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## *In situ* biomonitoring of PAH-contaminated sediments using juvenile coho salmon (*Oncorhynchus kisutch*)

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### Abstract

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous marine and freshwater sediment contaminants. Extensive data exist to confirm that PAHs are toxic to aquatic receptors. However, limited information is available regarding the bioavailability and genotoxicity of sediment PAHs to aquatic organisms. This study investigated an integrated biomonitoring approach using chemical analyses and biomarkers to characterize the bioavailability and genotoxicity of a complex PAH mixture in freshwater lake sediments associated with a former manufactured gas plant (MGP). Sediment PAH genotoxicity was assessed by flow cytometry (FCM), DNA adduct <sup>32</sup>P-postlabeling, and erythrocyte micronuclei in juvenile coho salmon (*Oncorhynchus kisutch*) caged in the water column. Significant PAH-induced genotoxicity was observed with FCM and <sup>32</sup>P-postlabeling, but not with erythrocyte micronuclei. Chromosome damage in peripheral blood and hepatic DNA adducts correlated with sediment, but not water column PAH concentrations. Total hepatic DNA adducts in salmon caged nearest the former MGP facility was  $39 \pm 6.5$  (RAL  $\times 10^9$ ), while salmon caged in a reference lake had  $28 \pm 2.3$  total hepatic DNA adducts per  $10^9$  nucleotides. These results indicate that *in situ* biomonitoring using biomarkers and caged fish can be a sensitive indicator of genotoxic PAHs in sediments.

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### 1. Introduction

Sharp declines in salmon populations throughout the Canadian and US Pacific Northwest have been attributed to global warming, inhospitable ocean conditions due to climate-related changes, overfishing, and degradation of freshwater, estuarine, and marine habitats by agricultural, industrial, and urban contamination (Bradford and Irvine, 2000; Noakes et al., 2000; Johannessen and Ross, 2002).

Recent studies suggest that pollution from polycyclic aromatic hydrocarbons (PAHs) can affect growth and reproductive viability in salmon populations (Sprong and Meador, 2005). Point sources (e.g., pulp and paper mills, manufactured gas plants (MGPs), creosote wood treatment facilities, municipal wastewater treatment plants) and non-point sources (e.g., urban storm water runoff, atmospheric deposition from vehicular emissions) have contributed considerable PAH loads to sediments. Since sediments are sinks and reservoirs of genotoxic PAHs, natural and anthropogenic perturbations may redissolve or resuspend sediment-bound PAHs, and cause continual,

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long-term exposure to salmon and other aquatic populations that inhabit or migrate through these contaminated freshwater, estuarine, and marine ecosystems.

Limited information is available to determine the effects that anthropogenic PAHs may have on the health and survival of salmon populations, particularly in freshwater habitats. A recent study observed that juvenile Chinook salmon (*Oncorhynchus tshawytscha*) in the upper Fraser River of Canada exhibited sublethal toxic effects that were most likely due to PAHs rather than polychlorinated biphenyls (PCBs), dioxins, or furans (Wilson et al., 2000). Arkoosh and Collier (2002) showed that Chinook salmon exposed to PAHs exhibited significantly suppressed immune response when challenged with a xenobiotic, and were more susceptible to the marine pathogen *Vibrio anguillarum* after exposure. They concluded that PAHs suppress immune function in salmon, and that they have the potential to adversely impact the health of Pacific salmon populations. Besides immunotoxicity, Stein et al. (1995) observed genotoxicity in outmigrant juvenile Chinook salmon exposed to PAHs in urban estuaries, even for brief residence times of several weeks. The salmon exhibited increased levels of PAH-induced hepatic DNA adducts and cytochrome P450 (CYP450) enzyme activity.

Additional research that integrates analytical data collected from sediment, water, and tissue samples with data from biomarkers of exposure and effect would help assess the bioavailability and potential health impact of PAH-contaminated sediments with respect to salmon populations (Bickham et al., 2000). Such research is particularly important as these migratory fish are forced to use progressively more urbanized and industrialized waterways and habitats. The primary challenge in conducting effective *in situ* biomonitoring is establishing a clear relationship between the analytical data, exposure dose, and biomarker response. This is particularly difficult when using feral (free-roaming) aquatic species since exposure duration and concentration are uncontrolled and exposure dose(s) can only be estimated from analytical data (Dunn et al., 1987; Willett et al., 1997; Ericson et al., 1998; Rose et al., 2000). On the other hand, *in situ* biomonitoring using caged species (DeFlora et al., 1993; Chappie and Burton, 2000; Barra et al., 2001) at pre-characterized locations allows contamination concentration gradients (doses) and geographic locations to be directly compared with biomarker responses, which would provide data to improve estimates of the dose response relationship and bioavailability of sediment contaminants. Haasch et al. (1993) also found that catfish caged in PCB contaminated environments exhibited elevated levels of hepatic microsomes.

Aquatic and benthic organisms typically are exposed to complex pollutant mixtures and may not express a simple dose response with respect to any biomarker pollutant combination. Thus, a battery of biomarkers is recommended to adequately characterize subtle, sublethal, or genotoxic effects (Altenburger et al., 2003). In particular, a

battery of biomarkers shown to be sensitive to PAHs and complex PAH mixtures (Pfau, 1997; Kirby et al., 2000; Van der Oost et al., 2003) may be used to assess the potential genotoxicity of PAH-contaminated sediments and ecosystems. Historically, three biomarkers that have effectively assessed PAH genotoxicity in aquatic organisms are erythrocyte micronuclei, which is a measure of clastogenicity and genotoxicity (Al-Sabti and Metcalfe, 1995); chromosome damage and DNA changes as detected using flow cytometry (FCM), which simultaneously assays numerous cellular and molecular endpoints (Bickham, 1990; Matson et al., 2005a, b); and <sup>32</sup>P-postlabeling which is used to quantify the formation of DNA adducts, a key process in early carcinogenesis (Maccubbin, 1994; DeKok et al., 2002; Akcha et al., 2003). The objectives of the current study was to compare a variety of internal and external measurements of PAH exposure to biomarkers of DNA damage.

