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SHORT COMMUNICATION

Differences in faecal profiles of porphyrins among river otters exposed to the *Exxon Valdez* oil spill

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River otters (*Lutra canadensis*) living in marine environments of Prince William Sound, Alaska, exposed to crude oil from the *Exxon Valdez* spill in March 1989, showed significantly elevated levels of faecal porphyrin over those of otters from non-oiled areas (oiled mean = 48.2, and non-oiled mean = 34.5 nmol g^{-1} dry faeces). Profiles of uro-, hepta-, hexa-, penta-, copro-, and protoporphyrin profiles were qualitatively characterized by high-performance liquid chromatography. These findings suggest that river otters may serve as a suitable indicator species in which porphyrin profiles can be used to monitor the effects of marine and freshwater crude oil exposure. Also, this is the first model showing the effects of an oil spill on porphyrins on a free-ranging mammal using a non-lethal methodology. These effects were detectable 1 year after the spill and following a major effort to clean oil from the shorelines of Prince William Sound.

Keywords: *Lutra canadensis, river otter,* Exxon Valdez *oil spill, porphyrins, Prince William Sound, Alaska.*

Introduction

River otters (*Lutra canadensis*) are widely distributed along coastal shores of the Pacific Northwest, including Prince William Sound, Alaska (Larsen 1984, Testa *et al.* 1994). As near-shore foragers on marine fishes and invertebrates in intertidal and subtidal zones (Larsen 1984, Bowyer *et al.* 1994), they are potentially excellent indicators of pollution (Baker *et al.* 1981, Clark *et al.* 1981) such as that from the *Exxon Valdez* oil spill of March, 1989. In that spill, over 39,000 metric tons of North Slope crude oil spread over > 3500 km of shoreline in Prince William Sound. We previously documented (Duffy *et al.* 1993, 1994a, b) that 2 years after the oil spill and an extensive effort to clean shorelines, river otters from a heavily oiled area (Herring Bay on northern Knight Island) had lower body mass and elevated levels of blood haptoglobins and liver enzymes than otters inhabiting a non-oiled area (Esther Passage). We hypothesized that elevated haptoglobin levels over so long a period could indicate chronic inflammation and liver injury after acute exposure to oil. Further sampling over large areas of Prince William Sound supported a hypothesis that river otters inhabiting oiled areas could be discriminated from those living in non-oiled areas using biomarkers, such as liver enzymes (Duffy *et al.* 1994a). Moreover, there were corresponding ecological effects from the spill including changes in diet, habitat selection, and sizes of home ranges for otters (Bowyer *et al.* 1994, 1995).

The pathway of prophyrin biosynthesis is sensitive to a number of xenobiotics and metals at low concentrations (Demathers and Lim 1993). Porphyrins are tetrapyrrolic pigments which are widely distributed in nature. These pigments possess a characteristic absorption spectrum with an intense band of absorbance at about 400 nm, which make their detection and estimation relatively specific. The main physiological significance of prophyrins lies in the pathway of haem biosynthesis, of which these substances can be considered as intermediary metabolites or oxidized by-products. Since this complex pathway of haem biosynthesis has been elucidated (Marks 1985, Woods 1989), porphyrins as indicators of pathology have been extensively used in human clinical chemistry (Bowers *et al.* 1992). Despite their potential usefulness as biomarkers, however, there are few studies where porphyrins have been used as a faecal biomarker in wild populations of mammals.

Several investigators in laboratory studies have described chemical-induced changes in patterns of porphyrins (i.e. profiles) in tissue and excreta of avian species resulting from exposure to halogenated aromatic hydrocarbons (Lambrecht *et al.* 1986, Miranda *et al.* 1987). In a wild population of herring gulls (*Larus argentatus*), Kennedy and Fox (1990) reported elevated levels of porphyrins in the livers of birds living on sites contaminated with halogenated aromatic hydrocarbons, but did not observe a correlation between the porphyrin response and the level of contamination. Akins *et al.* (1993) studied nestlings of European starlings (*Sturnus vulgaris*) and noted profiles for faecal porphyrin to be distinct in that six porphyrins were detectable during the development period of the nestlings. Levels of faecal porphyrins were elevated but not significantly so among nestlings treated with $HgCl_2$. In our study, we investigated the suitability of the river otter as a potential indicator species for evaluating field exposure to crude oil and the usefulness of both total porphyrins and porphyrin profiling as biomarkers.

