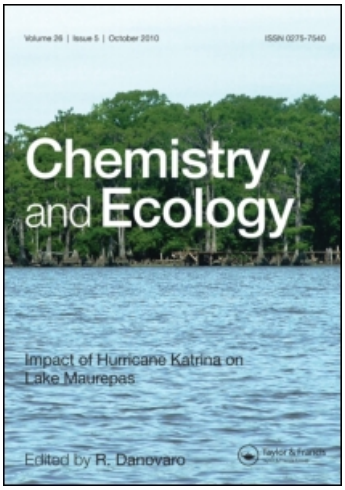


**Chemistry and Ecology**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713455114

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**To cite this Article** Wolfe, Douglas A. , Scott, K. John , Clayton Jr., John R. , Lunz, John , Payne, James R. and Thompson, Timothy A.(1995) 'Comparative Toxicities of Polar and Non-Polar Organic Fractions From Sediments Affected By the Exxon Valdez Oil Spill in Prince William Sound, Alaska', Chemistry and Ecology, 10: 1, 137 — 156
**To link to this Article: DOI:** 10.1080/02757549508035337
**URL:** http://dx.doi.org/10.1080/02757549508035337

*Chemistry and Ecology*, 1995, Vol. 10, pp. 137–156
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# COMPARATIVE TOXICITIES OF POLAR AND NON-POLAR ORGANIC FRACTIONS FROM SEDIMENTS AFFECTED BY THE EXXON VALDEZ OIL SPILL IN PRINCE WILLIAM SOUND, ALASKA

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*(Received 16 May 1994)*

Standardized tests were applied to aromatic and polar fractions of sediment extracts to determine whether polar constituents or oxidative degradation products contributed significantly to the toxicity of sediments oiled by the Exxon Valdez spill. Intertidal sediment and pore-water samples were collected in September 1990 from two heavily oiled sites and an unoiled site in Prince William Sound (PWS). Methylene chloride extracts from these samples were fractionated by liquid chromatography into aliphatic, aromatic and polar fractions, and the aromatic and polar fractions were tested for toxicity using the Microtox[R] test, bivalve larval mortality and development (*Mytilus*); several measures of genotoxicity in *Mytilus*, including SOS Chromotest[R], anaphase aberrations and sister chromatid exchange; and survival, anaphase aberrations and teratogenicity in coho salmon (*Onchorhynchus kisutch*). Microtox[R] and SOS Chromotest[R] protocols were applied in a screening mode to all samples, whereas other tests were applied only to selected fractions from two sites. Samples from Bay of Isles (oiled) were consistently more toxic (usually only 2 to 5-fold) than the Mooselips Bay (unoiled) samples, which gave very low responses in all tests. For both sites, however, responses to polar and aromatic fractions were about the same in most tests, suggesting that while the overall toxicity of the oil was low in these samples, at least part of that toxicity was derived from polar constituents. Compared to the parent hydrocarbons, polar oxidation products partition preferentially into pore-water and are more rapidly diluted and dispersed in the water column. These results suggest that polar oxidation products of petroleum hydrocarbons pose little risk to marine organisms, except possibly for infauna continuously exposed to pore-water in heavily oiled sediments. Independent surveys showed that sediment toxicity in PWS declined during 1989–1991 to near background levels, in accord with previous understanding of oil weathering and toxicity.

KEY WORDS: oil, degradation products, Exxon Valdez, toxicity tests, *Mytilus*, *Oncorhynchus kisutch*



## INTRODUCTION

The 24 March 1989 grounding of the Exxon Valdez in Prince William Sound (PWS) released approximately 35,500 metric tons of Prudhoe Bay crude oil (PBCO) into the Alaskan marine environment (Kelso and Kendziorek, 1991; Maki, 1991). During the next several weeks this oil was widely distributed, eventually affecting about 575 km of shoreline in PWS and about 1170 km in the Gulf of Alaska (Maki, 1991; Wolfe *et al.* 1993a, 1994a). Toxicity of sediments from lower intertidal and subtidal zones of selected oiled shorelines was surveyed each year from 1989 to 1991 in PWS

and (1989 only) in the adjoining Gulf of Alaska (Wolfe *et al.* 1993b, 1994b). Compared to unoiled Alaskan sediments or to contaminated sediments from urban areas elsewhere in the United States, the toxicity of these oiled sediments was not remarkably high in any year, as determined by Microtox$^R$ and by acute toxicity tests with amphipods and bivalve larvae. Nonetheless, low levels of toxicity were associated with heavily oiled sites in PWS in both 1989 and 1990, and this toxicity appeared to shift from lower intertidal sediments (0 m depth) into the shallow (3–6 m) subtidal zone during this time. While some oiled sites continued to exhibit significant toxicity, oiled and unoiled sites were indistinguishable from each other in 1991 (Wolfe *et al.* 1994b).

The work described here was undertaken to determine whether toxicity associated with oiled sediments in PWS was attributable in part to oxidized degradation products of petroleum. Earlier work demonstrated that photo-oxidation products generated in confined systems by irradiation of petroleum or refined petroleum products can enhance toxicity to test organisms (Lacaze and Villedon de Naide, 1976, Larson *et al.*, 1977, Malins *et al.*, 1985). Oxygenated petroleum derivatives have been documented in inshore waters of Bermuda (Burns *et al.*, 1990; Ehrhardt and Burns, 1990) and in coastal water contaminated by the 1992 Persian Gulf oil spill (Ehrhardt and Burns 1993). Because such compounds are not regularly measured in oil-contaminated environments, it is important to understand their contribution to the toxicity manifested after an oil spill, to avoid underestimates of toxicity potential based on parent oil alone. This paper describes results of toxicity tests on the aromatic and polar fractions from extracts of sediments and pore-water collected in 1990 at oiled and unoiled sites in PWS.



## METHODS

### *Sampling Locations and Sample Extraction*

Intertidal sediments and interstitial pore-water were collected from an unoiled reference site at Mooselips Bay (60° 12.45′ N,147° 17.90′ W), and two heavily oiled sites on Knight Island: Bay of Isles (60° 22.90′ N, 147° 42.75′ W) and Herring Bay (60° 27.25′ N, 147° 42.65′ W) (Figure 1). These three sites were sampled on September 11, 12, and 13, 1990, respectively.

Intertidal sediments were collected from depths up to 10 cm by scooping (with pre-cleaned metal utensils) the fine-grained material into 500 ml, 'certified clean' (I-Chem$^R$) glass jars. Two samples (40 jars each, ~20 kg total weight) were collected at each location. Samples were frozen within 12 h of collection and maintained frozen until extracted at the SAIC laboratory facilities in San Diego, California. Sediments were sequentially extracted in 500 g batches, using 400 ml methanol and then three 400 ml volumes of a 1:1 (v:v) mixture of methylene chloride-ethyl acetate. Combined extracts from different batches within a sample were concentrated to ~250 ml by evaporation over a water bath at 90–100°C in a round-bottom flask fitted with a Snyder column.

