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In Vitro Mammalian Mutagenicity of Complex Polycyclic Aromatic Hydrocarbon Mixtures in Contaminated Soils

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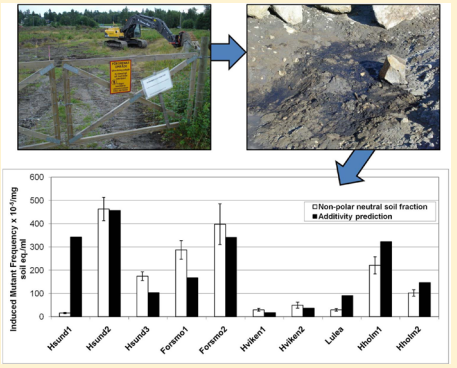
Supporting Information

ABSTRACT: This study employed an in vitro version of the *lacZ* transgenic rodent mutation assay to assess the mutagenicity of nonpolar neutral and semipolar aromatic soil fractions from 10 PAH-contaminated sites, and evaluated the assumption of dose additivity that is routinely employed to calculate the risk posed by PAH mixtures. Significant mutagenic activity was detected in all nonpolar neutral fractions, and 8 of 10 semipolar aromatic fractions (nonpolar > semipolar). Mutagenic activity of synthetic PAH mixtures that mimic the PAH content of the soils (i.e., 5-PAH or 16-PAH mix) were greater than that of the PAH-containing soil fractions, with 5-PAH mix >16-PAH-mix. Predictions of mutagenic activity, calculated as the sum of the contributions from the mutagenic mixture components, were all within 2-fold of the observed activity of the nonpolar neutral fractions, with one exception. Observed differences in mutagenic activity are likely the result of dynamic metabolic processes, involving a complex interplay of AhR agonsim and saturation of metabolic machinery by competitive inhibition of mixture components. The presence of hitherto unidentified polar compounds present in PAH-contaminated soils may also contribute to overall hazard; however, these compounds are generally not included in current contaminated site risk assessment protocols.



INTRODUCTION

Polycyclic aromatic hydrocarbons PAHs) are ubiquitous environmental pollutants formed via the incomplete combustion of organic material. Many PAHs have been classified as either known human carcinogens (e.g., benzo[*a*]pyrene, BaP), probable carcinogens (e.g., dibenz[*a*,*h*]anthracene), or possible carcinogens (e.g., benz[*a*]anthracene, benzo[*b*]fluoranthene).[1] Carcinogenic PAHs generally act via a mutagenic mode of action; they are first converted into electrophilic metabolites (e.g., benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide) that can react with DNA resulting in the formation of bulky adducts, which in turn can contribute to the formation of permanent sequence changes, that is, mutations. Many PAHs are also aryl hydrocarbon receptor (AhR) agonists that are capable of inducing their own metabolism via regulation of CYP enzymes controlled by AhR-mediated pathways.[2]

PAHs are almost always found in the environment as components of complex mixtures that contain hundreds, or even thousands, of substances, and many PAH-containing mixtures have also been classified as known human carcinogens (e.g., coal tar, tobacco smoke) or probable carcinogens (e.g., coal tar creosote).[1,3] Many former industrial sites contain soil that is contaminated with complex mixtures of PAHs and related substances. For example, industrial sites that were involved in the manufacturing of coal-tar creosote for wood preservation are often highly contaminated with a variety of PAHs and other polycyclic aromatic compounds (PACs) (e.g., O- and S- heterocyclics).[4] Industrial sites involved in the production of manufactured gas (also known as coal gas or town gas) and/or coke have also been shown to be contaminated with mixtures of PAHs and related compounds due to the production and improper disposal of coal tar.[5,6]

Hazard assessment of contaminated soils containing complex mixtures of PAHs and related compounds is not a simple task. The presence of unidentified compounds in the mixtures is a well-known stumbling block that hinders accurate assessment of hazard and risk. Moreover, there is a paucity of reliable information regarding the interactions of selected PAHs that have been prioritized for assessment and control. In recent years, some studies have employed methods such as bioassay-directed chemical fractionation to isolate and identify hazardous

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compounds, such as PACs, that are present at contaminated sites such as former manufactured gas plant (MGP) sites (for example, see Brooks et al, 1998[7]). In principle, these substances can be toxicologically assessed and subsequently included in determinations of hazard and/or risk. Other studies have investigated the validity of some of the assumptions and methods routinely used for the assessment of hazard and risk posed by PAHs present at these sites[8−10] (e.g., dose additivity, potency equivalency factors, etc.). Numerous mechanistic studies have also been carried out in an effort to understand the genotoxicity of PAHs in mixtures (for example, see refs 10−19).

The methodology routinely employed to assess the excess lifetime cancer risks posed by complex PAH-contaminated materials (e.g., soil) rarely involves biological assessment of the actual whole mixture. Moreover, although some groups have recommended the use of sufficiently similar "surrogate" mixtures to assess the risks posed by PAH mixtures, suitable data are rarely available.[20−23] Rather, cancer risk assessment methods for mixtures, which are currently advocated by governmental agencies such as the U.S. Environmental Protection Agency (U.S. EPA) and Health Canada (HC), calculate total risk as the incremental sum of the contributions from a targeted set of chemicals that are assumed to have the same mode of action.[21,24] For example, assessments for PAH-contaminated material typically calculate risk as the incremental sum of the contributions from a subset of PAHs highlighted by the U.S. EPA as priority PAHs.[25] The contributions of each monitored PAH to the total hazard of the mixture are generally inferred by employing the comparative potency of each compound relative to BaP, and an assumption of simple additivity is used to calculate the total concentration of BaP equivalents in the mixture. Although there is debate about the value of the potency equivalency factors (PEFs, also called relative potency factors and toxic equivalency factors) required to calculate total BaP equivalents for a PAH mixture, governmental agencies in several countries (e.g., U.S., Canada, Sweden, The Netherlands, the UK), as well as the WHO Internal Programme on Chemical Safety (IPCS), advocate the use of the PEF concept.[26] For a more detailed discussion of complex mixture risk assessment methods, the reader is referred to our companion paper.[27]

