# Environment Canada
## Water Science and Technology Directorate

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## Direction générale des sciences et de la technologie, eau
# Environnement Canada



Relative Genotoxicities of PAH of molecular weight 252 amu in coal tar-contaminated sediment

By:
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99-247

## MANAGEMENT PERSPECTIVE

- **Title:** Relative genotoxicities of PAH of molecular weight 252 amu in coal tar-contaminated sediment.

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- **NWRI Publication #:** 99-247
- **Citation:**

- EC Priority/Issue: This work was done as part of the Great Lakes 2000 Program and the Great Lakes University Research Fund, to aid in the assessment and remediation of contaminated sediments in areas of coal tar contamination such as Sydney Harbour and Hamilton Harbour. This work supports the EC priority of implementing ecosystem initiatives focused on ecosystems of national priority including AOCs in the Great Lakes and Atlantic Coastal Areas.

- Current Status: This work is an investigation of the genotoxicological potential of polycyclic aromatic hydrocarbons in coal tar-contaminated sediment. A combination of multi-dimensional chromatographic techniques and the Ames *Salmonella*/microsome assay was used to asses the contribution of the molecular mass 252 benzologue class and some individual compounds to the total genotoxic burden of coal-tar contaminated sediment. This work underscores the significant contribution of these compounds to the biological activity of complex environmental mixtures; their presence must be taken into consideration when planning remediation of PAH-contaminated sediments.

- Next Steps: To be presented at the 17th International Symposium on Polycyclic Aromatic Compounds (Bordeaux, France) and communicated to RAP processes.

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# RELATIVE GENOTOXICITIES OF PAH OF MOLECULAR WEIGHT 252 AMU IN COAL TAR-CONTAMINATED SEDIMENT

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Abstract. Bioassay-directed chemical fractionation methodology was used to calculate relative mutagenic potencies of polycyclic aromatic hydrocarbons (PAH) of molecular weight 252 amu in coal tar-contaminated sediment from Sydney Harbour, Nova Scotia. A normal phase HPLC technique was used to separate organic solvent extracts into fractions containing isomeric PAH of a single benzologue class. Bioassays with *Salmonella typhimurium* strain YG1025 with the addition of oxidative metabolism (S9) showed that approximately 50% of the mutagenic activity observed in the sediment extract was associated with PAH of molecular weight 252 amu. Further separation of the 252 PAH fraction using reversed phase HPLC yielded subfractions containing individual compounds; bioassay dose-response curves for these subfractions showed that benzo[a]pyrene was responsible for approximately 75% of the activity of the 252 PAH fraction.

Keywords polycyclic aromatic hydrocarbons; *Salmonella typhimurium*; bioassay-directed fractionation; genotoxicity; coal tar; sediments.

## INTRODUCTION

We previously used bioassay-directed fractionation to isolate and identify polycyclic aromatic hydrocarbons (PAH) including benzo[a]pyrene, indeno[1,2,3-cd]pyrene and PAH of molecular weight 302 amu as primary mutagens in extracts of coal tar-contaminated sediments from Hamilton Harbour, Lake Ontario[1] and Sydney Harbour, Nova Scotia[2,3]. In our most recent study[3], multi-dimensional chromatographic fractionation of crude extracts was carried out by open column alumina chromatography followed by normal phase HPLC to isolate fractions containing PAH of a single benzologue class. These fractions were bioassayed using the Ames *Salmonella typhimurium* microsome assay in a strain that detects base-pair substitutions (TA100-like strain) with the addition of oxidative metabolism in the form of an Aroclor 1254-induced rat liver supernatant (S9); PAH require oxidative transformations to reactive intermediates to manifest their biological responses. Selected PAH fractions exhibiting significant mutagenic activity were further separated by reversed phase HPLC and subfractions of the column effluent were collected. The subfractions were then bioassayed to identify individual PAH isomers within a molecular weight class which were responsible for the observed mutagenic response.