Interstitial pore-water was collected during receding tidal cycles from areas of ~144 m$^2$ at each intertidal location. Two samples (180 l each) were pumped from several excavations (15–30 cm deep) at each location into clean 20 l glass carboys. Samples were acidified in the field to pH 1–2 with hydrochloric acid, and extracted on board ship. Five hundred ml of 1:1 (v:v) methylene chloride-ethyl acetate and 200 ml of methylene chloride were added to each carboy and mechanically agitated

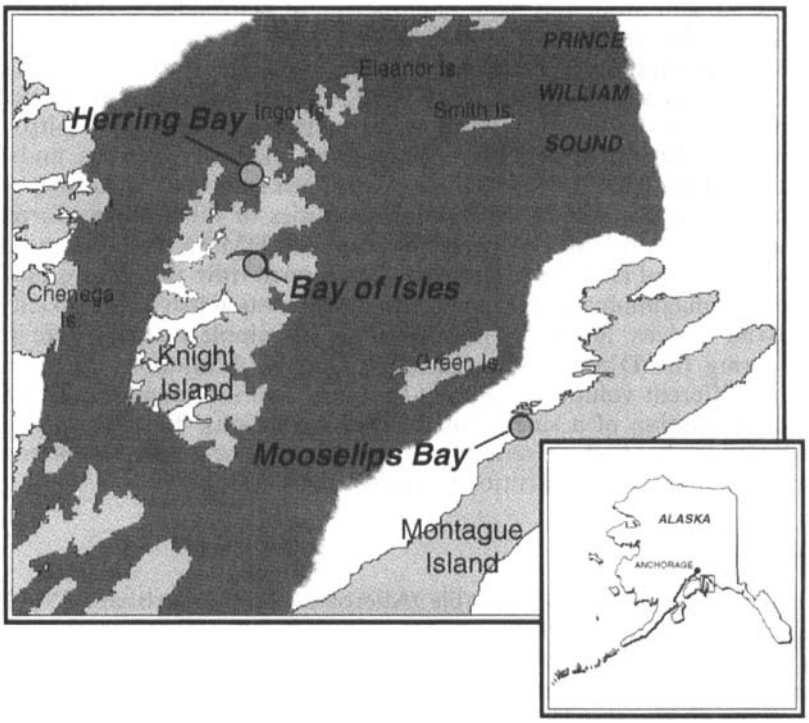


**Figure 1** Location of sampling sites for intertidal sediments and pore-water in Prince William Sound, Alaska. The shaded area represents the approximate extent of floating oil from the Exxon Valdez spill.



for 2 min with a stainless steel stirring device. Following phase separation, the solvent layer was transferred through stainless steel and teflon tubing to a one-litre separatory funnel, using nitrogen gas overpressure; and each carboy was re-extracted twice more with 500 ml of 1:1 (v:v) methylene chloride-ethyl acetate. Extracts were combined and transferred to clean 4-litre glass bottles for transport to the Kasitsna Bay field laboratory (operated for NOAA by the University of Alaska), where extracts were concentrated (at 80–100°C) in one-litre flasks fitted with Snyder columns to ~250–500 ml. Concentrated pore-water extracts were shipped (4°C, dark) to the SAIC laboratory in one-litre, 'certified clean' (I-Chem$^R$) glass bottles.

*Chemical Fractionation and Solvent Exchange*

Solvent extracts from sediment and water samples were separated into aliphatic, aromatic, and polar fractions by liquid chromatography (LC) on silica gel (70–140 micron, Sigma Chemical Co., St. Louis; column dimensions of 19 mm I.D. × 200 mm). About one ml of extract, representing less than 0.5 g extracted residue weight, was loaded on to the LC column; the aliphatics (F1) eluted with 30 ml hexane; the aromatics (F2) eluted with 45 ml hexane:benzene (1:1, v:v); and the polar constituents (F3) eluted (sequentially) with 80 ml methylene chloride/methanol (1:1, v:v), 80 ml methanol, and 80 ml methanol/ethyl acetate (9:1, v:v), which were then recombined. Elution patterns were checked with suites of analytical standards (aliphatics from

nC12 to nC32, and aromatics from naphthalene to benzo[ghi]perylene) to confirm that the desired LC separations were being achieved.

Prior to toxicity testing, fractions were solvent-exchanged into dimethyl sulphoxide (DMSO). A 30 ml aliquot of the fraction (F2 or F3) was concentrated to 1–5 ml by evaporation at room temperature under a stream of ultra-pure nitrogen gas, brought back to 30 ml with DMSO, re-evaporated under nitrogen gas, and replaced again with DMSO. The DMSO solutions were then subjected to toxicity testing.

Sample extracts containing substantial quantities of oil routinely formed two-phase systems (i.e., oil/DMSO) on partitioning with DMSO. Preliminary screening tests showed that not all toxic components partitioned from the oil into the DMSO. To ensure that maximum solubilization into DMSO was achieved for toxicity testing and to avoid toxicity losses, various dilutions of sample extracts were prepared directly during the DMSO exchange step. That is, dilutions were prepared by exchanging different volumes of extract fractions into set volumes of DMSO (and not by serial dilution of a single initial DMSO solution). Toxicity was estimated from a range in the dilution series that gave linear dose-responses relative to the quantity of sample extract partitioned against the DMSO.

### *Toxicity Testing*

Microtox$^{R}$ is a commercially available (Microbics Corp., Carlsbad, CA) bioassay based on the inhibition of bacterial luminescence (*Photobacterium phosphoreum*) (Chang *et al.*, 1981; Shiewe *et al.*, 1985). Prior to testing, extracts (at various initial dilutions) in DMSO were further diluted with 2% saline solution to a final DMSO concentration of 2%. For testing, 500 µl of 2% saline solution was dispensed into each cuvette followed by 10 µl of bacterial suspension. After 15 min an initial light reading was recorded and 500 µl of the diluted sample was added. Final light readings were recorded after a 15 min exposure period. The final DMSO concentration (1%) produced no light diminution in controls. Each sample dilution was run in triplicate, and a negative control containing 1% DMSO in 2% saline was tested along with each sample. Sample responses were corrected for blank response, and the effective concentration of extract yielding a 50% decrease in luminescence was calculated using either the trimmed Spearman-Karber method (Hamilton *et al.*, 1977), or probit analysis.

The SOS Chromotest$^{R}$ is a commercially available (Orgenics Corp., Israel) bioassay kit used to screen test samples for genotoxic activity. The test uses a strain of *Escherichia coli* (PQ37), genetically engineered to lack the enzyme beta-galactosidase, but in which the operon for that enzyme is linked to the cell's DNA repair mechanism (the 'SOS' system). When DNA damage occurs, the SOS system is activated, initiating production of beta-galactosidase, which is measured colorimetrically in the assay (Quillardet and Hofnung, 1985; Quillardet *et al.*, 1985). Viability of the test organism was checked by assays of alkaline phosphatase activity. Sample extracts in DMSO were diluted with DMSO/saline to yield a final DMSO concentration of 10% prior to testing. Genotoxic activity was calculated (Dayan *et al.*, 1987) as an SOS Induction Factor (SOSIF). Dayan *et al.*, (1987) suggested that a SOSIF >1.0 indicates genotoxicity, but we used more conservative criteria in this study, requiring that: (a) the SOSIF must be statistically different from the DMSO control; (b) the SOSIF must be >1.3; and (c) the observed response must be dose-dependent. Dunnet's procedure (Zar, 1984) was applied to SOSIF values to determine statistical differences.