MATERIALS AND METHODS

Subjects

Approximately 1 year after the *Exxon Valdez* oil spill, faeces of river otters (24–48 h old) were collected from about 80 km of shoreline in both oiled (Knight Island) and non-oiled (Esther Passage) areas of Prince William Sound from latrine sites of otters following the initial removal of faeces from these sites during June–September 1990 (Testa *et al.* 1994). These collections were facilitated by otters defaecating at latrine sites, where they congregate and engage in social activities (Rock *et al.* 1994). Faeces of river otters were haphazardly selected and analysed for total porphyrin levels using a diode array spectrophotometer; 117 samples were taken from oiled latrine sites at Herring Bay, and 84 were from non-

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oiled latrine sites at Esther Passage along the coast of Prince William Sound. Population estimates for the oiled study area (Knight Island) during summer 1990 ranged from 36 to 42 river otters; estimates from the non-oiled area (Esther Passage) ranged between 32 and 44 otters (Testa *et al.* 1994). These radiolabelling studies showed that up to seven otters can use a latrine site.

Faecal extraction

The protocol used for extraction of faecal porphryins is a modification of that developed by Lockwood (1985), and is summarized in Figure 1. Five millilitres of 12N HCL was added to approximately 1.0 g of dry (lyophilized) faeces. This mixture was vortexed, allowed to sit for 5 min, and vortexed again. Fifteen millilitres of both diethyl ether and distilled H_2O were added, and the mixture was vortexed after each addition. To ensure that the prophyrins were not denatured, the time elapsed between the addition of HCl and H_2O did not excced 10 min. This mixture was then centrifuged at 3000 RPM for 10 min. The aqeuous phase was centrifuged again at 4000 rpm for 5 min, and the supernatant refrigerated in the dark until time of analysis. The aqueous phase, which contains all prophyrins, was approximatley 20 ml, and exact volumes were recorded.