In our most recent bioassay-directed fractionation study of coal tar-contaminated sediment from Sydney Harbour (Nova Scotia, Canada), the molecular weight 252 amu PAH class of compounds was estimated to be responsible for almost 50% of the total mutagenic activity contained in the sample extract[3]; benzo[a]pyrene alone was estimated to be responsible for about 75% of the activity of the

molecular weight 252 PAH fraction. Relative contributions of the different molecular weight PAH classes isolated by normal phase HPLC to the total mutagenic activity of the extract were extrapolated from the initial linear portions of the dose-response curves. Estimates of the contributions of individual PAH, as in the case of benzo[a]pyrene, were estimated from single assays of subfractions collected during reversed phase HPLC separation of the parent molecular weight PAH fraction. Estimating the mutagenic potency or the relative contribution of a PAH to the total mutagenicity from a single dose value can be unreliable as the linear dynamic range of the assay is limited.

In this paper, we present a more detailed investigation of the contributions of individual PAH of molecular weight 252 amu to the mutagenic activity of an extract of coal tar-contaminated sediment from Sydney Harbour, Nova Scotia. A molecular weight 252 PAH fraction prepared using normal phase HPLC was separated by reversed phase HPLC and subfractions of the column effluent were collected. Each of the subfractions was assayed using the Salmonella typhimurium microsome assay with a TA100-like strain and metabolic activation. The fractions exhibiting positive bioassay responses were re-assayed using a dose range of five concentrations. The linear portions of the resulting dose-response curves were used to determine mutagenic potencies and to estimate the relative contributions of individual 252 PAH isomers to the mutagenic activity of the sample extract.

## EXPERIMENTAL

Dry sediment (5 g) was suspended in 50 mL of dichloromethane in a glass beaker. Eight consecutive ultrasonic pulses (15 seconds duration

each) were applied at full power using a 300 watt Fisher Sonic Dismembrator Model 300 with a 3/4 inch diameter titanium horn (Fisher Scientific, Fairlawn, NJ). The suspension was filtered and the procedure repeated with 50 mL of dichloromethane, followed by final sonication with 50 mL of methanol. Sediment free extracts were pooled to form a single extract. The extract was adsorbed onto neutral alumina (3 g, Brockman activity 1, 80-200 mesh) by solvent evaporation and applied to the top of fresh alumina (6 g) contained in a glass column. Organic components were eluted using solvents of increasing polarity; hexane (60 mL) afforded an aliphatic fraction followed by elution of non-polar PAC by addition of dichloromethane (70 mL). The PAC fraction was subjected to a Sephadex LH20 (Pharmacia Fine Chemicals, Uppsala, Sweden) column clean-up step (mobile phase, hexane/methanol/dichloromethane (6:4:3 v/v), flow rate 3 mL/min) to remove any remaining aliphatic compounds.

The non-polar PAC fraction was separated into PAH molecular weight classes using a normal phase 10 micron 25 cm X 9.4 mm i.d. Whatman Partisil M9 PAC (polyaminocyano) semi-preparative HPLC column (Whatman, Clifton, NJ). A 100 microlitre sample loop and a mobile phase flow rate of 4.2 mL/min was used with the following linear gradient elution program (elapsed time, composition of mobile phase): initial, 95% hexane and 5% dichloromethane; 10 min, 95% hexane and 5% dichloromethane; 30 min, 75% hexane, 25% dichloromethane; 45 min, 45% hexane, 55% dichloromethane; 60 min, 100% dichloromethane; 65 min, 100% acetonitrile; 70 min, 100% acetonitrile; 75 min, 100% dichloromethane; 80 min, 95% hexane, 5% dichloromethane. The column temperature was maintained at 40°C.