Toxicity was also assayed using 48 h static exposures of larval bivalve molluscs (Chapman and Morgan, 1983; ASTM, 1990; USEPA/ACOE, 1991), with percent

normal shell development (to the prodissoconch I stage) and percent survival as endpoints. Blue mussels (*Mytilus edulis*) were induced to spawn by raising water temperature from 15° to 20°C, and fertilization was accomplished in a clean 1 l beaker within two hours. Embryo density was determined on two 1.0 ml aliquots of each of five 1:99 dilutions of the homogeneous suspension. Embryos were inoculated into test vessels with a calibrated automatic pipette. Fractions were tested in 1% DMSO in sterile, filtered (0.22 μm) sea water adjusted with deionized water to 28 ppt salinity. After 48 h exposure, two 10 ml aliquots were removed from test vessels and preserved in 5% formalin-rose bengal; and normal (completely developed, straight-hinged, 'D'-shaped prodissoconch I stage) and abnormal larvae were counted. Mortality data are expressed as the ratio of the number of surviving larvae in test samples to that in sea water DMSO controls. Abnormality is expressed as % abnormal larvae, based on the total number recovered for each replicate. Statistics were performed on arcsin/square root-transformed data, using multiple paired t-tests.

Incidences of anaphase aberrations and sister chromatid exchange (SCE) were also determined in the exposed mussel larvae. Beginning twelve hours after fertilization, early trochophore larvae were exposed to the extract fractions in 1% DMSO. After 12 h exposure, larvae were concentrated by centrifugation, fixed (for 1 h) in Carnoy's solution (methanol/acetic acid), and then placed in 2% aceto-orcein stain for at least 15 min. Four slides were prepared from each replicate using a standard squash method, and sealed with clear nail polish. For each replicate, 100 anaphase cells were counted for aberrations (Hose, 1985), including stray chromosomes, lagging chromosomes, acentric fragments, attached fragments, unequal distribution of chromosomes, translocation bridges, side-arm bridges, and multipolar spindle formations.

For SCE counts, $10^{-5}$ M bromo-deoxyuridine was added to the exposure medium and colchicine was added to halt metaphase after 10.5 h of exposure. Larvae were collected on 10-μm mesh screens, then exposed successively to 2:1, 1:1, and 1:2 mixtures of sea water and 0.6% KCl for 10 min each, and fixed twice in methanol:acetic acid (3:1) solution. After centrifugation, excess fixative was decanted and 60% acetic acid was added to disaggregate the larvae. Larvae were pipetted on to a microscope slide, squashed with a second slide, then air dried and stained for 10 min with 33258 Hoechst stain in McIlvaine's buffer at pH 8. The slides were exposed to ultraviolet light for 60 min, rinsed in tap water, air-dried, and stained in 4% Giensa for 10 min before final rinsing, drying, and mounting. Ten second-division cells from each of two slides were scored for SCE under 1250× magnification for three replicates. Statistical analyses for SCE and anaphase aberrations were similar to those used for larval survival.

Fertilized eggs of coho salmon (*Onchorhynchus kisutch*) were exposed for 24 h (beginning 72 h after fertilization) to extract fractions to determine mortality, teratogenic and genotoxic effects. Following exposure, dead eggs were culled from test solutions and scored, and 25 eggs per treatment were placed in 5% buffered formalin for anaphase aberration scoring, using procedures similar to those for bivalves (Hose, 1985). An additional 25 eggs per treatment were rinsed three times with deionized water, and returned to glass dishes (covered with sterile gauze) randomly distributed in Heath trays in a recirculating hatchery system. Eggs were checked periodically for development to the 'eyed' stage, and scored weekly for survival. Dead eggs were recorded and removed. Six weeks after fertilization, the eggs were placed in partitioned Heath trays to accommodate emerging sac fry and monitored for hatching success. By 9 weeks after fertilization, 90% of the control fish had absorbed

their yolk sacs, and the test was terminated. Replicates were scored for final mortality, and the embryos were placed in 10% buffered formalin for subsequent teratogenic analysis. The embryos were scored for gross anomalies likely to preclude survival under natural conditions: stunted growth, spine deformities, fin deformities, aberrant optical lobes, protrusive neoplasms, and coagulated yolk (Birge *et al.*, 1983). The numbers of terata were noted per surviving embryo, and statistical analyses (multiple two-sample t-tests) were performed on arcsin/square root-transformed data.

### *Chemical Analyses and Characterizations*

To check on completeness of the LC separations and provide preliminary characterization of the oil constituents present in the samples, the aliphatic, aromatic, and polar fractions were analyzed on a Hewlett Packard 5890 gas chromatograph (FID-GC) equipped with flame ionization detector and a Model 7673A automatic sampler. The column (0.32 mm × 30 m) had a DB-5 stationary phase with a film thickness of 0.25 $\mu$m, and the flow rate of carrier gas was 2.0–2.5 ml $min^{-1}$. Following an initial 5 min period at 45°C, temperature was programmed to increase at 3.5°C $min^{-1}$ to 280°C.

Some fractions were also subjected to liquid chromatography and particle beam mass spectrometry (LC/PB-MS) (Miles *et al.*, 1992; Doerge *et al.*, 1993). The LC/MS measurements represented an experimental effort to detect and identify oxygenated polar metabolites (e.g. hydroxy and quinone derivatives) of the parent polynuclear aromatic hydrocarbons (PAH) in the oiled samples. Portions of selected fractions were also sent to Drs. Ed. Overton (Louisiana State University), and Manfred Ehrhardt (University of Kiel) for more detailed GC-MS analysis and comparison with previously identified mass spectra from petroleum oxidation products.



## RESULTS

Chromatograms are illustrated in Figures 2–3 for the three fractions of one pore-water sample each from Herring Bay (FOX WA), Mooselips Bay (LIPS WB), and Bay of Isles (BAY WA), respectively. Chromatograms for the fractions from one sediment sample (BAY SA) from Bay of Isles are shown in Figure 3. Repeating series of n-alkanes (along with pristane and phytane) were present in the aliphatic fractions of all samples (both water and sediments) from Herring Bay and Bay of Isles. Aliphatic fractions from these locations (Figures 2a, 3a,d) include $nC_{12}$ through $nC_{32}$, while the aromatic fractions (Figures 2b, 3b,e) include 2- to 6-ring PAHs, from substituted naphthalenes through benzo(ghi)perylene, reflecting moderately weathered residual oil. Standards demonstrated that neither aliphatics nor PAH were eluting with the polar fraction. Furthermore, comparison of chromatographic profiles for the aliphatic (F1), aromatic (F2), and polar (F3) fractions for the replicate samples from Bay of Isles and Herring Bay (Figures 2–3) indicated that prominent FID-GC peaks in polar factions were not observed in corresponding aromatic or aliphatic fractions. Appropriate separation of the aliphatic, aromatic, and polar constituents into their respective fractions was therefore confirmed.