Previous work in our laboratory used the Salmonella reverse mutation assay (i.e., the Ames test) to scrutinize the aforementioned approach for the assessment of excess lifetime cancer risk attributable to mutagenic PAHs in complex mixtures. More specifically, an additive, chemical-specific approach was employed to predict the mutagenic activity of complex PAH mixtures in ten contaminated soils, and these predictions were compared to the observed mutagenic activity. We demonstrated that the sums of the contributions from the individual priority PAHs detected in the soils, as well as the mutagenic activity of synthetic mixtures of priority PAHs that constituted sufficiently similar mixtures, were both greater than the observed activities of the corresponding complex PAH mixtures obtained from the contaminated material (i.e., organic fractions of the contaminated soils).[8] These results suggested that risk assessment methods currently employed for carcinogenic PAHs with a mutagenic mode of action, which express total risk as the sum of the incremental contributions from a small number of targeted substances, may be conservative (i.e., overestimate actual risk). However, the Salmonella test system employs an exogenous metabolic activation mixture with extraordinarily high cytochrome P4501A1 activity, and is therefore exceptionally sensitive to some priority PAHs (e.g., BaP). Furthermore, when tested in isolation, mutagenic PAHs have exclusive access to the enzymes required for metabolic activation. Thus, the resulting individual potency values can contribute to a sum that greatly exceeds the observed activity of the mixture, and the conclusions of our earlier study may not be relevant to the determination of risk for mammalian cells. In our previous study, we demonstrated that polar compounds also contribute to the overall mutagenic activity of soils contaminated with complex mixtures of PAHs and PACs. Such compounds are therefore expected to contribute to the overall hazard and risk of a contaminated site; however, their contribution is not addressed by current contaminated site risk assessment protocols.

The aim of the current study was to expand on our earlier work, and, employing an in vitro mammalian cell system for assessment of mutagenic activity, continue to evaluate the aforementioned assumption of dose additivity that is routinely employed to calculate the risk posed by PAH mixtures. We contend that a test system based on mammalian cells can provide a more realistic assessment of potential human health hazard, and a more robust evaluation of the assumptions routinely employed for risk assessment of PAH mixtures. More specifically, we measured the induction of mutations in a transgenic murine cell line following exposure to nonpolar neutral (i.e., PAH-containing) or semipolar aromatic fractions derived from ten PAH-contaminated soils. We then compared the observed levels of mutagenic activity to those of sufficiently similar synthetic PAH mixtures, and moreover, to predicted activities calculated as the sums of the contributions from the individual priority PAHs detected in each of the samples. Assessment of mutagenic activity employed the MutaMouse FE1 cell line, an in vitro version of the *lacZ* transgenic rodent mutation assay.[28] The FE1 cell line, which is a cytogenetically stable lung epithelial cell line derived from an adult male, is metabolically competent and can readily convert a variety of PAHs and PACs, including nitroarenes and aromatic amines, into DNA-reactive mutagens. FE1 cells are known to express CYP1A1, CYP1A2, and CYP1B1 and their activities are sufficient to metabolize PAHs to DNA-reactive metabolites.[29,30]

MATERIALS AND METHODS

Chemicals. *Safety Warning—Several PAHs Are Known or Suspected Human Carcinogens; They Should Be Handled with Extreme Care.* All chemicals used for the extraction, fractionation and chemical analysis of the soil samples were analytical grade (≥99%) and obtained from EMD Chemicals (Gibbstown, NJ). All reagents, cell culture media, and media supplements used for the mutagenicity assessments were obtained from Gibco-Invitrogen (Burlington, ON, Canada), unless otherwise indicated. PAHs were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada).

Soil Pretreatment, Extraction, Fractionation and Chemical Analysis. Ten soil samples, obtained from five PAH-contaminated industrial sites in Sweden, were examined in this study. The sites included three wood preservation facilities (Holmsund, Forsmo and Hässleholm), one MGP site (Husarviken) and one coke oven site (Luleå). All facilities, with the exception of the coke production facility in Luleå, were no longer in operation at the time of sampling. Detailed site information can be found in Lemieux et al. (2008).[8]

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The soils were previously characterized by our research group, and a complete description of the soil handling and extraction procedures is outlined in Lemieux et al. (2008).[8] These procedures have been extensively validated and show acceptable recoveries and reproducibility.[31−33] Briefly, the soil samples were air-dried and sieved (2 mm), and organic substances extracted via pressurized liquid extraction using an ASE 200 accelerated solvent extractor (Dionex, Sunnyvale, CA). The extracts were then fractionated on open silica columns (10% w/w deactivated) into three fractions: (i) aliphatics, (ii) nonpolar neutral compounds, and (iii) semipolar aromatic compounds using hexane, followed by hexane:dichloromethane (3:1), and dicholoromethane, respectively. Prior validation of the fractionation protocol confirmed that homocyclic, unsubstituted PAHs and alkyl-PAHs, including the U.S. EPA priority PAHs, are contained in the second fraction, and semipolar PACs, including oxy-PAHs and nitrogen heterocyclic PACs, are contained in the third fraction.[32] The first fraction was discarded, and the subsequent two fractions were evaporated to ∼1 mL using a gentle stream of ultrapure nitrogen. 500 μL dimethyl sulfoxide (DMSO) (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) was then added, and the samples reduced under nitrogen to a final volume of 500 μL. All samples were stored at 4 °C until mutagenicity testing. Characterization of the PAHs in the soils was carried out using gas chromatography−mass spectrometry as described previously.[8,32]