Reversed phase HPLC was performed using a 5 micron 25 cm X 4.6 mm i.d. Vydac Reversed Phase analytical column (201TP54, Separations Group, Hesperia, CA). A mobile phase flow rate of 1 mL/min was used with the following linear gradient elution programs (elapsed time, composition of mobile phase): Program A initial, 70% acetonitrile, 30% water; 30 min, 100% acetonitrile. Program B initial, 50% acetonitrile, 50% water; 30 min, 100% acetonitrile. The column temperature was maintained at 35°C.

The *Salmonella typhimurium* bacterial strain used was strain YG1025 which is similar to TA100 but is auxotrophic for histidine (his G46) and contains plasmids pKM101 and pBR322[4]. The bioassay protocol was adapted from Maron and Ames[5]. Bacteria were grown for 10 hours at 37°C in Oxoid Nutrient Broth #2 (15 mL) with ampicillin (50 μg/mL) and tetracycline (6.25 μg/mL). Extracts were dissolved in 50 μL DMSO and assayed in duplicate with metabolic activation (4% Aroclor 1254-induced rat liver S9). A dose range of five concentrations was assayed to produce a dose-response curve. After a 48 hour incubation at 37°C, the number of revertant colonies (histidine independent) was determined. Biological activity values were extrapolated from linear segments of the dose response curves. The average spontaneous reversion rate (background rate) of strain YG1025+S9 was 190 revertants per plate. Positive controls routinely used in this study were sodium azide and benzo[a]pyrene.

## RESULTS AND DISCUSSION

Sediment was sampled from Muggah Creek, Sydney Harbour, Nova Scotia in an area heavily contaminated as a result of coal tar

discharges[6,7]. Dry sediment was extracted sequentially with dichloromethane and methanol using an ultrasonication technique described previously[8]. The crude extract (8.0% by weight of dry sediment) was subjected to open column alumina chromatography, affording a non-polar aromatic polycyclic aromatic compound (PAC) fraction. This PAC fraction was further cleaned-up using a Sephadex LH20 gel column to remove remaining aliphatic compounds. The non-polar PAC fraction, which contained all of the PAH, accounted for 28% of the mass of the crude extract. Total PAH content of the sample was approximately 11,000 μg/g of dry sediment[3]. The non-polar PAC fraction contained over 90% of the mutagenic activity in the sample extract[3].

The non-polar PAC fraction was separated using normal phase HPLC into fractions containing PAH of molecular masses ranging from 178 to 352 amu. These fractions were bioassayed with *Salmonella typhimurium* strain YG1025 with the addition of rat liver supernatant (S9). The molecular weight 252 PAH fraction was found to be responsible for roughly 50% of the mutagenic activity displayed by the sediment extract[3]. Analysis of this fraction by probe electron impact-MS showed ions due almost exclusively to molecular masses of 252 amu PAH and their alkyl derivatives. The 252 PAH fraction was then analyzed by reversed phase HPLC (program A) and 30-second subfractions were collected. Each subfraction was subjected to bioassay with strain YG1025+S9. The resulting plot of the mutagenic responses of the molecular weight 252 amu PAH fraction and its UV absorption profile, called a mutation chromatogram, is shown in Figure 1. The mutation chromatogram characterizes the HPLC column effluent in