## 2. Samples and experimental methods

### 2.1. Research site

Sediments adjacent to a former MGP facility in the Pacific Northwest were chosen for study. Coal tar wastes from the MGP facility had previously been spilled or discharged into an adjacent freshwater lake. As a result, sediments and overlying lake water have been shown to be contaminated with PAHs. Although other contaminants have been identified in MGP lake sediment, this study focused on PAHs due to their high concentration and potential genotoxicity to aquatic organisms. The freshwater lake covers 235 ha and has mean and maximum depths of 10.4 and 15.2 m, respectively. The lake volume is about  $2.5 \times 10^7 \text{ m}^3$  and completely exchanges water about 52 times per year, particularly at high water flows during the rainy season and spring thaw in March or April. However, there is significant short-circuiting of flow when inflowing water does not completely flush the lake before flowing out, but rather flows mostly in the north part of the lake, where the former MGP is located. Lake water parameters were all within levels conducive to fish survival during the study. At a depth of 1.5 m, the lake water parameters were: temperature 6.9 °C; dissolved oxygen 10–11.5 mg/L; total dissolved solids (TDSs) 60.8–61.4 ppm and pH 8.0–8.1.

### 2.2. Caged salmon *in situ* biomonitoring experiment

A field experiment using caged juvenile coho salmon (*Oncorhynchus kisutch*) was performed in March 2002 to monitor the bioavailability and genotoxicity of lake sediment PAH5. The coho salmon was chosen for this study primarily due to its economic importance to the region. In addition, there is limited information regarding the effect sediment PAHs have on this salmon that migrates through the lake in which the study was

conducted. In mid March, juvenile fish about 15 months old were obtained from a Washington Department of Fish and Wildlife (WDFW) fish hatchery. On average, the salmon weighed 24.8 g ( $\pm 4.0$ ) and had total length of 11.6 cm ( $\pm 0.7$ ). They were transported to the MGP urban lake in chilled, well-aerated tanks, and transferred to a US Environmental Protection Agency (USEPA) research boat where they were maintained and gradually acclimated to lake conditions. The fish were placed in cages that were located at specific sampling stations to allow the fish to ingest PAHs from the water column for seven days. This is the approximate time they would spend in the study area during their outmigration to sea.

Based on previous analytical data, one reference and three contaminated sampling stations were selected for placement of caged fish. Two cages (separated by approximately 3 m) were placed at each station. The stations at the MGP site were selected to represent a PAH concentration gradient. Contaminated stations included near (NS;  $\sim 25$ – $35$  m), intermediate (INT;  $\sim 45$ – $55$  m), and far (FAR;  $\sim 65$ – $75$  m) distances from the former MGP facility shoreline. Two reference (REF;  $\sim 25$ – $55$  m) sediment station locations were also selected in a similar freshwater lake. The reference stations were upstream and approximately three miles east of the MGP facility. The eight lake sediment stations presented a PAH concentration gradients, from anthropogenic background (REF) to very high near shore (NS) to the former MGP facility. Each sediment station was marked using differentially corrected GPS and upland landmarks.

At each sediment station, about 20 salmon were net transferred to a  $45.7 \times 30.5 \times 20.3$  cm plastic-coated, wire mesh pinfish trap cage (Model 1264; FRABILL, Inc., Jackson, WI) that had been modified to prevent fish escape. The fish and cage apparatus were slowly taken to the lake bottom by SCUBA divers and clipped to an 18 lb lead anchor previously placed to mark the sampling station. Each cage was suspended approximately 15 cm from the bottom, which maintained the salmon within a 15–35 cm exposure zone in the water column above the sediment surface. The water depths at the sampling stations in the MGP lake at the NS, INT, and FAR locations were about 4.5, 7.5, and 12 m, respectively. During cage placement, lake water parameters directly pertinent to fish survival, such as dissolved oxygen, conductivity, pH, temperature, and TDSs, were measured using a Hydrolab multiprobe (Hydrolab21 Hach Company, Loveland, CO).

Following one week of exposure, salmon cages were retrieved from the lakes in the same order in which they were placed. Fish were sacrificed after a blood sample was obtained by caudal vein puncture. Blood samples were collected in heparinized hematocrit tubes from each fish from three groups: hatchery fish, the groups of fish caged in the reference lake, and the groups of fish caged in the MGP site lake. Each fish was sacrificed by cervical dislocation, placed in an alphanumerically coded zip-lock baggie, and quickly frozen on dry ice. Duplicate glass slide blood smears were made and air-dried in the field. The remaining

blood sample (approximately 2 mL) was placed in a cryovial and snap-frozen on dry ice. Blood and whole fish samples were returned to the laboratory on dry ice and stored at  $-80^\circ\text{C}$  until analysis.