PAHs and Synthetic Mixtures of Priority PAHs. Stock solutions of individual priority PAHs and synthetic PAH mixtures were prepared in DMSO. Two types of synthetic PAH mixtures were prepared, each containing PAHs in matched proportions to the PAH profiles of the nonpolar neutral soil fractions. The first type of mixture included all 16 U.S. EPA priority PAHs (i.e., naphthalene, acenaphthylene, acenaphthene, fluorene, anthracene, phenanthrene, pyrene, fluoranthene, benzo[*a*]anthracene, benzk[*k*]fluoranthene, chrysene, BaP, benzo[*b*]fluoranthene, indeno[*1,2,3-c,*d]pyrene, dibenz-[*a,h*]anthracene and benz[*g,h,i*]perylene). The second type of mixture included only the five PAHs that were previously observed to induce mutations at the transgenic *lacZ* locus in FE1 cells (i.e., benzo[*k*]fluoranthene, benzo[*b*]fluoranthene, BaP, chrysene, and dibenz[*a,h*]anthracene). The mixtures are hereinafter simply referred to as the 16-PAH and 5-PAH mixtures, respectively. All PAHs and PAH mixtures were stored in amber glass vials at −20 °C until use.

Mutagenicity of Soil Fractions, PAHs and Synthetic PAH Mixtures. *FE1 Cell Line.* A pulmonary epithelial cell line, denoted FE1, derived from the transgenic MutaMouse, was used to assess the mutagenic activity of the soil fractions, PAHs, and synthetic PAH mixtures. This cell line has been characterized, previously used for mutagenicity assessment, and is described in detail in White et al. (2003)[29] and Berndt-Weiss et al. (2009).[30] Note that FE1 cells also contain a functional P53 gene (i.e., are p53-competent) (unpublished results). FE1 cells were maintained in DMEM/F12 (1:1) supplemented with 2% v/v fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 ng/mL murine epidermal growth factor (Roche Diagnostics, Laval, QC, Canada). Treatment medium, prepared without FBS, was used to maintain the cells during all chemical exposures. All incubations were carried out at 37 °C, 95% humidity and 5% CO_2.

Exposure to PAHs, Synthetic Mixtures and Soil Fractions. FE1 cells were initially exposed to each of the 16 U.S. EPA priority PAHs. PAHs that induced a significant increase in *lacZ* mutant frequency at a concentration of 10 μg/mL or less were selected for further testing, at multiple concentrations (i.e., 4 or more), in duplicate. These included benzo[*k*]fluoranthene, benzo[*b*]fluoranthene, BaP, chrysene, and dibenz[*a,h*]anthracene. The cells were also exposed to each of the ten nonpolar neutral soil fractions, each of the ten semipolar aromatic soil fractions, and each of the 10 synthetic 5-PAH and 16-PAH mixtures. In each case, FE1 cells were exposed to at least four concentrations, in duplicate.

Preliminary experiments on PAHs included assays conducted with and without an exogenous metabolic activation mixture containing Aroclor-1254 induced rat liver S9 from male Sprague−Dawley rats (Molecular Toxicology Inc., Boone, NC). However, no significant difference in mutant frequency was observed upon the addition of an exogenous metabolic activation mixture (data not shown), and all subsequent exposures were carried out without S9. A positive control (0.1 μg BaP/mL) and a solvent (DMSO) control were run concurrently during each exposure, in duplicate.

Approximately 3×10^5 cells were seeded into 100 mm polystyrene culture dishes and grown overnight (∼16 h) to approximately 20% confluence. The following morning, the medium on each plate was replaced with 5 mL of serum-free medium containing 50 μL of the appropriate dilution of the desired test article (i.e., soil fraction, individual PAH, 5-PAH- or 16-PAH-mixture). All chemical dilutions were freshly prepared in DMSO on the morning of the exposure. FE1 cells were incubated in the treatment medium for 6 h, washed with Dulbecco's phosphate buffered saline (DPBS), and incubated for 72 h in serum-containing medium to permit mutation fixation.

Following the mutation fixation period, the medium was removed and cells incubated overnight in 3 mL of lysis buffer (10 mM Tris pH 7.6 (Caledon Laboratories Ltd., Georgetown, Canada), 10 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich Canada Ltd., Oakville, Canada), 150 mM NaCl, 1% (w/v) sodium dodecyl sulfate (SDS) and 1 mg/mL proteinase K (≥20 units/mg)). Total genomic DNA was then isolated from each plate using standard phenol-chloroform extraction, followed by precipitation in ethanol, as previously described.[29] DNA was stored at 4 °C until mutation scoring via the phenyl-β-D-galactoside (P-Gal) positive selection assay.

Detection of *lacZ* Mutations. The frequency of mutant *lacZ* loci in genomic DNA isolated from exposed FE1 cells was determined using the P-Gal positive selection assay as described elsewhere.[34] Briefly, *lacZ* transgenes were rescued from total genomic DNA and packaged into λ phage particles using the Transpack lambda packaging system (Agilent Technologies, Mississauga, ON, Canada). Phage particles were mixed with the host bacterium (*Escherichia coli lacZ*$^-$, *galE*$^-$, *recA*$^-$, pAA119 with *galT* and *galK*), plated on minimal medium containing the selective agent (i.e., 0.3% w/v P-Gal), incubated overnight at 37 °C, and scored for *lacZ* mutants (i.e., plaques). The total number of plaque-forming units (pfu) (i.e., number of λ vectors containing the *lacZ* transgene rescued from genomic DNA) was measured on concurrent titer plates without P-Gal. Mutant frequency (MF) was calculated as the ratio of mutant plaques to total pfu. Induced MF, calculated as the observed MF minus the experiment-specific spontaneous MF, was used in calculating all mutagenic potency values. This permits the

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elimination of interday variations in observed spontaneous mutant frequencies.

