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Determination and Genotoxicity of Polycyclic Aromatic Hydrocarbons Isolated from *Dreissina polymorpha* (Zebra Mussels) Sampled from Hamilton Harbour

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ABSTRACT. ***Polycyclic aromatic hydrocarbons (PAH) were determined in extracts of zebra mussels sampled from sites in Hamilton Harbour. The mussels were extracted using a tissue homogenizer method and the resulting solvent extracts were subjected to an alumina column and a Sephadex LH20 column clean-up procedure. Total PAH concentrations in Zebra mussel extracts ranged from 0.35 μg/g to 10.0 μg/g of mussel homogenate. The extracts were also found to exhibit significant mutagenic responses in the Ames Salmonella/microsome assay. The results indicate that zebra mussels might be used as biological indicators of PAH contamination in freshwater environments such as Hamilton Harbour,***

INDEX WORDS: ***Polycyclic aromatic hydrocarbons, zebra mussels, Hamilton Harbour, genotoxicity, PAH.***

INTRODUCTION

The use of mussels as bioindicators of pollution in aquatic environments has been described previously (Butler and Sibbald 1986, Dunn 1976, Dunn and Stich 1976). Both freshwater and marine mussels have been shown to display multi-xenobiotic resistance and to accumulate non-polar organic contaminants in their tissues (Hartley and Johnston 1983, Kurelec 1992). Mussels feed by filtering large volumes of water and their sedentary nature results in the sampling of the contaminants in a specific area. Mussels can also provide time-integrated data of contaminant levels in particle or water fluxes over long periods of time. Water sampling by either grab sampling or centrifugation permits the evaluation of contaminant concentrations in that sample matrix at that specific time. The sampling of suspended sediments requires sampling times which may vary from weeks to a year; the infrastructure requirements for suspended sediment collection can be prohibitive. In contrast, the analysis of contaminant levels in mussel tissue provides a time-integrated sample for the bioconcentration of xenobiotics by a living organism in the environment.

Hamilton Harbour, a body of water covering approximately 40 km^2, is an embayment located at the western end of Lake Ontario. Sediments in some areas of the harbor contain high levels of contaminants including PAH (Marvin et al. 1992, Murphy *et al.* 1990). These compounds, many of which are considered to be strong mutagens and/or carcinogens, entered the harbor through a number of routes, including discharges of industrial effluents, treated municipal sewage, atmospheric deposition, and roadway runoff.

In recent years the Great Lakes have been invaded by the exotic freshwater mussel *Dreissina*

polymorpha which is native to Europe. Since its initial detection in 1990, this mussel has become firmly established in Hamilton Harbour. The mussel's high spawning rate, great mobility, and high population densities (Griffiths ***et al.*** 1991, Cooley 1991, Mackie 1991) have resulted in an immediate impact on the harbor and its ecosystem. Research is currently underway to develop methods for the control of the zebra mussel and to project its future distribution in North American waterways (Neary and Leach 1992, Griffiths ***et*** al. 1991, Strayer 1991). In some areas, the zebra mussel population has risen to a level where it has become a major food source for fish and wildlife; this new food source has resulted in the disruption of bird migration cycles and exposure of fish and wildlife to the contaminants accumulated in mussel tissue.

In this paper we report our results in determining the PAH content and genotoxicity of organic solvent extracts of zebra mussels sampled from locations in Hamilton Harbour. We have used a simple and efficient method for the extraction of mussel tissues and for the clean-up of the resulting solvent extracts. This method results in the accurate determination of PAH in extracts that are free of many interfering compounds. The extracts were also tested for genotoxicity using the ***Salmonella typhimuriumlmicrosome*** assay. The results of this work suggest that zebra mussels may be an appropriate choice as bioindicators of PAH contamination in the harbor water column. As a result the need for the routine, systematic collections of water and sediment samples may in the future be obviated.