### 2.3. Sediment and lake water sampling and PAH analysis

The day before placement of salmon cages, sediment samples were collected at each station by USEPA SCUBA divers. Sediment samples were collected in 1 L glass jars with a Teflon-lined lid. To facilitate opening the jars under water, they were first filled with distilled water. To collect sediment, the divers first brushed away the “fluffy” layer of organic detritus on the surface. The jars were then filled with sediment by dragging the jar across the lake bottom. Sediments were oven-dried at  $60^\circ\text{C}$ , ground, and passed through a #20 (0.85 mm) sieve. Sediments were extracted with a 1:1 (vol) hexane:acetone solvent using a Tecator Soxtec<sup>TM</sup> Model HT-6 (FOSS Tecator, Eden Prairie, MN) automatic extraction system. Although low molecular weight PAHs (i.e., naphthalene, phenanthrene and alkylated compounds) were likely lost during the extraction, the focus of the analysis was the genotoxic high molecular weight PAHs.

A lake water sample was collected at each sediment station at the beginning and end of the fish exposure study. Water samples were collected using a 1.2 L Niskin sampler (General Oceanics Inc., Miami, FL) within the exposure zone (i.e., 15–35 cm above the sediment surface) of the caged fish. Water samples were transferred to 1 L amber glass jars with teflon-lined lids and shipped at  $4^\circ\text{C}$  to the laboratory for extraction and PAH characterization. Lake water extracts were prepared for chemical analysis using a modification of the USEPA 3535B solid phase extraction method (USEPA, 1997). This involved passing each water sample under small vacuum through two t-C18 Enviropak<sup>®</sup> cartridges (Waters Inc., Milford, MA). Each cartridge was eluted with 3 mL of a 1:1 (vol) dichloromethane:methanol solvent, the extract was dried with sodium sulfate, and the elutriates were combined, transferred to a pre-cleaned amber vial, adjusted to a final volume of five mL, and analyzed by GC-MS Method 8270C.

Extracts were subjected to GC-MS analysis using a Hewlett-Packard Model 5890 Series II gas chromatograph coupled with a Model 5972 mass spectrometer in selected ion monitoring mode at 70 eV with a source temperature of  $180^\circ\text{C}$ . A HP-5MS (J&W Scientific) fused silica capillary column (0.25 mm ID  $\times$  60 m, 0.25  $\mu\text{m}$  film) was employed. The column temperature started at  $35^\circ\text{C}$  (hold 0 min) and was temperature programmed at  $7^\circ\text{C}/\text{mm}$  to  $315^\circ\text{C}$  (hold 22 min). Helium was the carrier gas at a constant flow-rate of 0.7 mL/min.

### 2.4. Fish liver tissue extraction and PAH analysis

Hepatic PAH concentrations were measured to assess PAH uptake and bioavailability from sediments and lake water, and determine if *in vivo* biomarker responses and

hepatic PAH concentrations are correlated. Fish livers not used for  $^{32}\text{P}$ -postlabeling were composited according to their sediment station exposure group. Compositing was necessary since analysis of individual fish livers was prevented by small liver masses and analytical detection limits. Therefore, average tissue PAH concentrations for each exposure group (i.e., NS, INT, FAR, REF) were determined. Composite tissue samples were weighed wet, lyophilized overnight under vacuum using a Labconco Free Zone 12L Freeze Dry System (Labconco Corp., Kansas City, MO), and re-weighed prior to extraction.

Tissue samples were extracted with 40 mL of 1:1 (vol) acetone:hexane using a Dionex (Dionex Corp., Sunnyvale, CA) Model 200 Accelerated Solvent Extractor (ASE). A surrogate spike solution with deuterated PAHs was added to each sample to correct for PAH recovery efficiencies. The ASE was operated at 1500 psi, and each sample was solvent flushed in two cycles at 50% cell volume and 60 s purge time. The stainless steel extraction cells were heated to 100 °C with 2 min pre-heat, 5 min heat, and 5 min static times for each cycle. Sample filters (Part No. 049458) were grade D28 with a 1.983 cm diameter. A Zymark TurboVap LV evaporative solvent reduction apparatus (Zymark Corp., Hopkinton, MA) at 45 °C for 13 min was used to concentrate the extracts to 1 mL. Extracts were further reduced to 0.5 mL under purified nitrogen, and the PAHs analyzed using USEPA GC-MS Method 8270C as described for sediments and water.

## 2.5. Biomarker analyses

Genotoxicity was evaluated in the coho salmon using three biomarkers: micronuclei formation in erythrocytes, chromosome damage in peripheral blood measured by FCM, and DNA adduct formation in hepatic and gill tissues measured by  $^{32}\text{P}$ -DNA postlabeling.

**Erythrocyte micronucleus enumeration:** Blood smears were stained using Miles Stain (Stanbio Laboratory, Inc., San Antonio, TX) in a standard hematological staining procedure. Each slide was coded, examined microscopically (100–1000 $\times$  power), and scored twice for micronuclei using blind review by a single observer. The average number of micronucleated (MN) erythrocytes per 1000 erythrocytes was determined for each slide.

**Flow cytometric analysis:** About 15–20  $\mu\text{L}$  of blood was prepared for flow cytometric analysis according to the methods presented by Deitch et al. (1982) and Vindelov et al. (1983). In brief, this involves suspending blood cells in citrate buffer and lysing them with Nonidet P40. Cytoplasm is then digested with trypsin and RNase, and stained with propidium iodide. Propidium iodide intercalates between the bases and stains both RNA and DNA. Increased chromosomal damage is reflected by an increase in cell-to-cell variation in DNA content (Bickham, 1990). The flow cytometer calculates the full-peak and half-peak coefficient of variation (CV) in DNA content among cells in the  $G_1/G_0$  phase of the cell cycle.