In sharp contrast to samples from oiled sites, the aliphatic and aromatic fractions from Mooselips Bay pore-water (Figure 2) exhibited nearly flat baselines, similar to traces obtained with water method blanks. The Mooselips Bay polar fraction (F3) exhibited several peaks, but these were many fewer in number and much lower in

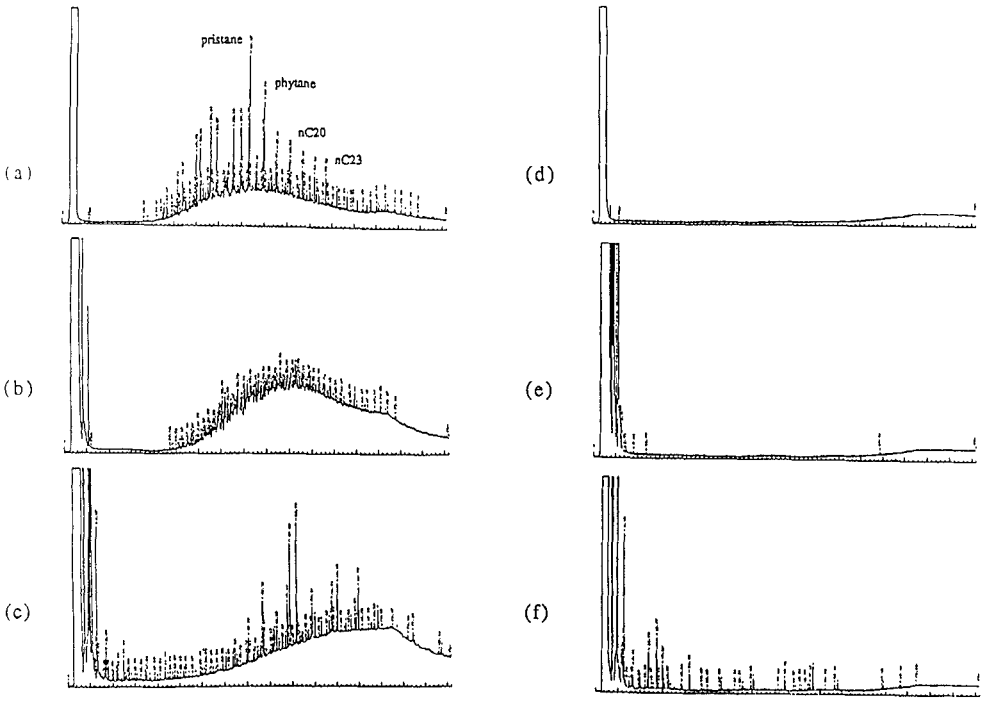


**Figure 2** Chromatograms (GC/FID) for pore-water extracts from Herring Bay (Sample FOX WA, chromatograms a – c) and Mooselips Bay (LIPS WB; d – f). The top charts in each series (a + d) are the aliphatic fractions; the middle (b + e) are the aromatic fractions; and the lower (c + f) are the polar fractions.



concentration than those in polar fractions from oiled sites (Compare Figure 2f with Figures 2c and 3c).

All samples were screened with Microtox$^R$ and SOS Chromotest$^R$, whereas only selected fractions were submitted to the other tests. Table I shows test results for various fractions and Table II shows statistically significant differences among selected samples and fractions. Microtox$^R$ results indicate that Bay of Isles samples were generally more toxic than corresponding samples from Mooselips Bay, while Herring Bay (FOX) exhibited intermediate toxicity. Samples BAY SBF3 and LIPS WBF2 were modest exceptions to this rule. While the toxicity of polar fractions (F3) was much lower than F2 for Mooselips Bay water, F2 and F3 gave notably similar results for water samples from Bay of Isles (Table I).

At the highest concentration tested (0.9%), four fractions, all from Bay of Isles (BAY SAF2, SAF3, SBF3, and WAF2), met our SOS Chromotest$^R$ criteria for genotoxicity, with an induction factor (SOSIF) > 1.3. The SOSIFs for these same four samples also exceeded 1.3 at the 0.45% dilution, but fell below that level at the 0.09% dilution. At the 0.9% dilution, the SOSIF for sample BAY WBF3 also approached 1.3. Both aromatic and polar fractions were represented in these genotoxic samples, and relative genotoxicity between F2 and F3 was mixed. The polar fraction from Bay of Isles (BAY WAF3), however, inhibited the activities of both beta-galactosidase and alkaline phosphatase, indicating acute toxicity without significant genotoxicity.

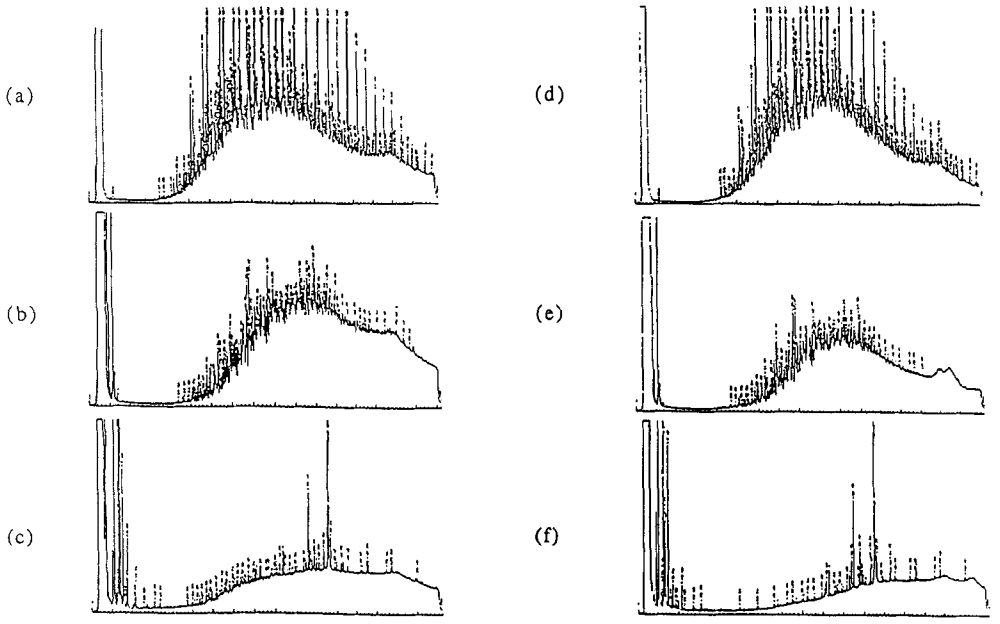


**Figure 3** Chromatograms (GC/FID) for pore-water extracts (Sample BAY WA, chromatograms a – c) and sediments from Bay of Isles (BAY SA; d – f). The top charts in each series (a + d) are the aliphatic fractions; the middle (b + e) are the aromatic fractions; and th lower (c + f) are the polar fractions.