Data Analysis. All data and statistical analyses were carried out in Microsoft Excel 2010. For each PAH, soil fraction, and PAH mixture, concentration response curves were constructed by plotting induced MF versus concentration. Mutagenic potency was calculated for each sample using ordinary least-squares linear regression on the linear portion of the concentration−response function. Only mutagenic potencies with $p < 0.05$ (i.e., slope significantly greater than zero) were considered significant.

The mutagenic potencies of the individual PAHs were used to calculate a predicted mutagenic potency for each nonpolar neutral soil fraction according to eq 1:[8]o

$$\text{total predictd mutagenic activity} = \sum_{i=1}^{n} \text{observed activity of PAH}_i \times \text{PAH}_i\text{concentration in soil fraction} \quad (1)$$

for priority PAHs 1 through n.

Where total predicted mutagenic activity is expressed as induced mutants $\times 10^{-5}$/mg eq dry soil/mL, the observed activity of each PAH is expressed as induced mutants $\times 10^{-5}/\mu$g PAH/mL, and the concentration of PAHs in the soil fraction is expressed as μg PAH/mg eq dry soil.

The mutagenic potencies of the synthetic PAH mixtures were compared to those of the corresponding nonpolar neutral fractions using the two-tailed Student t-test ($p \leq 0.05$) with the appropriate multiple test correction (i.e., Bonferroni).[8] Statistical comparisons of the mutagenic activities calculated using eq 1 and the corresponding observed activities of the soil fractions were not carried out due to the excessive variance associated with the predicted values. The high variance can largely be attributed to the variance associated with predicted contributions of each PAH to the total mutagenic activity of the mixture. Since it was not possible to include the detected concentrations of each PAH in the range of tested doses, the variance associated with each of the "new predictions" is expected to be large.[35]

RESULTS

PAH Composition of Soils. The results of the chemical analyses have been published elsewhere. For complete details the reader is referred to Lemieux et al. (2008).[8] Briefly, the soil samples were found to contain several PAHs and alkyl PAHs, including the 16 U.S. EPA priority PAHs. Total priority PAH levels ranged from 70 to 9300 μg PAH/g dry soil. The Supporting Information (SI) includes a table that provides a summary of the PAH levels for the soils investigated.

Observed Mutagenicity of Soil Fractions. Mutant frequencies of positive (BaP) and negative (DMSO) controls, run concurrently during each exposure, were well within established historical limits. More specifically, the mean mutant frequencies ($\times 10^{-5}$) were $578 \pm 29 \times 10^{-5}$ and $44 \pm 2.2 \times 10^{-5}$ mutants for positive and negative controls, respectively.

The mutagenic activity of the nonpolar neutral and semipolar aromatic fractions of the 10 PAH-contaminated soils were evaluated using the in vitro *lacZ* transgenic mutation assay in MutaMouse FE1 cells. Linear concentration−responses functions were analyzed, and the slope of the initial linear portion of the curve (i.e., the mutagenic potency) used as a measure of mutagenic activity for each fraction of each soil. The SI includes a figure that depicts a typical concentration−response plot for a nonpolar neutral soil fraction and its corresponding semipolar aromatic fraction. Table 1 summarizes the mutagenic potency values for each fraction of each of the 10 soils. The nonpolar neutral fractions from all 10 soils elicited significant positive responses, and the semipolar aromatic fraction from 8 of the 10 soils elicited significant positive responses. The mutagenic potencies of the nonpolar neutral fractions ranged from 16 to 463×10^{-5} mutants/mg soil eq/mL, and those of the semipolar aromatic fractions ranged from not detectable to 217×10^{-5} mutants/mg soil eq/mL.

Table 1. Mutagenic Potencies of Non-Polar Neutral and Semi-Polar Aromatic Soil Fractions Measured Using the *lacZ* Transgene Mutation Assay in MutaMouse FE1 Cells[a]

	mutagenic potency (induced mutant frequency ($\times 10^{-5}$)/mg soil eq/mL)	
soil	nonpolar neutral soil fraction	semipolar aromatic soil fraction
Holmsund1	16 ± 3	60.5 ± 17.5
Holmsund2	463.1 ± 50.1	3.9 ± 0.4
Holmsund3	174.6 ± 19.1	NM[b]
Luleå	29.9 ± 5.6	5.4 ± 1.3
Husarviken1	30.1 ± 6.1	3.8 ± 1
Husarviken2	49.7 ± 13	18.3 ± 4.3
Forsmo1	287.4 ± 4.3	216.6 ± 32.3
Forsmo2	397.7 ± 87.6	NM
Hässleholm1	221.2 ± 36.9	1.6 ± 0.4
Hässleholm2	102.1 ± 13.7	15.7 ± 4.5

[a]Mutagenic potency is expressed as induced mutant frequency ($\times 10^{-5}$)/mg soil eq/mL ± standard error. In all cases the slope values are significant at $p < 0.01$. [b]NM = not mutagenic.