METHODS

Sampling Sites

Seven zebra mussel sampling sites were chosen in Hamilton Harbour. Mussels were sampled in late November from the following locations (see Fig. 1): **Station 1.** Floating tire booms at a marina on the north shore of the harbor. **Stations 2, 3, 4, and** 5. Navigational buoys at selected sites in the Windermere arm of the harbor. **Station 6.** A buoy in an area east of Randle Reef commonly referred to as the Hamilton Harbour "tar pit" or "hot spot." Total PAH concentrations in sediments sampled from this area have been observed to be as high as 500-1,400 µg/g of dry sediment (Marvin et ***al.*** 1992, Murphy ***et*** **al. 1990). Station** 7. A bio-box at the East Side Filtration Plant at the Stelco Co. steel plant Hilton Works on the south shore of the harbor. The zebra mussels at this site had been continuously exposed to recirculated bay water from the harbor.

The zebra mussels sampled from stations 2 and 7 were smaller in size than those sampled at the other sites. Table 1 shows the size ranges of the mussels

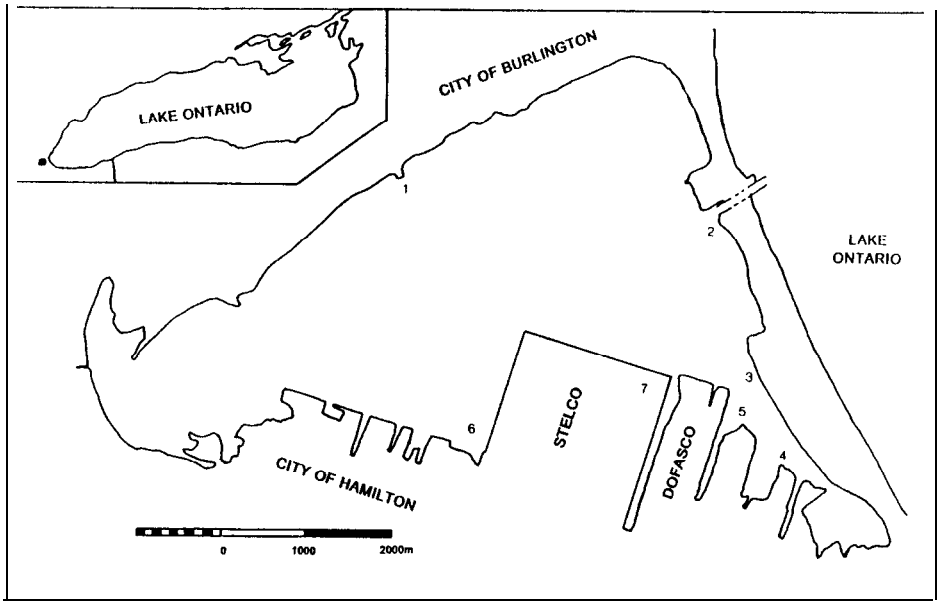

FIG. 1. Map of Hamilton Harbour area of western Luke Ontario showing the sites at which zebra mussels were sampled.

TABLE 1. Size ranges of zebra mussels sampled from stations in Hamilton Harbour. The sizes are expressed as the length of the shells in centimeters.

Station	Size range (cm)	Estimated Mean Size (cm)
1	1.7-2.0	2.0
2	0.3-0.8	0.7
3	0.3-3.0	2.5
4	0.3-2.5	2.2
5	0.3-2.5	2.2
6	1.0–2.5	2.0
7	0.3–0.8	0.7

sampled from each site. The mussels sampled at all sites did not have periphyton on the exterior of the shells and we have concluded that PAH contamination from this source was unlikely.

The mussels (250 g per station) were processed immediately after collection. The mussels were drained of excess water and then homogenized with the shells included in a Waring blender. The homogenate was centrifuged at 10,000 rpm for 15 min and the supernatant decanted and discarded.