Toxicity data for bivalve larvae (survival, development, and genotoxicity) are presented only for the 0.1% dilutions of each fraction (Tables I and II). Differences between Bay of Isles and Mooselips Bay samples were also discernible at the 1% dilutions, but generally not at 0.01% and 0.001%. At 1%, all Bay of Isles fractions except BAY SBF2 (77.9%) caused frequencies of 93–100% abnormal larvae. Except for LIPS WAF2, the Mooselips Bay fractions (1% dilution) elicited frequencies of 11–19% abnormality. LIPS WAF2 caused high mortality, and the few surviving larvae were all abnormal.

Larval mortality was usually higher for Bay of Isles fractions than for corresponding Mooselips Bay fractions, except for the comparison of F2 fractions from sediment samples (BAY SAF2 and BAY SBF2 versus LIPS S1F2), where the mortality for the LIPS sample was slightly higher than that for the BAY samples (Table I). In all cases where differences were statistically significant, larval abnormality was greater for Bay of Isles samples than for Mooselips Bay samples (Table II). At lower test concentrations (0.01% and 0.001%), mortality was low and variable, without any consistency of direction among samples, and abnormality was consistently low, with few statistically significant differences between samples.

Both anaphase aberrations and SCE exhibited statistically greater responses in bivalve larvae for Bay of Isles samples than for Mooselips Bay (Table I and II). The principal types of anaphase aberrations observed were translocation bridges, side-arm bridges, and attached fragments (Hose, 1985). The DMSO control, however, showed a significantly higher mean number of anaphase aberrations (15.8%) than the sea water control (7.0%). While all Bay of Isles samples were significantly higher than the sea water controls, no differences were detected between Mooselips Bay

**Table I** Summary of toxicity test results from polar (F3) and aromatic (F2) fractions of organic extracts of replicate sediments (SA,SB) and pore-water (WA,WB) from Bay of Isles (BAY), Herring Bay (FOX), Mooselips Bay (LIPS), and method blanks (MB). A dash (–) indicates that sample was not tested for that endpoint.

| Sample | Microtox | SOSIF | Bivalve (0.1% fraction) | | | | Salmonids (0.1% fraction) | | |
|---|---|---|---|---|---|---|---|---|---|
| | EC-50 (%) | (0.9% fraction) | Mortality (%) | Abnormal (%) | Anaphase Aberration (%) | SCE (# per chromosome) | Mortality (%) | Terata (# per fish) | Anaphase Aberration (%) |
| BAY SA F2 | 0.25 | 2.24 | 14.8 | 57.7 | 18.2 | 0.232 | 11.1 | 0.68 | 35 |
| BAY SA F3 | 0.22 | 1.58 | 25.6 | 92.8 | 25.3 | ** | 8.0 | 0.71 | 41 |
| BAY SB F2 | 0.5 | 1.19 | 16.2 | 16.5 | – | – | 17.3 | 0.43 | 33 |
| BAY SB F3 | 1.44 | 1.33 | 29.3 | 77.6 | – | – | 8 | 0.38 | 43 |
| BAY WA F2 | 0.67 | 1.66 | 28.6 | 99.5 | 21.0 | 0.217 | 2.7 | 0.59 | 32 |
| BAY WA F3 | 0.26 | 0.366 | 28.3 | 33.8 | 25.6 | 0.286 | 8.0 | 0.59 | 30 |
| BAY WB F2 | 0.09 | 0.855 | 32.0 | 22.8 | – | – | 12.0 | 0.84 | – |
| BAY WB F3 | 0.23 | 1.27 | 17.5 | 96.3 | – | – | 10.7 | 0.08 | – |
| LIPS S1F2 | 1.03 | 1.11 | 21.5 | 18.9 | 7.3 | 0.154 | 6.7 | 0.13 | 32 |
| LIPS S1F3 | 1.38 | 1.17 | 15.8 | 20.0 | 11.6 | 0.218 | 6.7 | 0.11 | 30 |
| LIPS S2F2 | 2.45 | 0.962 | – | – | – | – | – | – | – |
| LIPS S2F3 | 1.45 | 1.062 | – | – | – | – | – | – | – |
| LIPS WAF2 | 1.03 | 0.891 | 14.8 | 17.4 | 10.6 | 0.182 | 10.8 | 0.32 | 30 |
| LIPS WAF3 | 5.8 | 0.963 | 11.1 | 15.5 | 14.2 | 0.204 | 8.0 | 0.41 | 30 |
| LIPS WBF2 | 0.73 | 0.964 | – | – | – | – | – | – | – |
| LIPS WBF3 | 5.23 | 1.037 | – | – | – | – | – | – | – |
| FOX SB F2 | 0.54 | 1.049 | – | – | – | – | – | – | – |
| FOX SB F3 | 0.99 | 0.792 | – | – | – | – | – | – | – |
| FOX WB F2 | 0.12 | 1.070 | – | – | – | – | – | – | – |
| FOX WB F3 | 1.14 | 0.751 | – | – | – | – | – | – | – |
| FOX MB F2 | 0.12 | 0.827 | 32.7 | 20.0 | – | 0.220 | 10.7 | 0.63 | – |
| FOX MB F3 | 1.14 | 0.898 | 15.2 | 15.1 | – | 0.228 | 17.3 | 0.49 | – |
| MB269 F2 | 0.16 | 0.841 | 14.2 | 26.0 | – | – | 17.3 | 0.15 | – |
| MB269 F3 | 6.65 | 0.83 | 15.9 | 26.3 | – | – | 5.3 | 0.53 | – |

**Table II** Statistical differences between fractions at 0.1% dilution. Entries identify the samples that were significantly more toxic at p = 0.05, unless noted otherwise.

| Comparison | Bivalve (0.1% fraction) | | | Salmonids (0.1% fraction) | |
|---|---|---|---|---|---|
| | Abnormal | Anaphase Aberration | SCE | Terata | Anaphase Aberration |
| BAY versus LIPS | | | | | |
| SA F2 vs S1 F2 | 0 | BAY | BAY | BAY | 0 |
| SB F2 vs S1 F2 | 0 | – | – | 0 | – |
| SA F3 vs S1 F3 | BAY | BAY | BAY* | BAY | 0 |
| SB F3 vs S1 F3 | BAY | – | – | 0 | – |
| WA F2 vs WA F2 | BAY | BAY | BAY | (BAY) | 0 |
| WB F2 vs WA F2 | 0 | – | – | BAY | – |
| WA F3 vs WA F3 | (BAY) | BAY | (BAY) | BAY | 0 |
| WB F3 vs WA F3 | BAY | – | – | LIPS | – |
| F2 versus F3 | | | | | |
| BAY SA | F3 | 0 | F3* | 0 | 0 |
| BAY SB | F3 | – | – | 0 | – |
| BAY WA | 0 | F3 | F3 | 0 | 0 |
| BAY WB | F3 | – | – | F2 | – |
| LIPS S1 | 0 | 0 | F3 | 0 | 0 |
| LIPS S2 | 0 | 0 | 0 | 0 | 0 |
| SEDIMENT versus WATER | | | | | |
| BAY SA F2 vs WA F2 | WA | 0 | 0 | (SA) | 0 |
| BAY SA F2 vs WB F2 | 0 | – | – | 0 | – |
| BAY SB F2 vs WA F2 | WA | – | – | 0 | – |
| BAY SB F2 vs WB F2 | 0 | – | – | 0 | – |
| BAY SA F3 vs WA F3 | SA | 0 | SA* | 0 | 0 |
| BAY SA F3 vs WB F3 | 0 | – | – | SA | – |
| BAY SB F3 vs WA F3 | SB | – | – | 0 | – |
| BAY SB F3 vs WB F3 | WB | – | – | (SA) | – |
| LIPS S1 F2 vs WA F2 | 0 | 0 | WA | WA | 0 |
| LIPS S2 F3 vs WA F3 | 0 | 0 | 0 | WA | 0 |