The mutagenic potencies of the nonpolar neutral fractions were generally greater, and often far greater, than those of the corresponding semipolar aromatic fractions (e.g., 463.1×10^{-5} versus 3.9×10^{-5} mutants/mg soil eq/mL for Holmsund-2). The only exceptions were Holmsund-1, where the observed potency of the semipolar aromatic fraction (i.e., 60.5×10^{-5} mutants/mg soil eq/mL) was greater than the corresponding nonpolar neutral fraction (i.e., 16×10^{-5} mutants/mg soil eq/ml), and Forsmo-1, where the potencies of the two soil fractions were not significantly different at $p < 0.05$. Moreover, the mutagenic activity of the nonpolar neutral soil fractions was found to be significantly correlated with both total and priority PAH concentration (i.e., $r^2 = 0.47$ for both, $p < 0.03$). The mutagenic activity of the semipolar aromatic fractions was not correlated with PAH concentration.

Mutagenic Activities of Synthetic PAH Mixtures and Predicted Activities Based on Expected Contributions from Priority PAHs. The observed mutagenic potencies of the nonpolar neutral fractions were compared to (i) the observed activity of the synthetic priority PAH mixtures, prepared using the results shown in SI Table S1, and (ii) the predicted activity calculated using eq 1. Prediction of potency for the PAH-containing fractions, according to eq 1, required the mutagenic activity of each of the 16 U.S. EPA priority PAHs. As noted, only five priority PAHs (i.e., benzo[*k*]fluoranthene, benzo[*b*]-fluoranthene, BaP, chrysene and dibenz[*a*,*h*]anthracene) induced significant positive responses in the *lacZ* mutation assay in MutaMouse FE1 cells (data not shown). Figure 1

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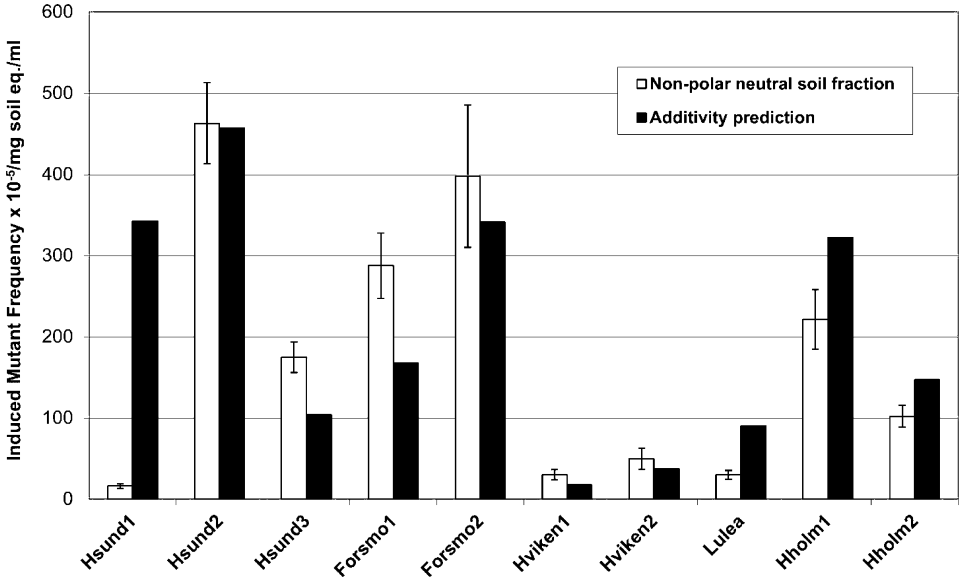

Figure 1. Comparison of observed mutagenic potencies for the nonpolar neutral soil fractions to predictions of their activity calculated using eq 1. All mutagenic potencies were measured using the *lacZ* transgene mutation assay in MutaMouse FE1 cells. Error bars represent standard error. Standard error values for predictions of mutagenic activity based on PAH additivity are not presented (see Materials and Methods for explanation). (Hsund = Holmsund; Hviken = Husarviken; Hholm = Hässleholm).

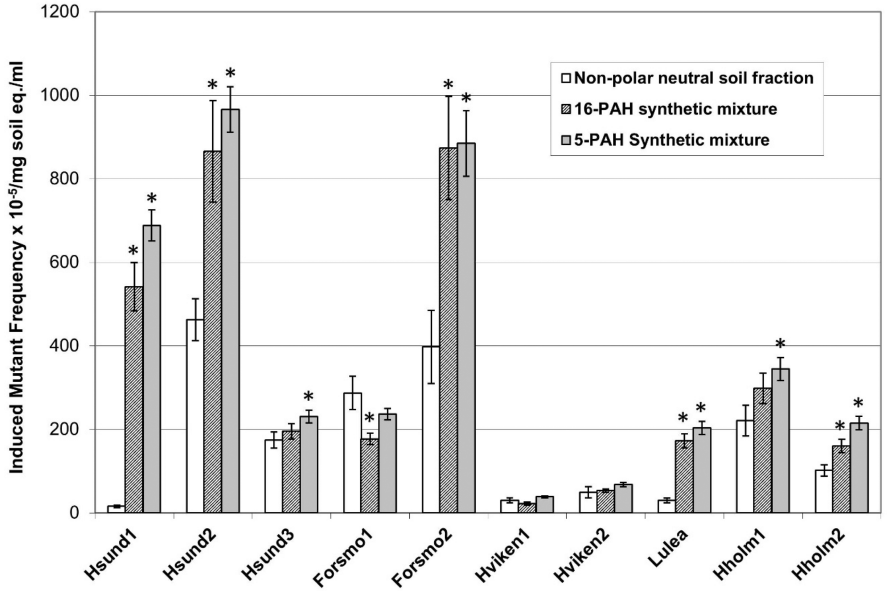

Figure 2. Comparison of observed mutagenic potencies for the nonpolar neutral soil fractions to the activity of synthetic PAH mixtures composed of either 16 priority PAHs or five mutagenic PAHs. All mutagenic potencies were measured using the *lacZ* transgene mutation assay in MutaMouse FE1 cells. Error bars represent standard error, and asterisks' (*) indicate where the mutagenic potency of the simplified, synthetic mixture was significantly different from the nonpolar neutral fraction ($p < 0.05$). (Hsund = Holmsund; Hviken = Husarviken; Hholm = Hässleholm).

shows a comparison between the observed mutagenic potencies of the nonpolar neutral fractions, and the predicted potencies calculated using eq 1. The comparison shows that the predicted potencies are all within 2-fold of the observed potencies, except for Holmsund-1, where the prediction was 21-fold higher than the observed potency. For 6 of the 10 soils studied, the predicted potencies were less than the corresponding observed values, whereas for 4 of the 10 soils the predicted potencies were greater than the observed values.

