions based on priority PAH mixtures were less than those based on additivity of each component PAHs. When tested individually, priority PAHs are more likely to yield a strong (i.e., maximal) mutagenic response, since they have *exclusive* access to the microsomal enzymes required for metabolic activation, and each *maximized* activity value contributes to over-prediction of mixture activity. In the actual mixture however, competition for microsomal enzymes by both mutagenic and non-mutagenic PAHs contributes to sub-additive mutagenic responses. This explanation is supported by observations of sub-additive effects observed for mixture samples with high concentrations of PAHs [12, 74, 75]. In the current study the largest overestimations of mutagenic activity (in *Salmonella* TA98) were made for the most heavily contaminated soils. Alternatively, it is also possible that electrophilic PAH metabolites bind to S9 proteins, thus reducing the effective

concentration of active metabolites that can react with DNA [74, 75]. Nevertheless, increased concentrations of S9 in the *Salmonella* assay system might alleviate the sub-additive responses of PAHs in complex mixtures, and the validity of these explanations could readily be tested experimentally.

The above discussion suggests that the concentration of microsomal enzymes (i.e., S9) confounds the use of the *Salmonella* assay for the study of additive effects. Since individual mixture components may require varying concentrations of S9 to induce a maximum mutagenic effect, and moreover, components compete for access to the microsomal enzymes, then the amount of S9 used in the *Salmonella* assay can greatly influence the measured mutagenic activities of complex mixtures [75, 124]. For example, assays using one concentration of S9 could suggest additivity, while assays using a different S9 concentration to examine the same mixture could suggest non-additive interactions. Therefore it might be prudent to repeat some of the *Salmonella* assays carried out for this study with varying S9 concentrations, and determine, for selected samples, the S9 concentration that maximizes the observed mutagenic response. Subsequent analyses could compare these new *maximized* activity values with the aforementioned predictions of mutagenic activity.

In 25 % of cases, the predicted mutagenic activities of the non-polar soil fractions were underestimations of the true mutagenic activity. It is possible that chemical interactions between PAHs could result in super-additive or synergistic effects in mixtures, however this is unlikely since PAHs themselves are generally unreactive compounds [122]. Since super-additive effects have only been observed when metabolic activation is present, it is more likely that synergism occurs due to biotransformation-

related processes. For example, some underestimations of mutagenic activity may be due to up-regulation of PAH-metabolizing enzymes in the FE1 system [120, 122, 125]. Recent unpublished experiments have shown that CYP1A1 is inducible by exposure to BaP [126], and increased levels of microsomal enzymes would be expected to increase the conversion of PAHs to reactive metabolites, and therefore an increase in mutagenicity [120, 125]. Alternatively, underestimations of the actual mutagenic activities may be related to contributions from the uncharacterized compounds in the non-polar soil fraction (i.e., O- and S-heterocyclics and alkyl PAHs) [27]. Coal-tar and creosote-contaminated sites are known to be composed of a vast array of PACs [91, 127], but the methods used in this study to predict mutagenic activity (i.e., those advocated by regulatory agencies such as the US EPA) only included contributions by priority PAHs. Thus, the possibility that unidentified compounds in the non-polar neutral fraction contribute to the mutagenic activity of the PAH-containing portion of the mixture cannot be discounted.

At first glance these results seem to suggest that, since most predictions were greater than the observed mutagenic responses, the methods advocated by governmental agencies to predict risk may provide cautious overestimations of soil mutagenic activity. However, predictions were only made for the non-polar neutral soil fractions and this conclusion would be restricted to the PAH-containing fraction of contaminated soils. Since most PAH-contaminated sites contain other PACs (e.g., heterocyclic compounds, nitroarenes and aromatic amines) [10, 27, 87], this conclusion may not be valid for an actual contaminated site. Risk assessments do not currently account for the hazards of polar PACs, such as those found in the polar aromatic soil fractions studied here, since

they are not classified as carcinogens or priority pollutants by the US EPA, IARC, or under CEPA. Nevertheless, it is known that these compounds are mutagenic, and they may actually remain in the soil long after the PAHs have been eliminated [10, 16, 87, 128]. Therefore, the exclusion of these compounds from risk assessments and predictions of mutagenic activity could result in underestimations of actual risk.

The identification of unknown mutagens in mixtures can be accomplished using *bioassay directed fractionation* techniques, whereby the complexity of the mixture is reduced by repeated fractionation and testing of fractions using bioassays [89, 129]. The mixture is iteratively fractionated in an effort to identify specific chemicals that are responsible for the mutagenicity and/or carcinogenicity of the mixture [130]. Individual compounds in mutagenic fractions can then be identified using analytical techniques such as mass spectrometry. Bioassay-directed fractionation of the soil fractions studied here would be a useful strategy for determining the identity of unknown mutagenic compounds in the soils.