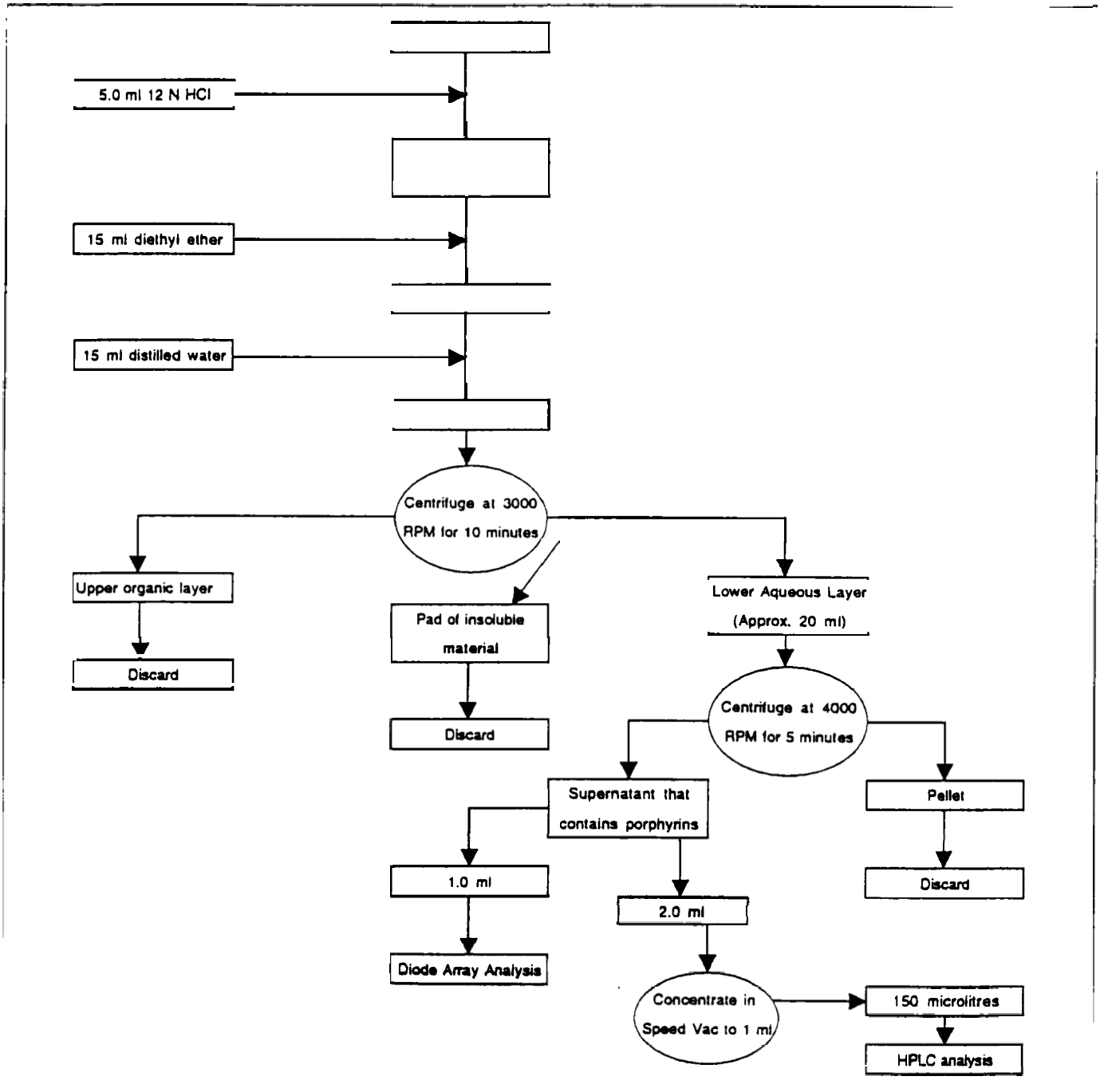

Figure 1. Porphyrin extraction procedure for river otter faeces.

Diode-array spectrophotometry

One millilitre of each faecal extraction was measured spectrophotometrically using a Perkin-Elmer diode-array spectrophotometer. Porphyrins have a characteristic absorbance in the Soret band, between 390 and 440 nm. The high noise created by the dark colour of aqueous phases complicates the spectra so the second derivative spectra (350–450 nm) were obtained for all samples and standards. The relative concentration of total porphryins was obtained by relating the trough depth (as measured from the baseline) of a standard porphyrin kit (Porphyrin Products, Logan, UT) to the trough depth of each sample. Porphyrin could be detected in every sample with 0.76 nmoles being the lowest level detected in the 201 samples analysed. The concentration of total porphyrins in each sample was calculated from the equation:

Total Porphyrins (nmole g^{-1} dry faeces) = TD × (6/std TD) × 20 ml\(DW × VU)

where: TD = trough depth of sample, measured from baselines; 6/st TD = trough depth of standard kit (6 nmol); DW = dry weight of sample initially used for extraction; VU = volume of sample used for diode array analysis.

HPLC analysis

Two millimetres of the initial aqueous phase were concentrated to approximately 1 ml using a SpeedVac concentrator. One-hundred fifty microlitres (ml) of each sample, which was selected abitrarily from the 201 extracted samples, was injected into a Waters HPLC system to determine porphyrin profiles. A Waters 441 UV detector with a 405 nm filter was used for sample analysis. A silica -C1 column with 5 μm packing was obtained from Phenomenex, Inc. (Torrance, CA). The gradient solvent system for the HPLC used was a modification of the procedure outlined by Lim and Peters (1984). Solvents for gradient elution were 10% (v/v) acetonitrile in 1 M ammonium acetate (solvent A) and 10% (v/v) acetonitrile in methanol (solvent B). All solvents were HPLC grade (Fischer Scientific, Inc.). Porphyrins were separated for 40 min with a linear gradient elution from 100% A to 100% B, followed by isocratic elution at 100% B for 20 min, then returning to 100% A over a 5 min period. The flow rate was 1 ml min^{-1} at room temperature. The detection limit for individual porphyrins was 1 nmol and the interassay variation was 7.83%.

Statistical analysis

We used a two-sample *t*-test to compare total porphyrins in the faeces (Zar 1984). A two-sample *Z*-test for proportions was used to compare selected porphyrins detected by HPLC (Remington and Schork 1970). Undetected samples were given a zero.