The observed potency values for the nonpolar neutral fractions were also compared to the potencies of the synthetic PAH mixtures (i.e., sufficiently similar mixtures). Both the 5-PAH mixture, which contained only PAHs that elicited a positive response in the MutaMouse FE1 assay, and the 16-PAH mixture, which contained all 16 U.S. EPA priority PAHs, were compared to the PAH-containing fractions. Each of the synthetic PAH mixtures induced a significant response; however, the 5-PAH mixtures consistently elicited a greater response than the 16-PAH mixtures. Figure 2 shows a comparison of the mutagenic activities for both types of synthetic mixtures, and the corresponding nonpolar neutral fractions. The 5-PAH mixture consistently yielded mutagenic activities that were significantly greater than, or not significantly different from, the corresponding soil fractions. Only a single

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simplified mixture yielded a response less than the corresponding nonpolar neutral fraction (i.e., Forsmo-1). Similar results were obtained for the 16-PAH mixtures. Again, 9 of 10 mixtures elicited responses that were greater than, or not significantly different from, the corresponding soil fractions, and only one mixture yielded a response significantly less than its corresponding nonpolar neutral fraction (i.e., Forsmo-1).

DISCUSSION

Observed Mutagenicity of Soil Fractions. To our knowledge, no studies have used cultured mammalian cells to evaluate the mutagenicity of soil extracts from a PAH-contaminated site; however, several studies have used cultured mammalian cells to assess the mutagenicity of PAH-containing extracts and/or fractions from other complex environmental samples such as urban air particulate matter,[36,37] diesel and gasoline exhaust particles,[38] and coal oil.[39] This study evaluated the mutagenicity of organic fractions from ten PAH-contaminated soils, and the results clearly show that both the nonpolar neutral and semipolar aromatic fractions from coal-tar and creosote contaminated soils induce a significant response in the in vitro *lacZ* transgenic mutagenicity assay in MutaMouse FE1 cells. The results follow those of our previous study, where we showed that these same soil fractions were mutagenic in a bacterial test system (i.e., the Salmonella reverse mutation assay).[8] Other studies of PAH-contaminated soils have also noted potent activity in soil extracts, and PAH-containing extract fractions using the Salmonella assay. For example, de Souza Pohren et al. (2012) found significant Salmonella mutagenic activity in organic, PAH-containing extracts of soil from a wood preservation site,[40] and Hughes et al. (1998) detected significant Salmonella mutagenicity in several soil fractions from a creosote-contaminated Superfund site.[41]

Here, and in our previous study,[8] the mutagenic activities of the PAH-containing soil fractions exceeded those of the corresponding semipolar aromatic soil fractions, indicating that the mutagenic activity associated with extractable organics is dominated by PAHs and similar nonpolar neutral compounds (e.g., alkyl-PAHs). This assertion is supported by the significant positive correlation between the mutagenic activity of the nonpolar neutral fractions and the PAH content of the soils examined. Moreover, several of the detected unsubstituted, homocyclic PAHs (e.g, BaP, benzo[*b*]fluoranthene) are recognized mutagens.[42−45] O- and S- heterocyclic compounds may also contribute to the mutagenicity of this fraction since earlier validation of the fractionation protocol showed that they can elute into the nonpolar neutral fraction.[32]

The fact that the semipolar aromatic soil fractions elicited significant positive responses, albeit weaker compared with those of the corresponding nonpolar neutral fractions, should not be disregarded. Mutagenic activity in this soil fraction indicates that the soils contain hitherto unidentified mutagens that are more polar than priority PAHs, yet the hazards posed by these substances cannot currently be included in a routine risk assessment. The identities of mutagenic polar PACs present at PAH-contaminated sites remain largely unknown; however, insight into the types of compounds that might be expected in this fraction has been provided by other studies of PAH-contaminated sites (e.g., refs 8, 32, and 46). For example, such compounds may include oxygenated PAHs (i.e., oxy-PAHs), nitroarenes, or aromatic amines, including *N*-heterocyclic compounds, some of which have been shown to be mutagenic in bacteria,[47] and mammalian cells.[44,48] Indeed, recent studies of soils from wood preservation and MGP sites have attempted, sometimes unsuccessfully, to identify hitherto unknown polar mutagens.[32] For example, Park et al. (2008) detected significant mutagenic activity in polar soil fractions from former MGP and wood preservation sites, but they could not identify the putative mutagens responsible for the activity.[49] Similar studies of contaminated sediments from industrial sites in Germany noted that polar PACs such as nitroarenes, azaarenes and keto-PAHs contributed to the observed mutagenic activity; however, a multitude of other PACs, including hydroxy compounds, lactones and quinones were also tentatively identified in mutagenic polar fractions.[50−52] The contributions of the latter substances could not be quantified due to a lack of chemical standards. Despite the obvious analytical challenge of positively identifying mutagenic polar PACs in contaminated soils, several of the researchers mentioned above have commented on the necessity, and moreover, have established the required analytical methodology.[33,53−55] The importance of investigating polar PACs in contaminated soils, particularly oxy-PAHs, has been highlighted by Lundstedt et al. 2007,[46] and the identification and hazard assessment of polar PACs in contaminated soils in general is among the stated objectives of the Polar PAC Network (http://www.mcnio.com/projects/the-polar-pac-network.html).