* BAY SA F3 judged toxic without statistical analysis
( ) = Probability between 0.05 and 0.10
0 = No significant difference
– = No data/samples not tested



and sea water controls. BAY WAF3 was the only test fraction significantly different from the DMSO control. The number of sister chromatid exchanges was lowest in sea water (0.121 ± 0.007) and DMSO controls (0.134 ± 0.02) and highest in BAY WAF3 (0.286 ± 0.05). However, the BAY SAF3 fraction caused such heavy chromosome damage that SCE could not be quantified, and it was presumed that this sample was the most genotoxic for this endpoint. All fractions but the sea water control, LIPS S1F2 and FOX MBF2 (0.220 ± 0.063), exhibited significant ($p<0.05$) toxicity relative to the DMSO control.

Mortality of larval salmonids was low in fresh water (9.3%) and DMSO controls (10.7%), and was generally low in all test samples with no significant differences in mortality between any of the samples (Table I). While the number of terata was higher in the DMSO control (0.25 per fish) than in the freshwater control (0.10 per fish), most of the Bay of Isles fractions elicited higher rates of teratogenicity. All

but one of the significantly different paired comparisons were higher for Bay of Isles samples than for Mooselips Bay samples (Table II). More than 90% of the observed teratogenic effects were stunted growth, bent spines, or irregular swelling of the optic lobes. The principal anaphase aberrations noted were translocation, side-arm bridges, and attached fragments; but the aberration rate was 24% in freshwater controls, and no significant differences were found between any of the fractions and the DMSO control (31%) (Table II).

Preliminary analysis by LC/PB-MS of F3 fractions from Bay of Isles did not identify oxygenated derivatives of benzanthracene, chrysene, triphenylene, benzo[e]pyrene, or benzo[a]pyrene in either the sediment or water extracts, though these parent compounds were easily detected in the corresponding F2 fractions (Doerge *et al.*, 1993). The sensitivity of LC/PB-MS was limited, however, especially for more volatile lower molecular weight constituents (PAH <4 rings), and oxygenated derivatives would probably not have been detected at concentrations much lower than the parent hydrocarbons. More detailed GC/MS analyses also failed to identify oxidized derivatives of petroleum hydrocarbons in the polar fractions of these sediment extracts (personal communication, M. Ehrhardt, University of Kiel). The F3 contained large numbers of aliphatic fatty acids, quite certainly of recent biogenic origin, along with a considerable amount of high-molecular weight material (possibly fulvic/humic acids, in part) that did not elute from the CP-Sil-8 CB (equivalent to SE54) column. This finding was similar to results obtained with sediment and water samples from the Persian Gulf after the Gulf War oil spill, where, although UV fluorescence indicated petroleum-derived constituents in polar fractions, structural elucidation proved impossible (Ehrhardt and Burns, 1993; and M. Ehrhardt, personal communication). The actual sources of toxicity in the various chemical fractions from PWS therefore remain unidentified.



## DISCUSSION OF PREVIOUS RELATED RESEARCH

The literature on the toxicity of Alaskan crude oil to Arctic and subarctic marine organisms is extensive (Anderson, 1977; Rice, 1985; Rice *et al.*, 1976, 1977, 1979, 1984; National Academy of Sciences, 1985; Wolfe, 1985; Karinen, 1988). Very little of this prior research was directed, however, toward the significance of either polar constituents of crude oil or intermediate oxidation products of petroleum hydrocarbons. Since these latter compounds have undergone preliminary oxidation and (sometimes) conjugation, they are more polar than their parent hydrocarbons, and would be expected generally to be less subject to bioaccumulation, more subject to excretion or depuration, more susceptible to further biodegradation, and more susceptible to dilution and dispersion in the water column (James and Kleinow 1994). Although the studies described in this paper suggest that such polar constituents may contribute an appreciable fraction of the residual total toxicity and mutagenicity associated with PBCO that has weathered *in situ* in intertidal sediments for 17 months, the total sediment toxicity measurable in most areas was already at a very low level by that time (Wolfe *et al.*, 1993b, 1994b; Boehm *et al., 1994).*

### *Toxicity of Oil and its Constituents*

Previous studies have shown that acute toxicity of oil diminishes as weathering progresses, and by the mid-1970's, it had been concluded that most oil toxicity was

attributable directly to the more water-soluble aromatic compounds (Moore and Dwyer, 1975; Neff *et al.*, 1976, Rice *et al.*, 1984). Interest at that time focused on which fractions of petroleum were most responsible for the toxicity observed. In short-term exposures, molar toxicity appeared to increase with number of aromatic rings (i.e., benzene <naphthalene <phenanthrene), at least up through 3-ring compounds, and also with the extent of substitution (i.e., benzene <toluene <xylene <ethylbenzene, etc.) (Neff *et al.*, 1976; Rice *et al.*, 1977; Caldwell *et al.*, 1977). In direct comparisons of various one-to three-ringed aromatics, the greatest toxicities (LC50-96h) were associated with dimethylnaphthalene (Caldwell *et al.*, 1977) or 1-methyl phenanthrene (Neff *et al.*, 1976). Chrysene, benzo(a)pyrene, and dibenzanthracene were not lethal to the test organism *Neanthes arenaceodentata* at their limits of solubility in such short-term exposures (Neff *et al.*, 1976).

Based on the relative concentrations of low-molecular weight constituents in crude oil, it is generally accepted that acute toxicity caused by oil in the environment is derived mainly from mono- and di-nuclear aromatics. When a water-soluble fraction (WSF) of oil was simulated, however, by mixing the 10 predominant aromatic hydrocarbons at the same concentrations and proportions found in a crude oil WSF, the toxicity of the resulting mixture was only 20–30% of the actual WSF, suggesting that either minor aromatic constituents, or other components, contribute significantly to the observed toxicity (Rice *et al.*, 1984). Korn *et al.* (1985), exploring possible contributions of phenol and p-cresol to the toxicity of crude oil WSF, found that toxicity of phenol (and p-cresol) was about twice that of toluene but only one-fifth that of naphthalene. Because the concentrations of toluene and naphthalene were respectively about 50 times and 2–7 times greater than that of phenolic compounds in the WSF, they concluded that the phenols were not likely to be major contributors to WSF toxicity.