This thesis examined only the mutagenic hazard of PAHs and related compounds; however, some of the studied soils also contain CCA (chromated copper arsenate), arsenic, zinc, cyanide and other heavy metals (Table 2.1). While the nature of the extraction methods employed suggest that the presence of such metals in the soil fractions is unlikely, it is possible that they can impact the actual hazard of the contaminated site. Several heavy metals are classified as definite human carcinogens (Class 1) and possible human carcinogens (Class 2B) by the IARC [131, 132], and some are listed as priority substances by both the US EPA and Health Canada [133]. Some

metals are also known to interfere with the function of PAH-metabolizing enzymes [123], and thus, can influence the mutagenic activity of PAHs.

A cautionary note is warranted regarding the extrapolation of mutagenic activities from bacterial and/or murine mutagenicity assays to humans. Inter-species toxicokinetic and toxicodynamic variations complicates such extrapolations [12]. Several metabolic and physiologic features of humans cannot be modeled using simple mutagenicity assays. For example, Van del Wiele et al demonstrated that human colon microbiota can hydroxylate PAHs thus resulting in the production of reactive metabolites in the lower gastrointestinal tract [134]. Such processes, which occur at the level of the whole organism, cannot be accounted for by using simple *in vitro* systems such as the *Salmonella* assay or the FE1 assay. Moreover, differences in PAH mutagenic activities have even been observed for different murine cell lines [135]. Other inter-species variations include differences in the expression of metabolic enzymes, and differences in the ability to conjugate and excrete mutagens. Confirmation of the results presented here using an *in vivo* mammalian model (e.g., a porcine system) could reduce the uncertainty associated with extrapolation to humans.

A note on the standard errors of predicted mutagenic activities

Rigorous analyses to determine the statistical significance of the differences between observed activities and predictions based on the additivity of the component PAHs were not carried out. This is due to the fact that the variances associated with the predicted mutagenic activities are quite large. The magnitude of these variance values can be traced to (i) the mean square error (MSE) values associated with each PAH

mutagenic potency (i.e., the slope of the initial concentration-response function), (ii) the large variance associated with each predicted contribution of each PAHs to the total mutagenic activity of the mixture, and (iii) the number of PAHs considered for each mixture prediction (i.e., 8). With respect to (ii), it was not possible to include the detected concentrations of each PAH in the range of doses tested in the mutagenicity assays (due to toxic effects in the assay). Consequently these are “new predictions”, which can be expected to have larger variances than predictions based on actual tested concentrations [77]. With respect to (iii), the magnitude of the variance associated with a prediction of total mixture mutagenic activity is proportional to the number of PAHs included in the calculation of predicted activity (or carcinogenic risk). Moreover, attempting to make the predictive model more accurate by including more mixture components will increase the magnitude of the variance on the mixture prediction. Thus it is not surprising that risk assessments rarely include any analysis of the error associated with predicted excess lifetime cancer risks. Nevertheless, it is important to remember, that risk assessment of contaminated sites are not used as a means to accurately quantify the exact magnitude of the risks associated with a site. Rather, it is used as a tool for the prioritization of sites for remediation, and for determining the suitability of a site for redevelopment.

4.6 Risk Assessment of Soils

The results presented in this thesis show that the studied soils are mutagenic, and since most mutagens are carcinogens [46, 47], it is likely that exposure to the PAHs and PACs in the soils will contribute to an elevated risk of cancer. The excess lifetime cancer risks of the soils studied here were calculated for construction workers and ordinary adults by assuming the primary route of exposure to be non-dietary (i.e., inadvertent) ingestion of soil particles (see Figure 3.8 and 3.9). Although lower molecular weight PAHs and polar PACs have increased water solubility, and may contribute to groundwater contamination [136] and drinking water exposure, carcinogenic PAHs and PACs are primarily sorbed to soil particles due to their low water solubility (i.e., high K_{ow}) and low vapour pressures [1].

Overall, the cancer risks posed by these soils were higher for a construction worker than for an adult. This can readily be attributed to the higher soil ingestion rates of construction workers: 100 mg/day versus 20 mg/day for ordinary adults. These values reflect Health Canada's current guidelines for preliminary quantitative risk assessment [81], however, it should be noted that other sources cite adult soil ingestion rates ranging from 10 to 480 mg/day [137-140]. No other soil ingestion rates were available for construction workers.