Comparisons of the Complex Soil Fractions, Synthetic PAH Mixtures, and the Sum of the Expected Contributions from Priority PAHs. Overall, the results show that predictions of mutagenicity based on the sum of the activity for each of the targeted, mutagenic PAHs are similar to the observed activities of the PAH-containing fractions, with the noteworthy exception of Holmsund-1 (Figure 1). In fact, most predictions are within 2-fold of the measured activity. Nevertheless, a combination of factors, which are addressed in more detail below, can account for differences that were observed. These include alterations in metabolic capacity, as well as contributions from unidentified mixture components.

Instances where the predicted mutagenic activities are less than those observed for the PAH-containing fractions (e.g., Holmsund-3, Forsmo-1) are likely attributable to the presence of unidentified mutagens in the complex soil fractions. Predicted activity values are based solely on the contributions from priority PAHs and, as already noted, other compounds present in the soils may be contributing to the mutagenicity of the nonpolar neutral soil fraction (e.g., alkyl PAHs, O- and S-heterocyclics). Information regarding the structure and mutagenic activity of such compounds is rarely available. Although these substances will contribute to the hazards posed by nonpolar neutral pollutants at PAH-contaminated sites, the significance of that hazard cannot be ascertained until the putative mutagens are identified.

Another likely driving force behind the greater-than-additive effects of the complex PAH-containing fractions is the induction of enhanced AhR-mediated metabolism of the mixture components. Conversion of mutagenic PAHs to DNA-reactive metabolites is known to involve cytochrome P450 isozymes such as CYP1A1, CYP1A2, and CYP1B1, all of which are known to be inducible in FE1 cells, and the production of these isozymes is known to be controlled by AhR agonism. Since PAHs are AhR agonists, and are thus capable of inducing the enzymes required for their own metabolism,[2] it is not unreasonable to expect that dynamic modifications in metabolic capacity plays an important role in determining the mutagenicity of a PAH mixture. For example, Mahadevan et al.

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showed that exposure to a complex PAH-containing mixture (i.e., coal tar extract) increased the levels of CYP1A1 and CYP1B1 proteins in human MCF-7 cells; however, the same study showed that DNA adduct levels observed following cotreatment with coal tar extract and either BaP or DBalP (dibenzo[*a,l*]pyrene) were lower than those observed for BaP or DBalP alone.[15] A similar study of urban air particulate matter showed that in vitro exposure can increase the expression of *CY1A1* and *CYP1B1* genes, and cotreatment with BaP and urban dust augments gene expression relative to BaP alone; again, the level of DNA adducts for the cotreatment was lower than that for BaP alone.[13] Related studies by Courter et al. showed increased EROD (ethoxyresorufin-*O*-deethylase) activity and *CYP1B1* expression following in vitro coexposure to diesel exhaust particulate matter extract and DBalP, compared to DBalP alone, and a slight increase in skin tumorigenicity following cotreatment with BaP and diesel exhaust particulate extract, compared to BaP alone.[17,18] Interestingly, several studies have also noted that in vitro exposures of human cells to complex PAH-containing mixtures such as cigarette smoke condensate[56] and diesel exhaust particulate matter extract,[17] or coexposure to diesel particulate extract and either BaP or DBalP, can augment the expression of aldo-keto reductase (AKR) genes (e.g., *AKR1C1*). AKRs catalyze the conversion of PAH dihydrodiols to *o*-quinones that are known to be capable of inducing abasic sites and oxidative DNA lesions.[57] In addition, nonmutagenic PAHs such as anthracene have been shown to enhance the mutagenic activity of BaP.[58] Thus, mutagenic and nonmutagenic PAHs in the soil fractions investigated is likely contributing to the augmentation of the metabolic machinery in FE1 cells (e.g., P450 isozymes, AKRs, etc.); this would enhance the metabolism of mutagenic PAHs to reactive metabolites, and thus augment the overall mutagenic activity of the mixture.

Conversely, instances where the predicted mutagenic activities calculated using eq 1 were greater than those observed for the PAH-containing fractions may result from competitive inhibition or saturation of enzymes involved in the metabolism and activation of PAHs. Such phenomena would contribute to a reduction in the mutagenic activity of a complex PAH-containing mixture relative to the theoretical maximum calculated as the sum of the contributions of each of the mutagenic priority PAHs. It seems likely that both mutagenic and nonmutagenic PAHs in the complex PAH-containing fraction compete for access to CYP isozymes. In fact, many nonmutagenic PAHs, such as phenanthrene and naphthalene are known P450 substrates,[59,60] and several PAHs have been shown to inhibit the activity of human CYP1A1, 1A2 and 1B1.[61] Moreover, some studies have shown that the presence of nonmutagenic PAHs in a mixture with mutagenic PAHs, such as BaP, reduces mutagenic activity. In contrast to inhibition of mutagenic activity by coexposure with nonmutagenic PAHs, in vitro exposures to mutagenic PAHs in isolation permit the mutagen to have exclusive access to the metabolic machinery required to convert the substance to a DNA-reactive metabolite. The resulting potency of each individual PAH can be viewed as a maximum, and this value may contribute to overestimation of mixture activity according to eq 1.