In a previous effort to determine which chemical constituents of PBCO exerted the greatest toxicity and mutagenicity, Warner *et al.* (1979) fractionated PBCO using a succession of solvent partitioning, gel permeation and adsorption chromatography. Toxicity of the resultant fractions was evaluated using the Ames bacterial mutagenicity test, a mammalian cell toxicity test, and a bioassay with mysid shrimp; the results suggested that aromatic hydrocarbon fractions were most toxic and probably represented the greatest biological hazard. Although most other fractions showed no toxicity at the levels tested, one high-molecular-weight polar oil fraction (otherwise uncharacterized) was both toxic and slightly mutagenic.

In the marine environment petroleum is decomposed, primarily through the processes of microbial biodegradation and photo-oxidation or auto-oxidation. These processes are effective for oil in surface slicks, the water column, sediments, and the atmosphere (photo-oxidation of evaporated compounds). The extensive literature on hydrocarbon metabolism by microorganisms was recently summarized by Bartha and Atlas (1987). Although the paraffinic and aromatic fractions of petroleum are quite readily degradable, the polar fractions as well as most nitrogen- and sulphur-containing fractions are essentially nonbiodegradable (NAS, 1985). The rate and final amount of biodegradation of any petroleum depend heavily on its composition and on specific abiotic environmental parameters. In general, the rate of petroleum biodegradation in marine waters or in surficial (1–2 cm) sediments is limited by the availability of inorganic nutrients and not by oxygen or temperature. Once oil penetrates into deeper sediments, however, oxygen may become the limiting factor for petroleum degradation. High-molecular weight PAH may remain indefinitely in aquatic sediments, and significant sublethal effects to benthic organisms have

been documented to accompany long-term persistence (Krebs and Burns, 1977; Jackson *et al.*, 1989).

Products of photo-oxidation include fatty acids, alkylated naphthols, substituted 1- to 3-ring aromatic and heteroaromatic acids, and alkylated benzothiophene sulphoxides (Overton *et al.*, 1979, 1980). Oxidized products of phenanthrene, including carbonyl, quinone, and carboxylic acid derivatives, were identified in sea water extracts after UV irradiation of a phenanthrene 'slick' for 120 h (Malins *et al.*, 1985). The asphaltene and resin fractions of crude oil appeared to inhibit production of photo-oxidized derivatives in these experiments; UV irradiation (120 h) of the separated aromatic/paraffinic fraction of PBCO caused a 20-fold increase in the partitioning of extractable organic material into underlying sea water and a 10-fold increase in sea water-soluble derivatives of $^{14}$C-phenanthrene added before irradiation. About half the phenanthrene metabolites in sea water after UV irradiation were not methylene chloride extractable, indicating oxidation to highly water-soluble products (Malins *et al.*, 1985).

Microbial biodegradation of alkanes, cycloalkanes, and mono-aromatics leads to the production of alcohols, aldehydes, and carboxylic acids that are generally of little concern from a toxicity standpoint. Highly condensed PAH, however, may be transformed by microbial metabolism to potential carcinogens or mutagens (James and Kleinow, 1994). Benzo(a)pyrene and benzo(a)anthracene, for example, are oxidized by eucaryotic organisms (macroorganisms, yeasts and moulds) to trans-dihydrodiols that are activated into oxides that are powerful mutagens. Procaryotic organisms (bacteria) oxidize the same compounds to cis-dihydrodiols which undergo oxidative fission of the ring structure without passing through these mutagenic intermediates (Gibson, 1977; Cerniglia and Heitkamp, 1989).

Some metabolic products of PAH are demonstrated mutagens or carcinogens known to bind to DNA (Ahokas *et al.*, 1979; Lech and Bend, 1980; Varanasi *et al.*, 1981). These same materials are associated with the prevalence of liver lesions, including neoplasms (Varanasi and Stein, 1991). These intermediary metabolites are the inferred likely mediators of biological damage in tissues that bioaccumulate and metabolize the parent hydrocarbons (Lech and Bend, 1980). Although it has been suggested that metabolites might contribute to the effects of hydrocarbon exposure through the food web (Carls, 1987; James and Kleinow, 1994), there is currently neither quantification nor even documentation of such an effect, nor is information available on the fate of hydrocarbon metabolites released into the environment. The data presented in this paper suggest that polar constituents do contribute part of the (albeit low) toxicity and genotoxicity associated with PBCO after more than one year of weathering in PWS sediments.

Asphaltenes and resins are heterogeneous, poorly characterized assortments of non-hydrocarbon compounds comprising respectively about 2% and 6% of PBCO (Clark and Brown, 1977). Asphaltenes are tar constituents that are highly resistant to biodegradation, and are not generally considered to be toxic to marine organisms. Resins include polar and heterocyclic NSO compounds, such as phenols, cresols, thiophenes, dibenzothiophenes, pyridines, and pyrroles. Very little work has been published on these compounds, but some of them are likely to undergo biodegradation, and very broad suites of NSO compounds have been found in oil-contaminated marine environments (Wolfe *et al.*, 1981; Krone *et al.*, 1986). Like hydrocarbon metabolites, these compounds exhibit moderate water-solubilities and are subject to dispersion in the water column. Although some of these compounds might be toxic at high concentrations, no studies have focused specifically on the

levels of toxicity under spill conditions in the marine environment. Since these fractions were not distinguished or separated from other polar constituents in this study, no statements are possible on their contribution to the observed low levels of toxicity.

### *Uptake-Depuration, Metabolism, and Toxicity of Oxygenated Hydrocarbon Derivatives Relative to Parent Compounds*

Bioavailability and bioaccumulation of hydrocarbons depend in large measure on whether the hydrocarbons are dissolved (or finely dispersed, or micro-colloidal) in the water column, adsorbed on to suspended particulate matter or sediments, or contained in food materials. The relative uptake of different hydrocarbons by organisms is related to hydrophobicity as reflected by octanol-water partition coefficients (Veith *et al.*, 1979; Dunn, 1980), as is their adsorption to sediments and suspended matter (Means *et al.*, 1979, 1980; Karickhoff, 1981; Wolfe, 1987). Accumulation potential and retention generally increase with increasing molecular weight (i.e., with number of aromatic rings and with the degree of alkyl substitution on the rings) (Roubal *et al.*, 1977; Melancon and Lech, 1979; Rice *et al.*, 1984). For the higher molecular weight PAH, however, this generalization may be obscured by their very low solubilities, associated slow bioaccumulation rates, and by simultaneous metabolism within the organism.

Phenol, cresol and toluene were more effectively excreted across the gills by Dolly Varden char (*Salvelinus malma*) than were naphthalene, anthracene, and benzo[a]pyrene, whereas metabolites of all of these compounds were excreted via the bile (Thomas and Rice, 1981, 1982). The bile is a major route of excretion for hydrocarbon metabolites in fish (Collier *et al.*, 1978, Varanasi and Gmur, 1981). Most bile metabolites of naphthalene are in the form of conjugates, but some excretion of naphthols and dihydrodiols also occurs (Varanasi and Gmur, 1981; Varanasi *et al.*, 1981). Similar metabolites are excreted by crabs and shrimp (Lee *et al.*, 1976; Sanborn and Malins, 1980). Metabolic conversion rates in fish and Crustacea must closely approximate the intake, since long-term accumulation of parent compounds does not occur. Prior exposure of Dolly Varden to naphthalene resulted in increased metabolism of orally-administered $^{14}$C-naphthalene (Thomas and Rice, 1985), reflecting the induction of cytochrome P450 by PAH (Stegeman *et al.*, 1981, Lee *et al.*, 1982). Prior exposure to toluene, however, did not induce accelerated metabolism of $^{14}$C-toluene (Thomas and Rice, 1985).