Nuclear DNA content was analyzed on a Coulter Epics Elite flow cytometer (Beckman Coulter Inc., Fullerton, CA) by quantification of nuclear fluorescence. A Coherent Innova 300 ion laser (Coherent, Inc., Santa Clara, CA), set for 514 nm emission with 0.5 W of power and equipped with a computer algorithm, determined full-peak (FPCV) and half-peak coefficient of variation (HPCV), mean, standard deviation, and number of cells. Cells are simultaneously analyzed for nuclear fluorescence forward scatter, side scatter, and time. Light scatter parameters indicate the amount of cytoplasm still attached to the nucleus, and time is used to insure stability of the instrument during the analysis. Cells with high levels of side scatter are excluded (gated) from the analysis to minimize variation resulting from sample preparation. Samples showing evidence of drift during analysis were reanalyzed.

At least 10,000 cells in the  $G_1$  peak were counted and the mean and standard deviation for each individual was recorded. The full and half peak CV of the gated  $G_1$  cell population was compared for each individual. Replicate blood samples were analyzed as separate experiments and all samples for a given experiment were analyzed consecutively on the same day to minimize variation. For all analyses, samples were analyzed in random order and the machine operator was unaware of sample identities. Alignment, focus, and instrumental gain are set prior to analysis using 0.097 mm fluorescent microspheres (Beckman Coulter Inc.). To account for instrument drift between runs, data were fractionally rank transformed within experimental runs to allow all samples to be included into a single analysis. According to Levene's equality of variance test, experimental groups had homogeneous variances ( $p = 0.486$ ). A non-parametric ANOVA and Dunnett (> control) *post hoc* comparisons were used to evaluate differences between each experimental site and the reference. The probability level determining significance was  $p \leq 0.05$ .

**$^{32}\text{P}$ -Postlabeling assay:** Tissues were excised from individual fish from sampling 10 stations in the MGP site and reference lake. The DNA was isolated by solvent extraction combined with enzymatic digestion of the protein and RNA (Reddy and Randerath, 1986). The isolated DNA was stored at  $-80\text{ }^\circ\text{C}$  until analysis.

DNA adducts were quantified using nuclease P1-enhanced bisphosphate  $^{32}\text{P}$ -postlabeling (Stout et al., 2004). Briefly, approximately 10  $\mu\text{g}$  of DNA from each tissue was digested by enzymatically hydrolyzing the DNA to normal (Np) and modified (Xp) deoxyribonucleoside 3'-monophosphates using micrococcal endonuclease (Sigma) and spleen phosphodiesterase (WBC). The hydrolyzed DNA is treated with nuclease P1 (Calbiochem), which selectively hydrolyzes normal 3'-mononucleotides to nucleosides, thereby enriching the mixture in modified (adducted) 3'-monophosphates. The enriched modified nucleotides are converted to 5'- $^{32}\text{P}$ -labeled 3',5'-bisphosphate derivatives by incubation with carrier-free [ $\gamma$ - $^{32}\text{P}$ ]ATP (MP) and polynucleotide kinase (USB) mediated

phosphorylation. The  $^{32}\text{P}$ -labeled DNA adducts were purified and partially resolved by one-dimensional development using 2.3M  $\text{NaH}_2\text{PO}_4$  (pH 5.75). An original area ( $2.8 \times 1$  cm) of the lower portion of cellulose map containing adducts was excised and the  $^{32}\text{P}$ -labeled products were contact-transferred to fresh polyethyleneimine (PEI)-cellulose sheets. Labeled products were then resolved by two-dimensional thin-layer chromatography (TLC). The first dimension used 3.82 M lithium formate + 6.75 M urea (pH 3.35). The second dimension was obtained using 0.72 M  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O} + 0.4$  M TRIS + 7.65 M urea (pH 8.2).

The  $^{32}\text{P}$ -labeled products or radioactive spots on each chromatogram were quantitated by Instantimager (PerkinElmer). Screen-enhanced autoradiography of the chromatograms was performed on Kodak XAR-5 X-ray (gray) film for 25 h at  $-80^\circ\text{C}$ . The count rates of individual radioactive spots from individual fish from each exposure group were used to calculate relative adduct labeling (RAL) values  $\pm$  standard error of the mean (SEM) for each spot. RAL values were calculated according to the following:

$$\text{RAL} = \frac{\text{Sample count rate [cpm]} + (\text{DNA-P}[\text{pmol}] \times \text{Spec. Act.}_{\text{ATP}}[\text{cpm}/\text{pmol}])}{\text{DNA-P}[\text{pmol}]}$$

where DNA-P represents the amount of DNA assayed (expressed as pmol DNA monomer units) and specific activity (Spec. Act.) of [ $\gamma$ - $^{32}\text{P}$ ]ATP used in the labeling reaction. Thus, for 10  $\mu\text{g}$  DNA, and ATP with a specific activity of  $4.5 \times 10^6$  cpm/pmol, 145 cpm corresponds to an RAL value of  $10^9$  (i.e., an estimated level of one modification in DNA nucleotides). Total RAL values are calculated by summing the values for individual spots from each DNA sample. Differences of mean DNA adduct levels between exposure groups or cage locations were tested with ANOVA, with Student–Newman–Keuls (SNK)

test for multiple comparisons, and unpaired Student's *t*-test. The probability level determining significance was  $p < 0.05$ .