This notion of "metabolic insufficiency" for mutagenic PAHs in complex mixtures is supported by several studies. Courter et al. showed that the tumorigenicity of BaP is significantly delayed by cotreatment with a PAH-containing complex mixture (i.e., urban particulate matter extract), and that this effect is likely associated with dose-related, noncompetitive inhibition of CYP1A1 and CYP1B1 activity.[16] Related studies noted that in vitro cotreatment with diesel particulate extract and BaP elicited significantly decreased levels of DNA adducts, compared with BaP alone; cotreatment of Sencar mice with DBalP and diesel particulate extract markedly reduced tumorigenicity, compared with DBalP alone.[17,18] The latter observation is thought to be related to the stronger inhibitory effect of PAH-containing complex mixtures on CYB1B1 activity, relative to CYP1A1.[14,18] The aforementioned Mahadevan et al. studies showed that a complex PAH-containing mixture derived from coal tar can decrease the levels of DNA adducts formed by BaP or DBalP.[15] Follow-up work by the same group demonstrated competitive inhibition of human CYP enzymes in V79 cells, and moreover, that this competitive inhibition resulted in a reductions of BaP- and DBalP-induced DNA adduct formation; the inhibitory effects of the complex mixture on CYP1B1 activity was found to be much stronger in comparison with CYP1A1.[14] Similarly, a study by Binkova & Sram showed that environmental PAH mixtures containing BaP (i.e., extractable organic matter from respirable air particles) induced lower levels of DNA adducts in human embryonic lung fibroblast cells compared to BaP alone.[19]

The comparisons between the mutagenic activity of the PAH-containing nonpolar neutral fraction and the synthetic PAH mixtures showed that the activity of the 5-PAH and 16-PAH mixtures is the same or higher than their corresponding complex mixtures. More specifically, the overall trend shows that the complex PAH-containing fractions have the lowest activity, followed by the 16-PAH mixture, and then the 5-PAH mixture. In other words, increased mixture complexity is associated with decreased mutagenic activity. This pattern is consistent with the aforementioned hypothesis of "metabolic insufficiency", that is, mutagenic PAHs in a more simplified mixture can be more effectively metabolized than mutagenic PAHs in a complex fraction. Comparison of the 5-PAH and 16-PAH mixtures revealed that the more simplified mixture containing only PAHs that elicit a significant positive response in the FE1MutaMouse assay (i.e., benzo[*k*]fluoranthene, benzo[*b*]fluoranthene, BaP, chrysene and dibenz[*a,h*]-anthracene) is always more mutagenic, thus providing additional support for the contention that competition with nonmutagenic PAHs for the limited metabolic machinery likely limits the mutagenic activity of a PAH mixture in vitro.

The results of this work provide only circumstantial evidence for competitive inhibition and/or metabolic saturation of CYPs by nonmutagenic PAHs in a complex mixture, and additional research would be required to confirm the hypothesis of "metabolic insufficiency". For example, follow-up experiments could involve augmentation of enzymatic capacity in vitro exposure via the addition of exogenous microsomes isolated from FE1 cells or MutaMouse tissue. In theory, the addition of microsomal enzymes should boost the mutagenic activity of the nonpolar neutral soil fractions, or the 16-PAH mixtures, to the level observed for the 5-PAH mixture. Measurements of AhR agonism, and the induction of various CYP isozymes, following exposure to single compounds and PAH mixtures would also contribute to improved understanding regarding the metabolic alterations induced by PAHs and PAH mixtures. Regardless, critical examination of the "metabolic insufficiency" hypothesis is a profitable area for follow-up research since improved understanding of the toxicological behavior of PAHs in

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complex mixtures may impact contaminated site risk assessment.

The results of this study indicate that the mutagenic activity of PAHs in complex mixtures, and by extension, potential carcinogenic hazard, is influenced not only by the levels of known mutagens in the mixture, but also by the presence of nonmutagenic PAHs and related compounds. Moreover, the dynamic metabolic processes that catalyze the conversion of PAHs to oxidized metabolites, some of which are DNA reactive, are controlled by a complex, dynamic interplay of AhR agonism and stimulation of P450 isozyme production, and competitive inhibition of P450 isozymes by both mutagenic and nonmutagenic PAHs in the mixtures. In addition, soils contaminated with complex mixtures of PAHs and related compounds, such as those examined in this study, also contain more polar mutagens that may be contributing to overall hazard. These are currently not included in routine contaminated site risk assessment protocols. It is important to emphasize that whereas most of the aforementioned studies of complex PAH mixtures investigated changes in DNA damage frequency and/or metabolic capacity, the current study examined induced mutant frequency, which is determined by the complex dynamic interplay between metabolism and DNA damage processing. Several studies have investigated factors that influence the formation, persistence and mutagenicity of DNA damage induced by individual PAHs and PAH-containing complex mixtures.[17,62−64]

In our companion paper,[27] we describe a novel bioassay-based approach to calculate levels of BaP equivalents for use in the assessment of excess lifetime cancer risk, and compare the results obtained to those generated using the standard risk assessment paradigm based on a small number of targeted PAHs and an assumption of additivity.

Follow-up work, which is currently underway, is employing subchronic (i.e., 28-day), repeat-dose oral MutaMouse exposures, and subsequent quantification of *lacZ* mutant frequency in multiple tissues (e.g., stomach, small intestine, liver, bone marrow), to extend investigations into the mutagenic activity of PAH mixtures and the degree to which observed responses are consistent with those expected based on the concentrations and activities of priority PAHs. Tissue-specific alterations in metabolic capacity and DNA damage processing are also being examined. This work will ultimately contribute to an improved understanding regarding the mutagenic and carcinogenic hazards posed by PAHs in complex mixtures.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information includes the concentration (μg/g dry wt) of 24 PAHs in the soil samples examined (Table S1), as well as an example of typical concentration−response plots of induced *lacZ* mutant frequency (x 10^{-5}) in FE1 cells exposed to a nonpolar neutral fraction of an organic soil extract and its corresponding semipolar aromatic fraction (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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