Both conjugated and nonconjugated metabolites occur also in muscle tissue (Varanasi and Gmur, 1981; Thomas and Rice, 1982). Consumption of such metabolites represents a potential mode of exposure to predatory macro-organisms, but only limited insight on the potential amounts of such metabolites in edible tissue is available from experimental results with labelled compounds. Twenty four h after intragastric administration of $^{14}$C-labelled naphthalene, anthracene, and benzo(a)pyrene to Dolly Varden char, 15%, 11%, and 7.5% of the respective radioactivities were found in muscle tissue. Of those amounts, only 5.1%, 3.3% and 8.8%, respectively, were associated with a polar metabolite fraction (as opposed to parent compound) (Thomas and Rice, 1982). Similar studies could be used to estimate the relative proportions of metabolites and parent PAH in edible tissues (e.g. Varanasi *et al.*, 1990).

Malins *et al.* (1985) compared a number of toxic responses to sea water accommodated fractions (SWAF) of weathered and unweathered oils, including PBCO. Hatching success of English sole eggs (*Parophys vetulus*) was lower in

unirradiated SWAF than in irradiated SWAF generated under low-flow conditions with fuel oil. When SWAFs of no. 2 fuel oil were produced in this agitated flow-through system, UV irradiation caused less than a 2-fold increase (over the unirradiated SWAF) in total extractable organic materials, and no differences were observed in mortalities of English sole embryos exposed for 48 h. When no. 2 fuel was irradiated under static conditions, however, the extractable organic material in the SWAF was enhanced about 23-fold (to 161 ppm), and mortality was substantial, with an apparent EC-50 of about 25 ppm. Preparation of SWAFs from PBCO under identical conditions, however, produced no differences between flow-through and static conditions either in levels of total extractable organic materials or in mortalities of English sole embryos. English sole larvae were more sensitive than the embryos, showing significant mortality after 48 h exposure to fuel oil SWAF (in the low-flow system). The toxicity to larvae of the non-irradiated SWAF was slightly greater than that of the irradiated SWAF. Mortalities of newly hatched surf smelt larvae exposed in the low-flow system were not different for irradiated oil, nonirradiated oil, and control (no oil) treatments. At sublethal levels of total hydrocarbon (0.25–0.35 ppm) in the unirradiated SWAF, however, the larvae showed pronounced effects on swimming behaviour. This effect diminished with time (probably due to loss of volatile hydrocarbons from the SWAF), and was reversible. Cytopathological examination of the affected larvae showed necrosis of sensory tissues and compression of muscle bundles. Larvae exposed to irradiated SWAF were apparently not so affected. These data suggest that the potentially toxic products of hydrocarbon oxidation are more readily dispersed in the water column than their parent hydrocarbons, thereby reducing bioavailability and toxicity potential. Malins *et al.* (1985) concluded that these studies provided no evidence that photo-oxidation would significantly enhance the toxicity of petroleum under most conditions in the marine environment.



## CONCLUSIONS

The toxicity tests described here confirmed and extended the results of a survey that showed low residual sediment toxicity in 1990 in lower intertidal and shallow subtidal zones of heavily oiled sites in PWS. Fractions from Bay of Isles were consistently more toxic than corresponding fractions from Mooselips Bay for a variety of tests, including Microtox$^{R}$, SOS Chromotest$^{R}$, and abnormality, anaphase aberration, and sister chromatid exchange in bivalve larvae. However, samples from Bay of Isles (which showed obvious signs of heavy oiling) were only slightly more toxic (usually 2 to 5-fold) than samples from Mooselips Bay, which was not oiled and which gave very low responses in all tests.

The salmonid teratogenicity endpoint exhibited about the same sensitivity to oiled sediments (ability to distinguish Bay of Isles from Mooselips Bay samples) as the bivalve larval abnormality, anaphase aberration, and sister chromatid exchange endpoints, whereas the salmonid anaphase aberration was much less sensitive.

Toxicity of polar fractions from Bay of Isles was similar to, or higher than that of corresponding aromatic fractions. Polar fractions from pore-water were much more toxic to Microtox$^{R}$ at Bay of Isles than at Mooselips Bay, consistent with the observation that oxidized petroleum derivatives were more concentrated (relative to parent compounds) at oiled sites in pore-water extracts than in sediment extracts (personal communication, M. Ehrhardt, University of Kiel). Although the overall

toxicity of these samples was very low, we concluded that an appreciable part of that toxicity was attributable to polar constituents. No other patterns in relative response of corresponding sediment and water fractions were observed for any test or for either site.

Results from multi-year sediment toxicity surveys suggested that acute chemical toxicity (including that attributable to oxidized constituents) declined between 1989 and 1991, and shifted concomitantly from the lower intertidal zone (0 m) into shallow (3–6 m) nearshore sediments. The results are consistent with earlier published conclusions that the chemical toxicity of oil is associated primarily with the lower molecular weight aromatic compounds being lost most readily through weathering. The most significant toxic effects probably occurred during the first days after the spill, when the most toxic aromatic constituents were abundant. Nonetheless, the low levels of toxicity and genotoxicity demonstrated here in relation to polar constituents and/or derivatives of oil cannot be dismissed entirely (Burns, 1994 in press). Our samples were collected approximately 17 months after the spill, and the contribution of polar materials to the toxicity observed over that time period is unknown. Although the overall toxicity has been described, its origins are not known in detail; and for some organisms (e.g. intertidal infauna), prolonged exposure to polar materials in pore-water could contribute significantly. For practical purposes, however, the existing cumulative data bases on acute and sublethal oil toxicity (e.g. NAS, 1985) appear appropriate for assessing relative sensitivities of marine species and estimating ranges of potential response to oil spills.



*Acknowledgements*

The authors gratefully acknowledge the assistance of the Washington Department of Fisheries for supplying the broodstock coho salmon. We also thank the Lummi Indian Tribe of Washington for the loan of their Heath trays for raising the juvenile salmonids. We thank Dr. Aimee Bakken (University of Washington, Seattle) for advice on methodologies and assistance in quality control on the bivalve larval anaphase aberrations tests; and Dr. Dan Doerge (U.S. Public Health Service, Jefferson, Arkansas) and Dr. Manfred Ehrhardt (University of Kiel, Germany) for chemical analytical support. The able assistance of Annette Zukowski, Ann Thornton, Cornelia Mueller, and Bill Peters is also acknowledged. This work was carried out with support from projects Air/Water 4, Air/Water 6, and Subtidal 4 under the State/Federal Natural Resources Damage Assessment for the Exxon Valdez oil spill.

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