Comparison of the estimated excess cancer risk attributable to the various sites to some level of acceptable risk (i.e., as determined by a regulatory body) was employed to determine if the sites are likely to pose a significant, unacceptable level of risk for the exposed receptors (i.e., adults and construction workers). The level of acceptable excess lifetime cancer risk varies, depending on the source consulted, from 10^{-6} to 10^{-4} . For

example, the level of acceptable incremental lifetime cancer risk varies between Canadian provinces from 10^{-6} to 10^{-5} [81], and US regulatory agencies use values between 10^{-6} and 10^{-4} [138, 141]. For the soils evaluated here, the excess lifetime cancer risk for an adult range from 10^{-6} to 10^{-4} , while the risk for a construction worker range from 10^{-6} to 10^{-3} . In general, the soils with higher levels of B2 PAHs (i.e., creosote-contaminated sites) showed higher levels of excess cancer risk. A summary of the incremental excess lifetime cancer risks for the soils evaluated in this study, relative to three common levels of acceptable risk, is provided in Table 4.1.

Table 4.1 Percentage of contaminated soils that would be considered unsafe using acceptable levels of incremental excess lifetime cancer risk of 10^{-4} , 10^{-5} or 10^{-6} for adults and construction workers. Risks were calculated using the B2 method as well as the MP-Salmonella and MP-FE1 methods (see Section 2.8).

Receptor	Method *	Percent of soils exceeding level of acceptable level of risk		
		10^{-4}	10^{-5}	10^{-6}
Adult	B2	0	70	100
	MP-Salmonella ^a	50	100	100
	MP-FE1 ^b	0	0	33
Construction worker	B2	70	100	100
	MP-Salmonella	100	100	100
	MP-FE1	0	33	33

* Note that the same number of samples were not evaluated for each method (N = 3 for BaP-FE1 method; N = 10 for BaP-Ames method; N = 10 for B2 method).

^a MP-Salmonella refers to the non-targeted method which used *Salmonella*-derived mutagenicity data to calculate risk.

^b MP-FE1 refers to the non-targeted method which used FE1-derived mutagenicity data to calculate risk.

Table 4.1 shows that the level of risk that is considered acceptable, and the method employed to calculate excess risk, greatly influence the interpretation of the results. Consider the targeted B2 method and the non-targeted MP-Salmonella method. Applying the most conservative level of acceptable cancer risk (i.e., 1 cancer case in 10^6 individuals), and either the B2 method or MP-Salmonella method, indicates that all soils should be considered unsafe for both adults and construction workers. However, if one applies a less conservative level of acceptable cancer risk (i.e., 1 cancer case in 10^5 individuals), the B2 method would identify 3 soils as relatively *safe* for ordinary adults. Not surprisingly, the soils that would be considered relatively safe are those with the lowest levels of PAHs (Hviken-1, Hviken-2 and Luleå). Interpretations are quite different when considering a risk level of 1 case in 10^4 individuals. Using this level of acceptable risk, all soils would still be considered unsafe for construction workers using the MP-Salmonella method, however only 70% of the soils would be considered unsafe using the B2 method. Conversely, using this level of acceptable risk, none of the soils would be categorized as unsafe for adults using the B2 method, and only half of the soils would be considered unsafe for adults according to the MP-Salmonella method.

The MP-Salmonella method consistently yielded higher predictions of risk than the B2 method. This a result of the higher dose of BaP equivalents calculated using the slope ratio method (see Equation 4, Section 2.8). This indicates that cancer risk based on mutagenic potency data is higher than that based solely on detected carcinogenic PAHs. These results suggest that a targeted, chemical-specific approach to risk assessment may actually underestimate the actual cancer risk posed by a contaminated site. However, it should be noted that risk estimates based on *Salmonella* mutagenic potency data and

calculated BaP equivalents may be overestimations that result from the assumption that all components of the non-polar neutral and polar aromatic fractions are in fact mutagenic carcinogens. This assumption may be reasonable for mutagenic PAHs, since most are in fact categorized as animal carcinogens by agencies such as IARC, however, mutagenic PACs can be extremely mutagenic (e.g., nitroarenes), but are not clearly carcinogenic in experimental animals (e.g., 1-nitropyrene, 1,3-dinitropyrene).

Although BaP was used as a reference compound in the absence of complete chemical data and/or PEF values [9], the results presented in this thesis support the contention that BaP's contribution to the carcinogenic hazard of a contaminated soil (i.e., a complex mixture) is uncertain. BaP may not in fact be a suitable point of reference for overall mutagenic and/or carcinogenic hazard since there was no correlation between BaP concentration and the *Salmonella* mutagenic activity of the soils. Moreover, the actual suitability of BaP as a reference compound will be dependent on the route of exposure and the type of cancer monitored [142]. For example, the oral slope factor used here is based on measurements of BaP-induced carcinomas and papillomas of the gastrointestinal tract (GI) tract [82], and this endpoint and its accompanying slope factor may not be relevant for other target tissues that may be effected by other PAHs and unidentified PACs.