Results

There were 117 oiled and 84 non-oiled samples of otter faeces analysed by the diode-array spectroscopy (Figure 2). The total concentration of porphyrins in the oiled samples ranged from 48.7 to 119.2 nmol g^{-1}. The total concentration of porphyrins in the non-oiled samples ranged from 0.76 to 178.3 nmol g^{-1}. The mean±SE of total porphyrins for faeces of river otters living in oiled areas was 48.2±2.45 nmol g^{-1} dry faeces (n = 117), whereas the concentrations in faeces was lower for faeces of otters from non-oiled areas (34.5±3.42 nmol g^{-1} dry faeces); this difference was highly significant (t = 4.21, d.f. = 204, $P < 0.001$).

There were 31 oiled and 19 non-oiled samples randomly selected and analysed using HPLC. The individual porphyrins present in the samples were detectable by HPLC, but, because the values were at the limit of detectability, porphyrin profiles were scored for the presence of the individual porphyrins. In the oiled areas, the number of times uroporphyrin and

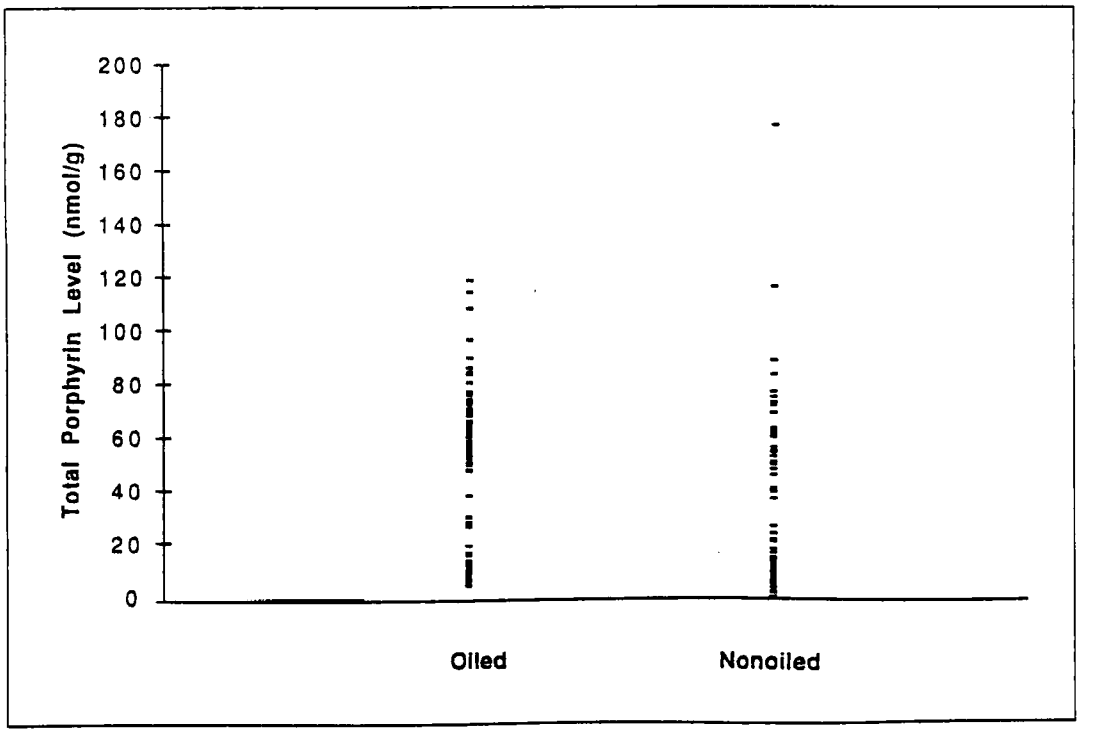

Figure 2. Comparison of total porphyrins in oiled and non-oiled otter faecal samples from Prince William Sound, Alaska. Mean porphyrin level; non-oiled samples: 34.5±3.24 nmol g^{-1} dry faeces, mean±SEM, n= 84; oiled samples: 48.2±2.45 nmol g^{-1} dry faeces, mean±SEM, n = 117. $P < 0.001$, *t*-test.