Tissue Homogenizer/Ultrasonication Extraction

The extraction procedure using the tissue homogenizer was based on a National Oceanographic and Atmospheric Administration standard procedure for the extraction of toxic organic compounds from marine mussels (MacLeod et al. 1985). Aliquots of homogenate (125 g) were suspended in centrifuge bottles by the addition of 50 mL of HPLC grade methylene chloride and were extracted using a Janke and Kunkel Ultra-Turrax T50 tissue homogenizer with an S50 head (Terochem Inc., Toronto, Ontario). The homogenizer was operated for several cycles at 100 rpm for 45 seconds each. The sample was then centrifuged at 4,000 rpm and the methylene chloride was decanted and reduced in volume by rotary evaporation under reduced pressure.

The homogenate of the zebra mussels sampled from station 7 was extracted a second time using a Fisher Model 300 sonic dismembrator apparatus (ultrasonicator, Fisher Scientific, Fairlawn, NJ) in an attempt to extract additional PAH. We have previously' utilized ultrasonication for the determination of PAH in a variety of sample matrices (Marvin *et* al. 1992, Bryant *et* al. 1989). Aliquots of previously extracted homogenate were transferred to a beaker, suspended in 75 mL of methylene chloride, and subjected to eight consecutive 15-second pulses using the sonicator at full power. The beaker was partially immersed in ice and a time interval of 1 minute was maintained between pulses to minimize solvent heating. The solvent was removed by filtration using a Buchner funnel and the process repeated with 75 mL of fresh methylene chloride. The extraction was then repeated a third time with 75 mL of HPLC grade methanol. The solvent extracts from the ultrasonic extraction procedures were combined, reduced in volume, and subjected to the alumina clean-up procedure.

The secondary extraction procedure employing ultrasonication yielded on average an additional 18% of PAH from the mussel homogenate. These results indicate that extraction using the tissue homogenizer alone is adequate for preparing extracts for preliminary determinations of PAH in mussel tissues and for bioassays. The mussels sampled at the other sites were extracted with methylene chloride using the tissue homogenizer alone.

Alumina Clean-up and Chromatography

The alumina clean-up and fractionation procedure has been described in detail in a study comparing the efficiencies of ultrasonic and Soxhlet extraction of PAH from sediments and air particulate materials (Marvin *et* al. 1992). Solvents used in this study were either HPLC grade or were distilled in glass in the laboratory. The organic compounds from the sample extracts were adsorbed to alumina by the addition of alumina (3g, Brockman activity 1, 80-200 mesh, Fisher Scientific) to the extract followed by solvent evaporation under reduced pressure. The sample adsorbed to alumina was then applied to the top of fresh alumina (6 g, dried at 170°C for 48 hrs) contained in a glass column (1 cm x 30 cm). Organic components were eluted using solvents of increasing polarity. Hexane (60 mL) was added to the column to elute aliphatics. The non-polar polycyclic aromatic hydrocarbons (PAH) were eluted by the sequential addition of benzene (50 mL) and methylene chloride/ethanol (70 mL, 99:1 v/v). This PAH-containing fraction was then subjected to an additional chromatographic step using Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden) to remove the remaining aliphatics (mobile phase hexane/methanol/methylene chloride (6:4:3 v/v), flow rate

3 mL/min). This PAH-containing fraction is referred to in the text as fraction A23/LH20.

Gas chromatography-mass spectrometry (GC-MS) experiments were performed using a splitless injector on a Hewlett-Packard Model 5890 Series II gas chromatograph equipped with a Hewlett-Packard Model 597 1A mass selective detector (Hewlett-Packard Co., Mississauga, Ontario). The instrument was operated in selected ion monitoring mode (SIM) for the detection of the following ions; m/z 178, m/z 202, m/z 228, m/z 252, and m/z 276.