### 3. Results

#### 3.1. Lake sediment PAH concentrations

Sediment PAH concentrations at the reference and MGP site lake sediment stations collected before the caged salmon experiment are summarized in Table 1. The USEPA has identified 16 *priority pollutant* PAHs due to their particular toxicity to mammals and aquatic organisms. Of the 16, seven are classified as *probable human carcinogens* (Group B2) based on a sufficient weight-of-evidence of carcinogenicity from multiple animal species and exposure routes. These seven PAHs are assumed to account for most of the genotoxicity observed in human and ecological populations. Due to their genotoxicity, widespread occurrence, and potential health threat to the salmon in this study, the carcinogenic PAHs are presented individually in the analytical data tables.

As anticipated from prior sampling, a concentration gradient was observed with PAH levels in sediments substantially decreasing from the near (NS) to intermediate (INT) to far (FAR) shore sampling stations. The mean sediment PAH concentration of about 8 mg/kg for the reference lake (REF) falls within sediment anthropogenic PAH levels for the Pacific Northwest region (Stout et al., 2004). Mean PAH concentrations for the MGP site lake were 694, 175, and 58 mg/kg for the NS, INT, and FAR sediment stations, respectively. In addition, the proportion of carcinogenic to non-carcinogenic PAHs in the reference lake sediments averages about 8%, while in the MGP site lake it averages about 70%. This indicates the MGP

Table 1  
Sediment PAH concentrations (mg/kg) at the reference and MPG site lake sampling stations

Sediment PAHs (mg/kg dry wt)	Reference lake (REF)		MPG site lake					
	REF 1	REF 2	Near shore (NS)		Intermediate (INT)		Far (FAR)	
			Station 3	Station 4	Station 1	Station 2	Station 5	Station 6
Total noncarcinogenic	4	11	394	431	143	56	40	28
Carcinogenic								
Benzo[a]anthracene	0.1	0.2	38	43	18	5	4	2
Benzo[b]fluoranthene	0.1	0.2	39	54	14	6	5	3
Benzo[k]fluoranthene	0.0	0.1	28	26	14	5	3	2
Benzo[a]pyrene	0.0	0.1	53	64	24	9	5	4
Chrysene	0.1	0.2	45	48	19	5	5	3
Dibenzo[a,h]anthracene	0.0	0.0	5	9	2	1	1	0
Indeno[1,2,3-c,d]pyrene	0.1	0.1	54	57	20	9	6	4
Total Carcinogenic PAHs	0.3	0.9	262	301	111	40	29	18
Total PAHs	4.3	11.9	656.0	732.0	254.0	96.0	69.0	46.2

REF-1 and REF-2, reference lake stations 1 and 2, respectively; NS, INT and FAR, MGP site lake sampling stations that were nearest to the shore, an intermediate distance from the shore, and farthest from the shore, respectively.

facility, and likely other PAH sources, have impacted this urban lake's sediments with PAHs significantly above typical anthropogenic levels.

### 3.2. Lake water PAH concentrations

The PAH concentrations in the water column where the cages were placed at the reference and MGP sampling stations are summarized in Table 2. Water samples were not filtered prior to analysis in order to measure both dissolved and suspended PAHs. This concentration should more closely approximate total PAH exposure for the caged salmon. The mean total PAH concentration detected in water column samples from the reference lake stations was 13 ng/L. The mean total PAH concentrations for fish caged near the MGP site were 37, 587, and 34 ng/L for the NS, INT, and FAR stations, respectively; while mean carcinogenic PAHs in water column samples from the REF, NS, INT, and FAR stations were 2, 7, 230, and 9 ng/L, respectively (Table 2).

The increased PAH concentrations detected in INT water column samples compared with stations closer to or further away from the shore line may reflect increased suspended sediments or solids re-suspended by boat traffic and/or snowmelt water flow (Table 2). Concentrations of carcinogenic PAHs in water column samples from stations near the MGP site were three to more than ten times greater than concentrations detected at the reference lake stations. These data indicate that salmon caged at the stations near the MGP site were likely to have been exposed to higher concentrations of genotoxic PAHs than salmon caged at the reference lake stations.

Lake water parameters pertinent to fish survival in the cages, such as dissolved oxygen (DO), conductivity, pH, temperature, and TDSs were measured during the March

experiment. Lake water quality parameters were well within the ranges necessary for salmon survival, which was confirmed by having 100% salmon survival in all cages after the seven day exposure period (data not shown).

### 3.3. Fish liver tissue PAH concentrations

The PAH concentrations in salmon livers from fish caged at the reference and MGP site lake stations for seven days are summarized in Table 3. Total PAHs averaged 1650, 1729, and 1744 ng/g in salmon livers from the REF, INT, and FAR lake stations, respectively. Only one NS composite liver sample was available, which contained 1186 ng/g PAHs. Liver PAH concentrations for reference and MGP site lake stations exhibited a large amount of variability. Liver PAH concentrations also appeared to demonstrate no relationship to source as REF-1 was highest, REF-2 was lowest, and livers from NS Station 4 exhibited the lowest PAH concentration for MGP site lake fish. The data from the current study, similar to other studies (Payne et al., 2003), suggest that liver PAH concentration is not a good biomarker of exposure or effect.