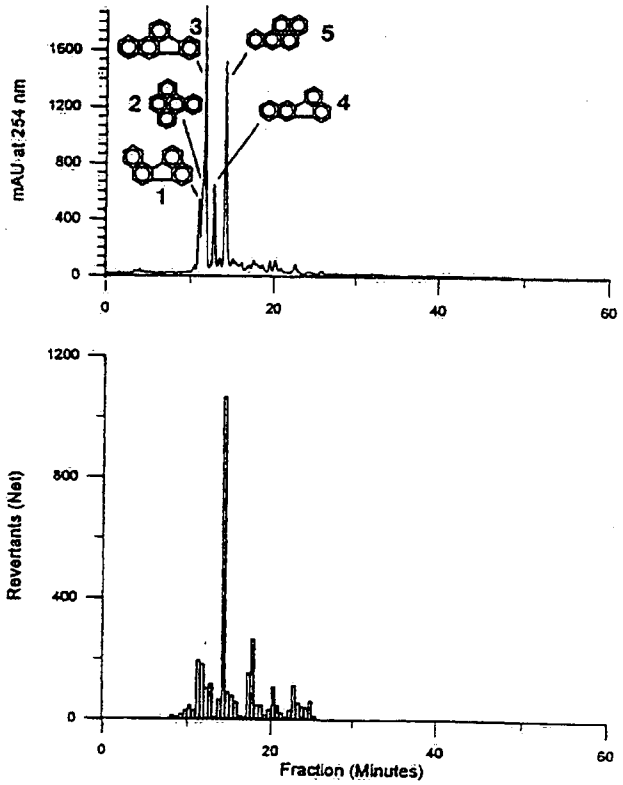


FIGURE 1. Molecular weight 252 amu PAH fraction mutation chromatogram. Thirty-second subfractions were collected during reversed phase HPLC separation and then subjected to bioassay with strain YG1025+S9. The top panel shows the HPLC UV absorption profile with PAH identified by number: 1. benzo[j]fluoranthene 2. benzo[e]pyrene 3. benzo[b]fluoranthene 4. benzo[k]fluoranthene 5. benzo[a]pyrene. The bottom panel shows net mutagenic responses exhibited by single doses of each of the individual subfractions.

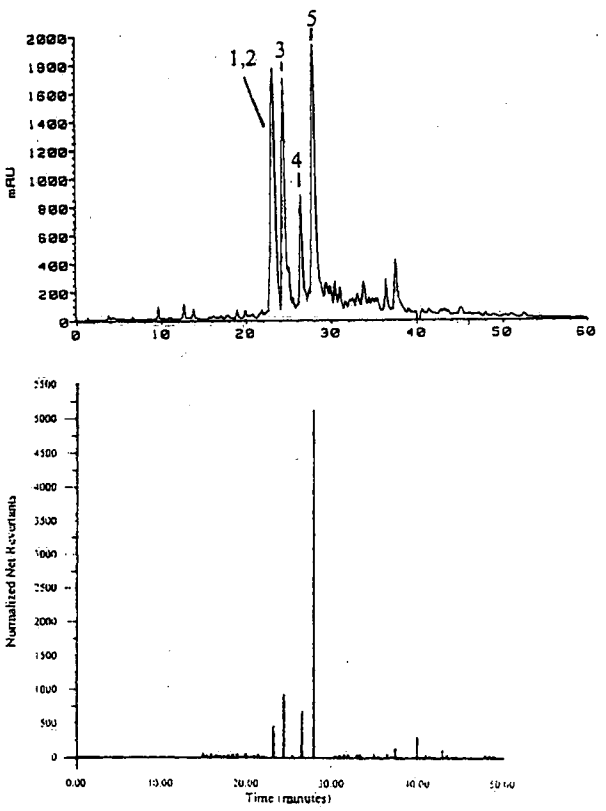


FIGURE 2. Dose-corrected molecular weight 252 amu PAH mutation chromatogram. Thirty-second subfractions were collected during reversed phase HPLC and subjected to bioassay with strain YG1025+S9. The top panel shows the HPLC UV absorption profile with PAH identified by number: 1. benzo[j]fluoranthene 2. benzo[e]pyrene 3. benzo[b]fluoranthene 4. benzo[k]fluoranthene 5. benzo[a]pyrene. The bottom panel shows the mutagenic responses calculated from dose-response curves for each compound.

terms of both a conventional UV absorption profile, and a mutagenic activity profile. This experimental approach enables isolation of biologically active compounds in single subfractions to facilitate their identification. The mutagenic activities of the individual subfractions are expressed as net revertants, reflecting background subtraction of the spontaneous reversion rate of YG1025+S9 (190 revertants). Subfractions judged to exhibit a positive mutagenic response were those that equalled or exceeded 190 net revertants (equivalent to a doubling of the spontaneous reversion rate of YG1025+S9). The molecular weight 252 mutation chromatogram exhibited a peak of high mutagenic activity that co-eluted with benzo[a]pyrene (Figure 1). Other active subfractions contained the benzofluoranthenes (b, j and k) and benzo[e]pyrene. The identity of these compounds was confirmed by comparison of their reversed phase HPLC retention times and UV absorption spectra with authentic standards, and by GC-MS analyses of the individual subfractions (data not shown).