The following temperature program was used for GC-MS analysis: 50°C to 160°C at 20°C/min; 160°C to 290°C at 3°C/min; final time at 290°C, 10 min. The column was a 30 m x 0.25 mm i.d. DB-5 with a 0.25 micron stationary phase film coating (J and W Scientific, Folsom, CA).
Injector temperature: 300°C
Mass transfer line temperature: 300°C
Carrier gas: Helium at 8 psi

Bioassays

In this study two *Salmonella* ***typhimurium*** bacterial strains were used (Watanabe ***et*** al. 1990,1991). Strain YG1029 is a TA100-type strain which is auxotrophic for histidine, contains pKM 101, and has been modified by the addition of plasmid DNA pBR322 containing the gene for the activating enzyme *O*-acetyltransferase. Strain YG1024 is a TA98-type strain which is auxotrophic for histidine, contains pKM 101, and has been modified by the addition of pBR322 plasmid DNA containing the gene for the activating enzyme 0-acetyltransferase. The multicopy plasmid increases the number of copies of the enzyme from one per cell to approximately 50 per cell.

The protocol used for the bioassays was adapted from that of Maron and Ames (1983). Bacteria were grown for a period of 10 hours at 37°C in Oxoid Nutrient Broth #2 (15 mL) with ampicillin (50 µg/mL) and tetracycline (6.25 µg/mL). Dilutions of organic extracts dissolved in 50 µL DMSO were assayed with and without metabolic activation (4% Aroclor 1254-induced rat liver S9). A dose response relationship was determined using a range of seven concentrations assayed in duplicate. After a 48 hour incubation period at 37°C, the number of revertant colonies (histidine independent) was determined using a Biotran II colony counter (New Brunswick Scientific). Biological activity values were extrapolated from the linear segments of the dose response curves. The positive controls routinely used in this study were benzo[a]pyrene and 2-aminofluorene (Sigma Corp, St. Louis, MO). The values for these positive controls and the spontaneous reversion rates were similar to those previously reported (Marvin et al. 1993).

RESULTS AND DISCUSSION

Determination of Polycylic Aromatic Hydrocarbons

Figure 2 is a GC-MS selected ion monitoring (SIM) chromatogram from the analysis of the PAH-containing fraction (fraction A23/LH20) from the zebra mussels sampled at station 4. The chromatogram is relatively free from interfering compounds which simplifies the accurate quantitation of the PAH. Table 2 shows the homocyclic PAH determined in the mussel extracts from each site, and the individual concentrations expressed in ng per gram (ppb) of wet mussel homogenate. The total PAH concentrations, expressed as the sum of the individual PAH concentrations, ranged from 0.35 µg/g for the mussels sampled at station 1 to 10.0 µg/g for the mussels sampled at station 6. The precision of the analyses is typically 5% to 8% and the limits of detection for the individual PAH under the chromatographic conditions used ranged from 40 to 200 picograms/g of mussel homogenate.

The profiles of the GC-MS chromatograms were very similar to chromatograms we have obtained from the analysis of extracts of material collected in sediment traps in Hamilton Harbour (data not shown). The resuspension of PAH-contaminated benthic sediments and industrial discharges result in total PAH concentrations in suspended sediments in some areas of the harbor that are comparable to those of the bottom sediments. Mayer and Nagy (1992) have determined total PAH concentrations (sum of 16 priority pollutant PAH) in suspended sediments as high as 106 yglg at a site near station 3 and we have quantified total PAH concentrations of 92 µg/g (sum of 16 priority pollutant PAH) in this same area. High concentrations of PAH have also been determined in the harbor water (Ontario Ministry of the Environment and Energy 1985). PAH concentrations in Hamilton Harbour sediments, suspended sediments, and water have been observed to exhibit dramatic spatial variations.

The GC-MS chromatograms of the extracts of mussels from all sites were similar in profile. This