It should be noted that risk estimates based on *Salmonella* mutagenic potency data differed from those calculated using FE1 potency values. The FE1-based calculation consistently provided dramatically lower estimations of excess cancer risk than the *Salmonella*-based estimation. This is caused by the fact that the amount of BaP equivalents required to induce the mutagenic response observed in the assay systems was

consistently lower for the FE1 assay than the *Salmonella* assay. These differences translate into lower risk estimates and highlight the effect of using different mutagenicity assays to estimate the risk posed by complex mixtures. It should be noted that the FE1-based risk estimates may in fact be more reasonable since metabolism and activation of PACs (e.g., nitroarenes and heterocyclics) in the FE1 assay are more closely related to that which would be expected in the risk receptors (i.e., human adults). Conversely, metabolism and activation pathways in *Salmonella* are known to elicit extremely potent mutagenic responses [35].

The targeted B2 method assumed additivity of PAH risk, while the non-targeted MP methods used genotoxicity data on the mixture itself, and therefore, did not require an assumption of PAH additivity. The assumption of dose additivity is commonly used in risk assessment for compounds that have similar mechanisms of action and differ only in their potencies [4, 12]. Thus, this assumption is valid since the PAHs and PACs detected in the studied soils are converted to mutagenic metabolites via the similar biochemical pathways (i.e., oxidative metabolism by CYP enzymes) [8, 36, 108, 143]. Dose-additivity is the basis of both the PEF approach used in the B2 method [118], and the MP approach that required conversion of potency values to their BaP equivalence, and adding the activities of the non-polar neutral and polar aromatic soil fractions. However, the risk estimates calculated using the MP methods must be interpreted with caution. As already noted, the methods employed assume that the ingredients in the non-polar neutral and polar aromatic fraction are not only mutagenic, but also carcinogenic in experimental animals. Verification of actual carcinogenic activity would require chronic animal bioassays. Moreover, the results presented here and elsewhere, suggest that non-

additive interactions can influence the overall mutagenic and carcinogenic activity of complex PAH mixtures such as the non-polar neutral and polar aromatic fractions [12, 74, 75, 120-123, 125].

When interpreting the risk assessment results, one should bear in mind that the results are estimates of excess lifetime cancer risk. The actual risk posed by a PAH mixture in soil will vary depending on the actual amount of soil ingested, the frequency of exposure to the site, the carcinogenic activity of the mixture constituents, the interactions of the carcinogenic PAHs and PACs in the mixtures, as well as several factors that may alter the susceptibility of the exposed receptors (e.g., age, gender, life stage, illness, etc.). Furthermore, it should be noted that the carcinogenic PAHs and PACs in a contaminated soil are only partially bioavailable and they are unlikely to be completely absorbed by the GI tract. Unfortunately, the gastrointestinal absorption of PAHs in soil is poorly understood and long term experiments on pure PAHs, such as those used to generate oral slope factors, do not provide any information on the effect of soil particles on PAH carcinogenic activity [81]. The mitigating effect of soil particles on gut absorption and carcinogenic activity is not considered in the current risk assessment framework, and actual evaluation of the carcinogenic risk posed by soil contaminated with carcinogenic PAHs and PACs would require epidemiological studies (e.g., case-control or cohort studies) that relate cancer incidence to actual levels of human exposure.

Overall Conclusions

Ten coal tar- and creosote-contaminated soils were found to contain several mutagenic PAHs, and the concentrations exceed Canadian regulatory thresholds for industrial or residential use. Extraction and fractionation of the soils yielded non-polar neutral and polar aromatic extracts that exhibited significant mutagenic activity in both bacterial and *in vitro* mammalian assays. Non-targeted approaches, based on similar synthetic PAH mixtures, and targeted approaches, based on an assumption of additivity of priority PAHs, yielded overestimations of the observed mutagenic activities of the non-polar neutral (i.e., PAH-containing) soil fractions. This suggests non-additive interactions of mutagenic PAHs in complex mixtures. However, these overestimations may simply be due to a saturation of the metabolic capacity of the genotoxicity assays systems employed. These results suggest that targeted risk assessments based on the additivity of identified priority PAHs will provide conservative estimates of risk for PAH-containing portion of contaminated soils. However, the total risk associated with a contaminated soil risk may be underestimated obtained if the samples also contain unidentified mutagenic PACs such as aromatic amines and heterocyclic compounds.

Furthermore, risk assessments based on observed mutagenic activities of the soils varied depending on the mutagenicity assay used to calculate dose and estimate risk. Calculations based on *Salmonella*-derived mutagenicity data yield estimates of excess cancer risk that are higher than those calculated by the more traditional B2 carcinogen method. Risk estimates that were calculated using FE1-derived mutagenicity data showed the opposite trend (i.e., lower than B2 estimates). Risk estimates must therefore be interpreted with caution, and one must consider the validity of the methods used to

calculate risk, as well as the identity and concentrations of chemicals included in the more traditional risk assessment framework.

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