### 3.4. Erythrocyte micronucleus results

The relationship between PAH exposure concentration and response, in terms of erythrocyte micronucleus (MN) counts, did not show a dose–response relationship in the coho salmon. That is, there was no difference in MN between the hatchery fish, reference lake fish, and fish at any location in the MGP site lake (data not shown). Although the micronucleus assay has been successfully used in *in situ* biomonitoring of the aquatic environment to assess the genotoxicity of PAHs in fish, it has exhibited a

Table 2  
Lake water PAH concentrations (ng/L) at the reference and MPG site lake fish cage stations

Lake water PAHs (ng/L)	Reference lake (REF) (n = 2)		MPG site lake					
	REF 1	REF 2	Near shore (NS) (n = 4)		Intermediate (INT) (n = 4)		Far (FAR) (n = 4)	
			Station 3	Station 4	Station 1	Station 2	Station 5	Station 6
Total noncarcinogenic	15	7	33	27	609	106	19	31
Carcinogenic								
Benzo[a]anthracene	2	0	2	1	56	8	1	2
Benzo[b]fluoranthene	0	0	0	2	63	10	1	3
Benzo[k]fluoranthene	0	0	0	1	40	6	0	2
Benzo[a]pyrene	0	0	0	2	83	10	0	3
Chrysene	2	0	3	2	71	8	1	3
Dibenzo[a,h]anthracene	0	0	0	0	10	1	0	0
Indeno[1,2,3-c,d]pyrene	0	0	0	1	86	7	0	2
Total Carcinogenic PAHs	4	0	5	9	409	50	3	15
Total PAHs	19.0	7.0	38.0	35.9	1018.4	156.5	22.2	46.1

REF-1 and REF-2, reference lake stations 1 and 2, respectively; NS, INT and FAR, MGP site lake sampling stations that were nearest to the shore, an intermediate distance from the shore, and farthest from the shore, respectively.

Table 3  
Liver PAH concentrations (ng/g) for salmon caged seven days in the lakes

Salmon liver PAHs (mg/kg dry wt)	Reference lake (REF)		MPG site lake					
	REF 1	REF 2	Near shore (NS)		Intermediate (INT)		Far (FAR)	
			Station 3	Station 4	Station 1	Station 2	Station 5	Station 6
Total noncarcinogenic	2032	703	Salmon lost during cage recovery from lake	1092	1143	1609	1835	1327
Carcinogenic								
Benzo[a]anthracene	25	2		6	18	9	8	6
Benzo[b]fluoranthene	157	11		26	100	50	67	27
Benzo[k]fluoranthene	124	4		18	76	44	41	19
Benzo[a]pyrene	105	5		20	222	56	61	17
Chrysene	53	3		12	35	18	21	12
Dibenzo[a,h]anthracene	8	0		2	4	3	4	1
Indeno[1,2,3-c,d]pyrene	65	3		10	44	27	32	9
Total Carcinogenic PAHs	537	28		94	499	207	234	91
Total PAHs	2569	731		1186	1642	1816	2069	1418

lack of sensitivity in some fish species, even when they were exposed to elevated levels of PAHs (Al-Sabti and Metcalfe, 1995).

Important factors that may affect the sensitivity of the micronucleus assay in fish species as a measure of genotoxicity include interspecies differences; particularly differences in xenobiotic metabolism, DNA repair, and cell proliferation in the target organ. Other factors that may affect the formation of micronuclei include: fish age, sex, diet, health, reproductive status, and genetic strain. Sanchez-Galan et al. (1998) successfully employed the micronucleus assay for *in situ* biomonitoring of fish in contaminated freshwater ecosystems. They attribute the sensitivity of the assay to counting MN in cells removed from the cephalic kidney, where continuous mitotic divisions occur, as opposed to evaluating MN induction in erythrocytes from peripheral blood, as was done in this study. Further research is needed to assess the relevance and validity of the MN assay as an *in situ* biomonitoring tool.

### 3.5. FCM results

Results of FCM on peripheral blood obtained from caged salmon are summarized in Fig. 1. An ANOVA confirmed significant differences in transformed full-peak CVs (FPCV) among experimental groups ( $p = 0.022$ ). Near-shore (NS,  $p = 0.005$ ) and far-shore (FAR,  $p = 0.024$ ) experimental group FPCVs were significantly elevated relative to reference (REF) fish, whereas, the intermediate group was borderline non-significant (INT,  $p = 0.061$ ). FCM data reveal a clear trend in genotoxic response that corresponds with the PAH concentration gradient observed in sediments. That is genotoxicity, as measured by mean FPCVs from individual fish in each

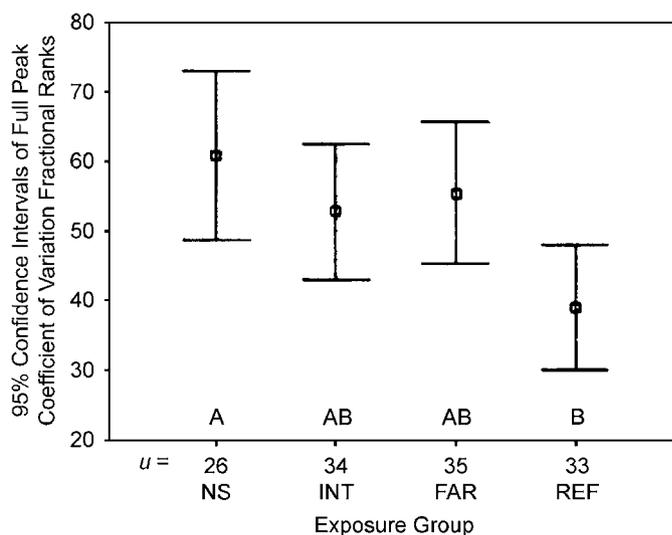


Fig. 1. Flow cytometric analysis of DNA damage in juvenile coho salmon exposed to manufactured gas plant (MGP) site lake sediments. Graph of fractionally rank-transformed full-peak coefficients of variation (FPCV). Data are presented as mean  $\pm$  standard error of the mean. NS, near shore in MGP site lake; INT, intermediate distance from shore in MGP site lake; FAR, farthest point from shore sampled in MGP site lake; REF, reference lake. MGP sites that are significantly elevated relative to the reference group are denoted with the following: \* $p \leq 0.05$  and \*\* $p \leq 0.01$ .

exposure group, generally increased with increasing sediment PAH concentration for each sediment station location closer to the former MGP site.