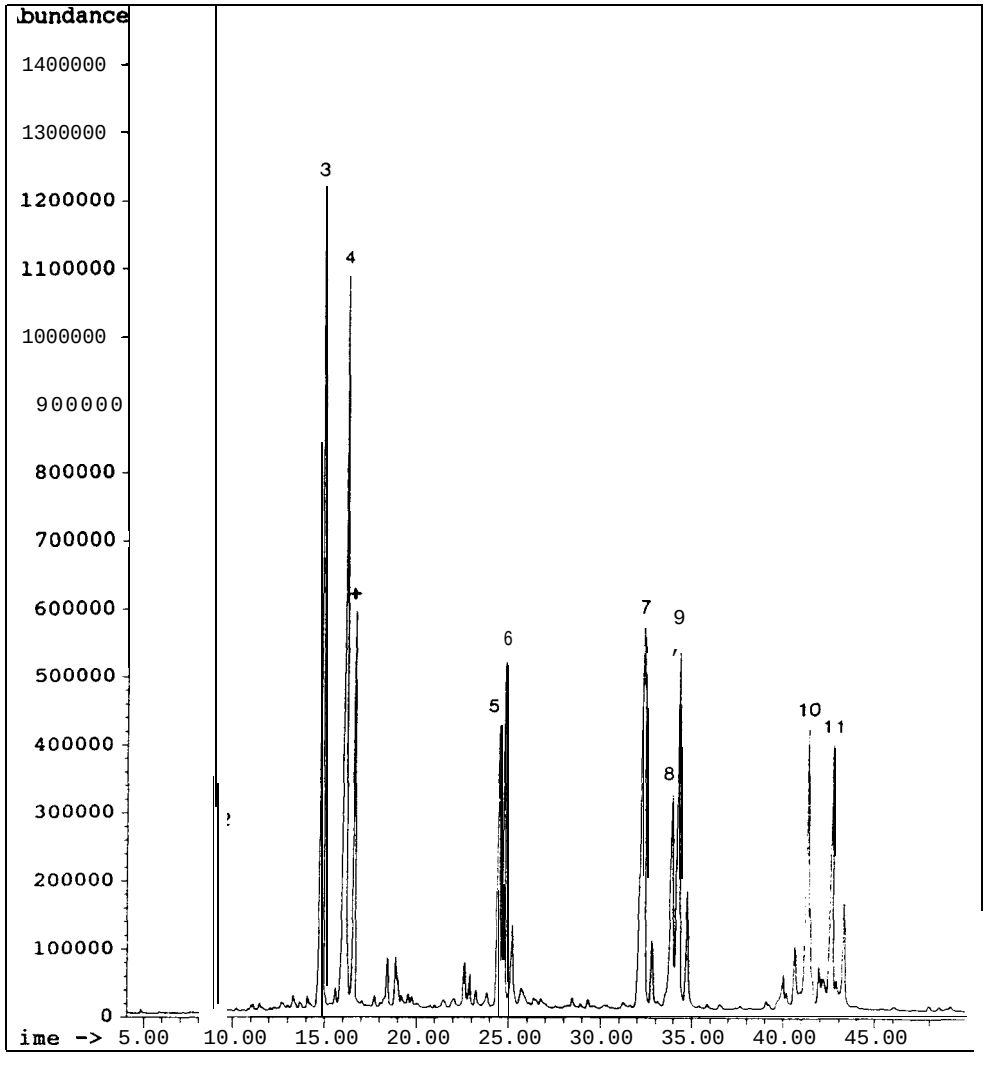

FIG. 2. GC-MSD selected ion monitoring chromatogram of fraction A23/LH20 of the extract of the zebra mussels sampled from site 4. The peaks are numbered to correspond to those PAH compounds listed in Table 1. The + identifies the internal standard peak of 9,10-dimethylanthracene.

similarity in profile is illustrated in Figure 3 which shows the relative concentrations of the individual PAH normalized to the concentration of benzo-[e]pyrene in each sample. The highest PAH levels determined in the extracts were found in mussels sampled from station 6 (near Randle Reef). This area of the harbor is grossly contaminated with PAH as a result of coal tar discharges. High levels of PAH have been determined in benthic sediments (Murphy *et* al. 1990 and Marvin *et* al. 1992) and in suspended sediments (Mayer and Nagy 1992) sampled from this area. The smaller average lengths of the zebra mussels sampled at stations 2 and 7 do not appear to have an affect on their ability to accumulate PAH in their tissues.