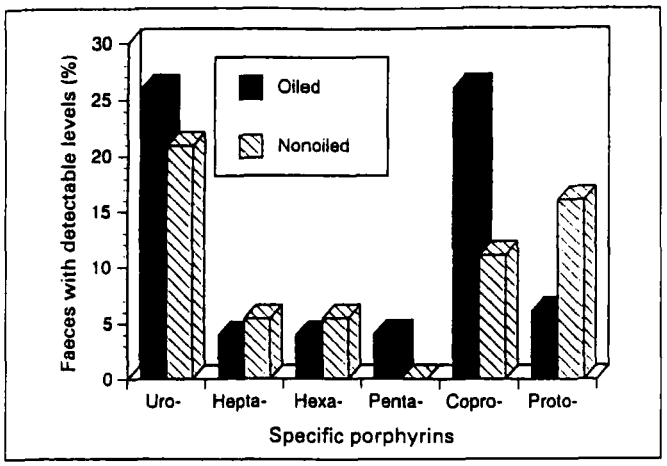

Figure 3. Comparison of the percentage of oiled and non-oiled faecal samples from Prince William Sound, Alaska which have detectable levels of individual porphyrins. $n = 31$ faeces from the oiled area, $n = 19$ faeces from the non-oiled area.

coproporphyrin could be detected were increased, whereas protoporphyrin detection was decreased compared with the non-oiled areas (Figure 3). Only the difference for the proportion of coproporphyrin, however, was significant ($Z = 1.97$, d.f. = 1, $P < 0.05$).

Discussion

Shorelines in Herring Bay received extensive oiling following the *Exxon Valdez* spill; 73% of shorelines showed evidence of some oil, and 26% were oiled heavily by August 1989 (Bowyer *et al.* 1995). River otters continued to inhabit oil areas in Herring Bay (Testa *et al.* 1994), and both their reliance on intertidal and subtidal habitats (Bowyer *et al.* 1994, 1995) as well as large home ranges (20–40 km of shoreline) indicated that otters were probably exposed to crude oil. The difference in total porphyrin concentrations in samples of otters faeces from oiled and non-oiled areas of Prince William Sound strongly suggests that there is an effect from the oil on the haem synthesis of river otters. The HPLC analysis also shows qualitative differences in porphyrin profiles of otter faeces between the oiled and non-oiled ares. Coproporphyrins appeared to be mostly responsible for differences observed in total porphyrins.

We believe the faecal-porphyrin profile could reflect the effect of oil on the hepatogastric system. Normally, porphryins other than protoporphyrin are formed in small amounts and the pathway of haem synthesis is carefully controlled (Kappas 1987). When the regulatory process is disturbed, however, a variety of other porphyrins and porphyrin precursors accumulate (i.e. piling up; Marks 1985). In animals, these porphyrins and precursors are usually formed in the liver and excreted in the urine and faeces. In our study, faeces in the oiled area showed a higher percentage having coprophyrin present than those in the non-oiled area, while there was a relative but non-significant decrease in the percentage of faeces showing protoporphyrin from the non-oiled area (Figure 3). Coproporphyrinogen oxygenase is localized in the mitochondrion and there is a close relationship between mitochondrial structure and biochemical functionality (Hackenbrock 1972, Fowler *et al.* 1987). Because the disruption of liver mitochondrial respiration and any change in the normal flow of electrons will influence the activity of this enzyme, the piling up and subsequent presence of coproporphrin in a greater percentage of faeces of river otters living in oiled areas support the previous findings that indicated liver damage in animals living in oiled areas (Duffy *et al.* 1994a).

Biochemical markers provide a link between the inferences of exposure assessments and the extrapolation of future risk (Silbergeld, 1987, 1995). Haem synthesis responds to genetic, nutritional and chemical factors in addition to being influenced by feedback mechanisms. For instance, we know the diet of river otters differed between oiled and non-oiled areas in 1990, with otters from oiled areas consuming more perciformes (fish) and otters from non-oiled areas eating more achaeogastropda (gastropods) and malacostraca (crabs) (Bowyer *et al.* 1994). Although we noted a difference between the total means of porphyrins in faeces collected from oiled and non-oiled areas, HPLC profiling or fingerprinting appears to be a more distinctive biomarker for comparison of river otters faces from oiled and non-oiled areas. Further studies are needed on animals in Prince William Sound to evaluate the specificity and sensitivity of porphyrin profiles as a useful biomarker in low-level chronic chemical exposures. More research is also needed to determine whether exposure to oil directly affects porphyrin production (as opposed to a pollution-mediated dietary shift) and further investigation into temporal and spatial variation in faecal porphyrin levels are needed to validate the use of faecal porphyrin as a biomarker.

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