The large positive response exhibited by the subfraction containing benzo[a]pyrene indicated this compound was responsible for the majority of the mutagenic activity contained in the 252 PAH fraction. Previously, we had estimated that benzo[a]pyrene was responsible for 75% of the activity of this fraction[3]. However, this estimate was based on mutagenic activity values determined from single bioassay analyses. The doses of these mutagenic compounds may not fall within the linear range of the dose-response curve; if so, the calculated activities may not accurately reflect the true potencies of the fractions. A second reversed phase HPLC separation was performed on

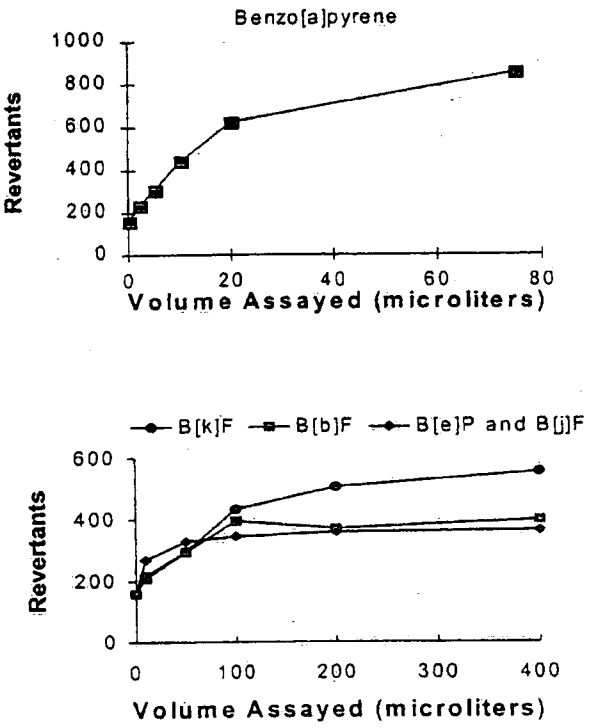


FIGURE 3. Dose-response curves exhibited by molecular weight 252 amu PAH in thirty-second subfractions collected during reverse phase analysis when assayed with strain YG1025+S9.

the 252 PAH fraction using a modified gradient elution program (program B) which resulted in improved separation of benzo[b]fluoranthene (Figure 2). Column effluent subfractions corresponding to each of the four major chromatographic peaks were collected in addition to 30-second subfractions from 15-50 min; the resulting mutation chromatogram (Figure 2) was produced by

determining the dose-corrected bioassay response in strain YG1025+S9 of each subfraction. Those subfractions which showed responses of 350 revertants or greater were then re-assayed using a dose range of five concentrations (Figure 3). Bioassays of the subfraction containing benzo[a]pyrene were repeated at greater dilution to afford linear dose-response data (Figure 3). The non-linear nature of the dose-response curves at the higher doses (Figure 3) are typical of PAH-rich extracts[9].