Genotoxicity of Mussel Extracts

Mussel extracts from stations 2, 3, 4, 5, and 6 were subjected to bioassay with ***Salmonella*** strain YG1024 (TA98-like) and strain YG1029 (TA100-like), both with and without the addition of metabolic activation in the form of a rat liver supernatant (S9). The 0-acetyltransferase-rich strains were cho-

TABLE 2. Concentrations of homocyclic PAH determined in extracts of zebra mussels sampled from Hamilton Harbour. Concentrations are expressed in ng/g of mussel homogenate. Quantitation was achieved using an internal standard.

	Station 1	Station 2	Station 3	Station 4	Station 5	Station 6	Station 7
1. Phenanthrene	81	22	66	774	5	472	30
2. Anthracene	11	6	19	165	3	115	16
3. Fluoranthene	49	143	237	1,318	65	1,399	109
4. Pyrene	44	136	237	1,002	74	1,312	144
5. Benzo[a]anthracene	32	79	160	486	50	747	88
6. Chrysene	48	93	172	501	56	753	132
7. Benzofluoranthenes (UNR)*	38	170	404	1,178	134	1,957	162
8. Benzo[e]pyrene	14	72	169	467	52	686	76
9. Benzo[a]pyrene	27	82	227	701	64	1,076	54
10. Indeno[1,2,3-cd]pyrene	4	35	181	613	37	802	7
11. Benzo[ghi]perylene	3	32	131	466	31	720	8
TOTAL (ng/g)	351	870	2,003	7,671	571	10,039	826

*UNR denotes peaks unresolved by GC-MS

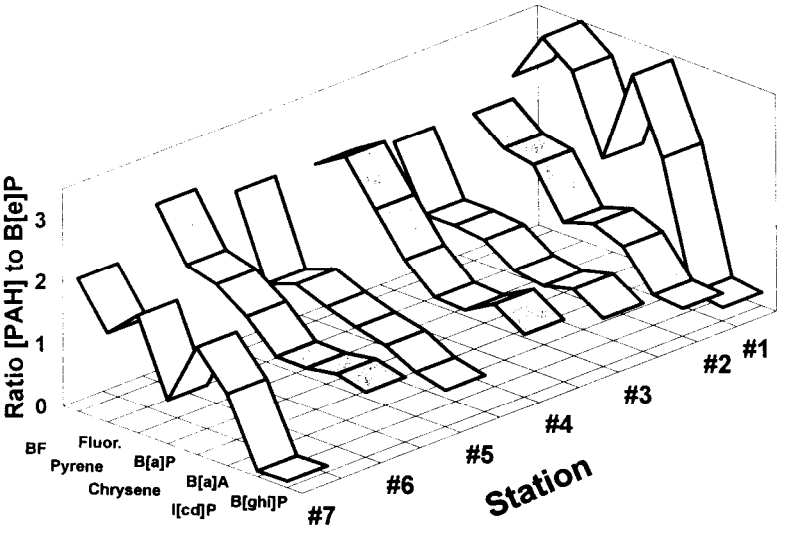

FIG. 3. Graph showing the relative concentrations of PAH in extracts of mussels sampled from each site. The concentrations of the individual PAH were normalized to the concentration of benzo[e]pyrene (given the value of 1) in each of the extracts.

sen for this study because we believe them to be superior as general detectors of mutagenic activity in complex environmental mixtures. A range of seven concentrations of each A23/LH20 fraction was assayed in duplicate. All of the extracts assayed exhibited positive responses in both strains in the presence of S9 (e.g., Fig. 4). No response was detected at the highest doses in the absence of S9.