The exposure scenario used in this study for the juvenile salmon closely resembles typical conditions for juvenile coho salmon that normally are in the MGP site lake for about one week during their seaward migration in middle to late March. Although significant genotoxicity was observed in caged juvenile salmon, additional data are

needed to assess the potential long-term impact such genotoxicity will have on this salmon population. However, studies have shown that chronic exposures to similar environmental contaminants can impact genetic diversity that results in population reduction (Bickham et al., 2000; Theodorakis et al., 2000; Peterson et al., 2003).

### 3.6. $^{32}\text{P}$ -postlabeling assay results

DNA adduct levels were measured in hepatic and gill tissue in a subset of reference and MGP site lake (INT and NS) salmon. Representative autoradiographs of hepatic and gill DNA adducts are presented in Fig. 2. The autoradiographs indicate that a minimum of 11 hepatic DNA adduct spots and 20 gill adduct spots were consistently present in all fish. Fish from the MGP site lake and the reference lake exhibited qualitatively similar adduct patterns.

The total hepatic DNA adduct levels (mean  $\pm$  SEM) in the salmon in the MGP site lake and the reference lake were  $38.9 \pm 6.5$  and  $28.2 \pm 2.3$  in  $10^9$  nucleotides, respectively. These levels were not significantly different ( $p = 0.07$ ). However, the RAL values of the individual adduct spots 2, 3, and 6 were significantly higher in the MGP site lake fish than in the reference lake fish ( $p < 0.05$ ); Fig. 3). This supports a dose response effect, suggesting there was an increase in salmon hepatic DNA adducts from exposure to MGP site lake PAHs.

The RAL values of individual gill DNA adduct spots were also measured. Levels of total gill adducts were  $70.9 \pm 22.5$  and  $71.3 \pm 20.3$  per  $10^9$  nucleotides for the MGP site lake and reference lake fish, respectively. The levels of both total and individual DNA adducts in gills were very

similar ( $p > 0.05$ ). Similar trends were observed among the individual DNA adducts in gills (data not shown). Although gill DNA adducts are generally higher than liver adducts in fish, these data suggest that liver adducts are the more sensitive measurement of exposure.

Mean total RAL values increased for each fish cage station location closer to the former MGP site, or as the sediment PAH concentration increased. Both hepatic and gill DNA adduct data exhibited a consistent PAH dose–DNA adduct response trend. Although gill DNA adduct and water column PAH levels were not significantly correlated, this

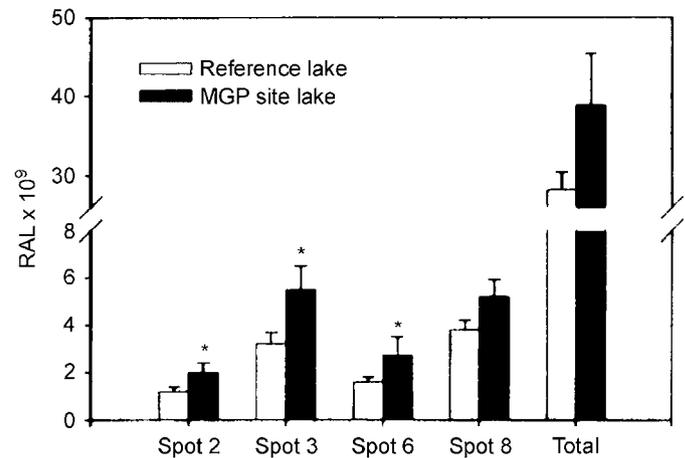


Fig. 3. Hepatic DNA adduct levels in the salmon in the manufactured gas plant (MGP) site lake and reference lake. Data are presented as mean  $\pm$  standard error of the mean. The adduct spots marked with an asterisk showed significantly higher adduct levels in the MGP site lake salmon than in the corresponding control group ( $p \leq 0.05$ ). There was not a significant difference in total hepatic DNA adduct levels between the MGP site lake and reference lake fish ( $p = 0.07$ ).