TABLE 1. Mutagenic potencies of molecular weight 252 amu PAH calculated from dose-response curves produced from a) assays of standard compounds in strain YG1025+S9 and; b) from assays in strain YG1025+S9 of subfractions collected by reversed phase HPLC analysis as shown in Figure 2.

| **Compound** | **Bioassay Responses (rev/μg)** | | |
|---|---|---|---|
| | Stds | Subfrns | % of total |
| 1. Benzo[j]fluoranthene | 33 | 40* | 2% |
| 2. Benzo[e]pyrene | 43 | 52* | 7% |
| 3. Benzo[b]fluoranthene | 43 | 43 | 13% |
| 4. Benzo[k]fluoranthene | 55 | 57 | 10% |
| 5. Benzo[a]pyrene | 310 | 350 | 68% |

*activities were calculated using PAH concentrations in the subfraction and standard bioassay responses using the following equation:

$$1.30[A]\ \text{rev}/\mu g \times 4.9\ \mu g + [A]\ \text{rev}/\mu g \times 2.4\ \mu g = 350\ \text{revertants}$$

where 4.9 μg and 2.4 μg were the quantities of benzo[e]pyrene and benzo[j]fluoranthene, respectively, in 100 μL of the subfraction; 350 revertants was the estimated response of 100 μL of the subfraction; 1.30 was the ratio of standard activities of benzo[e]pyrene to benzo[j]fluoranthene, and; [A] was the response of benzo[j]fluoranthene. By calculation, [A] = 40 rev/μg.

The potential inaccuracies resulting from calculation of mutagenic activities from single-dose experiments are apparent when considering the flat nature of the dose-response curves at the higher concentrations.

The molecular weight 252 PAH fraction was characterized quantitatively by GC-MS prior to reversed phase HPLC separation. Therefore, the quantity of individual PAH collected in the subfractions that were subsequently bioassayed was known. Authentic standards of 252 PAH were also assayed in strain YG1025+S9 and these data were found to have standard deviations of roughly 15%; these data were then compared with mutagenic activities extrapolated from the dose-response curves produced for the dose-response mutation chromatogram (Table 1). For HPLC subfractions containing single compounds, the calculated activities were within 12% of the standard values, indicating that these data were indistinguishable from data for the standards and that these compounds were primarily responsible for the activities in the subfractions. Benzo[e]pyrene and benzo[j]fluoranthene co-eluted in the reversed phase HPLC separation (Figure 2); mutagenic activities were calculated from the dose-response curve resulting from bioassays of the HPLC subfraction containing the two compounds (Figure 3) using the known concentrations of the two compounds in the parent fraction and their standard bioassay responses. Both of the experimentally derived mutagenic activities were within approximately 17% of the responses exhibited by the corresponding standards. A statistical comparison of the experimentally derived and standard responses would require an additional number of replicate experiments.

The GC-MS analyses of the subfractions showed that perylene co-eluted in the subfraction containing benzo[b]fluoranthene. However,

bioassays of a perylene standard solution yielded no response in the *Salmonella* assay. The percent contributions to the overall mutagenic activity of the 252 PAH fraction were based on the five compounds listed in Table 1. Both mutation chromatograms (Figures 1 and 2) indicated the presence of additional mutagenic compounds in the 252 PAH fraction that eluted after benzo[a]pyrene. The peak of mutagenic activity at 40 min (Figure 2) has tentatively been attributed to anthanthrene, a molecular weight 276 amu PAH. Other bioactive compounds may be alkyl derivatives; probe mass spectrometry analyses of the 252 PAH fraction showed the presence of compounds of molecular weights of 266 amu and 280 amu. Work is currently underway to identify additional potential mutagens in the extract.

## CONCLUSIONS

Using the data obtained from the dose-response curves shown in Figure 3, it was estimated that benzo[a]pyrene was responsible for approximately 70% of the mutagenic activity exhibited by the 252 PAH fraction, which was close to the value of 75% previously estimated using single-dose data[3]. Both the reversed phase HPLC subfraction containing benzo[a]pyrene and a standard solution exhibited a strong response when assayed with YG1025+S9. Other compounds exhibited considerably weaker responses; benzo[e]pyrene and the benzofluoranthene isomers were estimated to individually contribute less than 15% of the activity associated with the molecular weight 252 PAH fraction.

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