The mutagenic potencies of the extracts are expressed in revertants per gram of wet mussels and were obtained from the linear regression of the linear portions of the dose response curves (Table 3). The error estimates for the assays are expressed as the standard deviations of the slopes of the dose response curves. The requirement for oxidative metabolism to manifest a positive response and the non-linear nature of the dose response curves at the higher doses is typical of PAH-rich mixtures. We

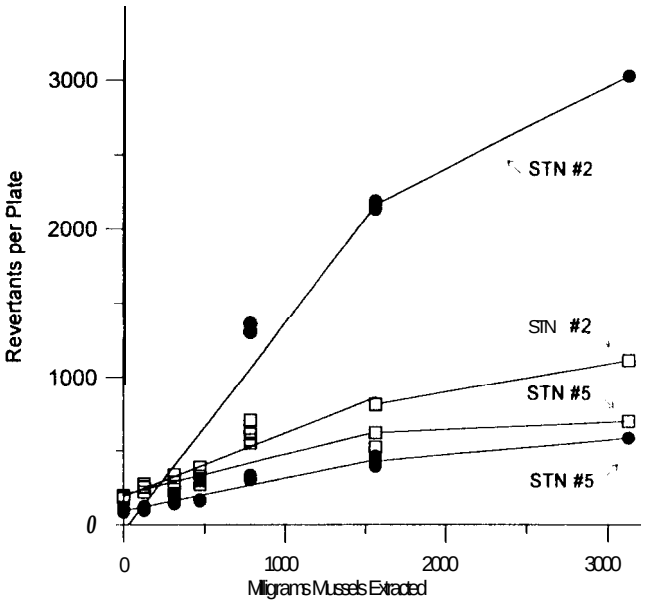

FIG. 4. Dose response curves exhibited by the PAH-containing fractions (A23/LH20) of selected zebra mussel extracts when assayed with* Salmonella typhimurium *strains with the addition of 4% rat liver S9. The doses are expressed in milligrams of mussels extracted. ●strain YGI024 (TA98-like) □ strain YG1029 (TA100-like).

TABLE 3. Mutagenic potency of fraction A23/LH20 expressed in revertants per gram of zebra mussels extracted.

Sampling Station	YG1024 O-acetyl-transferase (TA98)		YG1029 O-acetyl-transferase (TA100)	
	(–)	(+S9)	(–)	(+S9)
Station #2	nd[1]	1,792±(163)[2]	nd	259±(18)
Station #3	nd	202±(22)	nd	117±(27)
Station #4	nd	179±(30)	nd	46±(18)
Station #5	nd	170±(18)	nd	165±(35)
Station#6	12±(12)	421±(45)	3±(5)	330±(67)

[1]Not done. No activity was detected at the highest dose so dose response curve was not completed.

[2]Values (± standard deviation) were determined from slope of the linear portion of the dose response curve (seven doses of extract). Mutagenic potency of the A23/LH20 fraction was based on the original wet weight of zebra mussels (including shells) extracted with dichloromethane. Values for positive controls (not shown) were similar to those previously reported (Marvin *et al.* 1993).

have observed similar behavior in extracts of sediment sampled from the Randle Reef area of the harbor (Marvin *et al.* 1993).

The mussel extract from station 2 showed significantly higher activity in the TA98-like strain with S9 than did the extracts from other sites but exhibited relatively low levels of PAH contamination. These results suggest the presence of a mutagenic compound(s) not present in the extracts of mussels sampled at the other sites or the presence of a compound(s) at higher concentrations than in mussels sampled from the other sites. There appears to be no correlation between the magnitude of the observed mutagenic responses and the PAH concentrations in extracts of zebra mussels sampled from any of the sites. The presence of other mutagenic compounds, including PAH, in these complex extracts may account for this poor correlation. We have previously shown that significant amounts of mutagenic activity observed in extracts of sediment from Hamilton Harbour may be due to high molecular weight PAH that are not designated as priority pollutants (Marvin *et al.* 1993).

CONCLUSIONS

The described methodology affords extracts of zebra mussels that are free of interfering compounds and allows for the accurate quantitation of PAH. All of the mussel extracts analyzed in the study contained significant levels of PAH contamination and all of the extracts that were tested using the Salmonellalmicrosome assay exhibited positive mutagenic responses with the addition of oxidative metabolism. These results show that zebra mussels in Hamilton Harbour are accumulating genotoxic contaminants and could potentially be used as bioindicators of contamination.

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