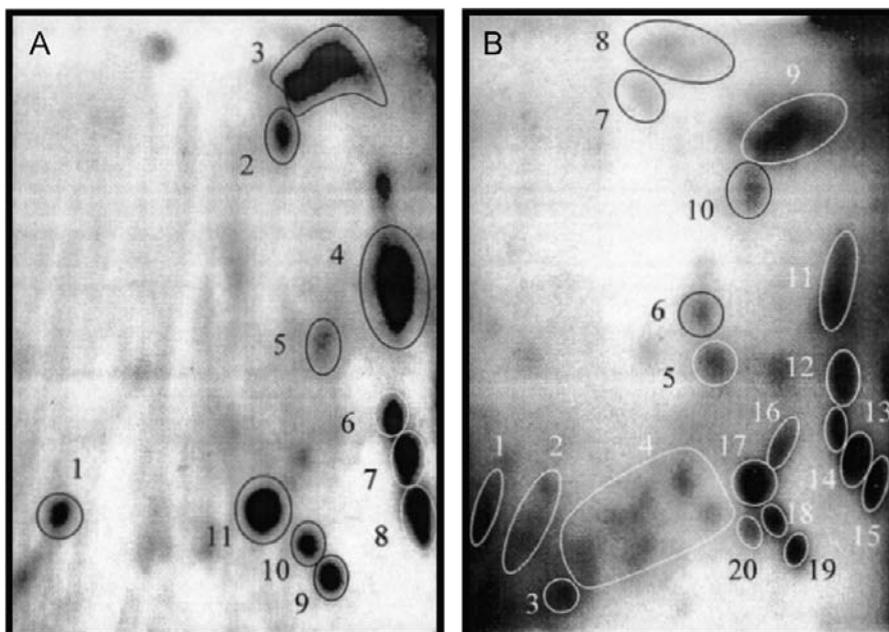


Fig. 2. Representative autoradiographs of individual hepatic (A) and gill (B) tissue DNA adducts in juvenile coho salmon exposed to manufactured gas plant site lake sediments.

may reflect the analysis of unfiltered water. These data identify a DNA adduct response gradient that corresponds to the sediment, but not water column, PAH concentration gradient. Comparable results were observed using FCM. However, none of the total adducts levels from the exposure groups were statistically significant from one another.

#### 4. Discussion

The results from this study corroborate those of Stein et al. (1995), who observed that outmigrant juvenile salmon exposed to PAHs for relatively brief residence times of several weeks in urban estuaries had increased induction of cytochrome P450 enzymes and associated damage to hepatic DNA, as evidenced by increased levels of xenobiotic-induced DNA adducts. Whether the increased exposure and biochemical responses in juvenile salmon indicate altered fitness and ability to survive is not known. The current and previous studies suggest the potential exists for an incremental effect of increased exposure to anthropogenic chemicals on the physiological fitness of juvenile salmon as they migrate through contaminated environments. Additional research is needed to assess the potential health risks migratory fish might endure along their entire migratory route. The importance of this problem may expand as migratory pathways become increasingly urbanized and industrialized.

It is reasonable to think that the caged juvenile coho salmon in this experiment experienced PAH exposures that are similar to feral juvenile salmon on their seaward migration. The caged fish were suspended approximately 15–30 cm above PAH-contaminated sediments in the reference lake, and NS, intermediate (INT), and far (FAR) distances adjacent to the MGP site for seven days. In a similar river-lake system, Swales et al. (1988) observed that feral juvenile coho salmon showed a strong association with shoreline areas and almost all were captured near the bottom substrate or sediment. In addition, juvenile coho salmon do not necessarily exhibit avoidance responses away from petroleum-contaminated waters, either on the water surface or dispersed in the water column (Maynard and Weber, 1981). Thus, the exposure scenarios (e.g., location and duration) selected for the caged fish in this *in situ* biomonitoring study appear to be sufficiently accurate to assess if PAH contaminated sediments and lake water would be bioavailable to, and have a genotoxic impact on, migratory juvenile coho salmon. In fact, a strong, fairly predictable, PAH dose–genotoxic response was observed using FCM and DNA <sup>32</sup>P-postlabeling, which indicates that weathered, sparingly soluble, sediment-bound PAHs can be bioavailable to and genotoxic in such migratory and other non-migratory feral fish.

#### 5. Conclusions

When incorporated into a well-designed *in situ* biomonitoring plan, the biomarkers used in this research appear

to provide sensitive measurements of the bioavailability and genotoxicity of PAH-contaminated sediments to aquatic species. Such biomarkers of exposure and effect can be used to establish quantitative correlations of the cause and effect relationship of a toxicant at any level of biological organization, from an individual organism up to a population or ecosystem (Shugart et al., 1992; Theodorakis et al., 1992; Bickham and Smolen, 1994; Den Besten, 1998). *In situ* biomonitoring has a great potential for linking biomarker data with community and ecosystem level responses. Biomarkers respond to toxicant exposures in a time-dependent manner and have varying levels of longevity and stability. Thus, using multiple biomarkers of exposure and effect should improve the quality of the risk assessment. It may be useful to combine the measurements of genotoxicity (e.g., DNA adduct formation), as a molecular dosimeter, with an analysis of carcinogen metabolism, exposure dose, and the determination of tumor formation in order to provide insight into the dose–response relationship and the mechanisms involved in chemical carcinogenesis.

In the current study, genotoxicity as measured by DNA postlabeling and FCM was significantly increased in an exposed population of coho salmon when compared to a reference population. Although genotoxicity correlated with PAH concentrations in sediment, it was not correlated with PAH levels in the water column. Water column data may have proved a more useful metric of exposure if samples had been filtered. In addition, DNA adduct levels in gill tissue (possibly representing both dissolved and solid phase PAHs) were not significantly different, whereas adduct levels for specific spots in the liver DNA were significantly different between contaminated and reference stations.

Juvenile coho salmon released from the hatchery where the fish for this study were obtained take approximately 6–8 weeks to travel from the hatchery through a series of rivers and lakes, including the MGP site lake, to the sea. During this seaward migration, the salmon have an estimated residence time in the MGP site lake of about one week. However, exposure to PAH-contaminated lake sediments from the former MGP facility is only one segment along these juvenile salmon's migratory pathway through highly urbanized and industrialized waterways and estuaries to the sea. It is possible that additional exposure duration and/or exposure doses to PAHs and other genotoxins during their migration would exacerbate the effects seen in this research.

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