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Estimation of Hemosiderosis in Seabirds and Fish Exposed to Petroleum

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Many seabirds die each year after exposure to petroleum hydrocarbons (Piatt and Lensink 1990; Khan and Ryan 1991). Crude oil, following ingestion, induces hemolytic anemia which occurs 4 to 5 d later (Leighton et al. 1983). A recent study reported the presence of hemosiderin in the liver of common murres (Uria aalge) at intervals up to 42 d after oil-contamination (Khan and Ryan 1991). Hemosiderosis has also been reported in plaice, Pleuronectes platessa, following the Amoco Cadiz oil spill off the coast of France (Haensly et al. 1982) and after experimental exposure of Atlantic cod, Gadus morhua, and longhorn sculpin, Myoxocephalus octodecemspinosus, to petroleum (Khan and Kiceniuk 1984; Khan 1991).

Hemosiderosis is an abnormality characterized by excessive deposition of a yellow-brown pigment, hemosiderin, in the tissues of vertebrate animals. It results after excessive destruction of erythrocytes following hemorrhage from trauma, chronic congestion, hemolytic disorders, parasitic infections, and exposure to some toxic chemicals. The pigment is usually located in Kupffer cells of the liver, reticulo-endothelial cells of organs and tissues and less often in parenchymal cells in birds. It can be satisfactorily demonstrated by Perl's Prussian blue staining method, a monospecific test that distinguishes it from hemoglobin, bile pigments (i.e., bilirubin, hematoidin) malarial pigments and porphyrins (Drury et al. 1967).
Hemosiderin, in tissues, may be concentrated in discrete areas, i.e., melanomacrophage centers in fish or it may be distributed in a diffuse manner as in most other vertebrates. Estimation in fish by counts per unit area in tissue sections has been used previously (Haensly et al. 1982) but this method is inaccurate when the centers vary in shape and size or the pigment is distributed diffusely. A recent study reported the use of image analysis to quantify hemosiderin in the spleen of rats after treatment with chlorotoluran (Nyska et al. 1989). This technique was used in the present study to ascertain the extent of hemosiderosis in tissues of birds and fish exposed to petroleum.

MATERIALS AND METHODS

Tissues were obtained from birds and fish to ascertin the extent of hemosiderosis.

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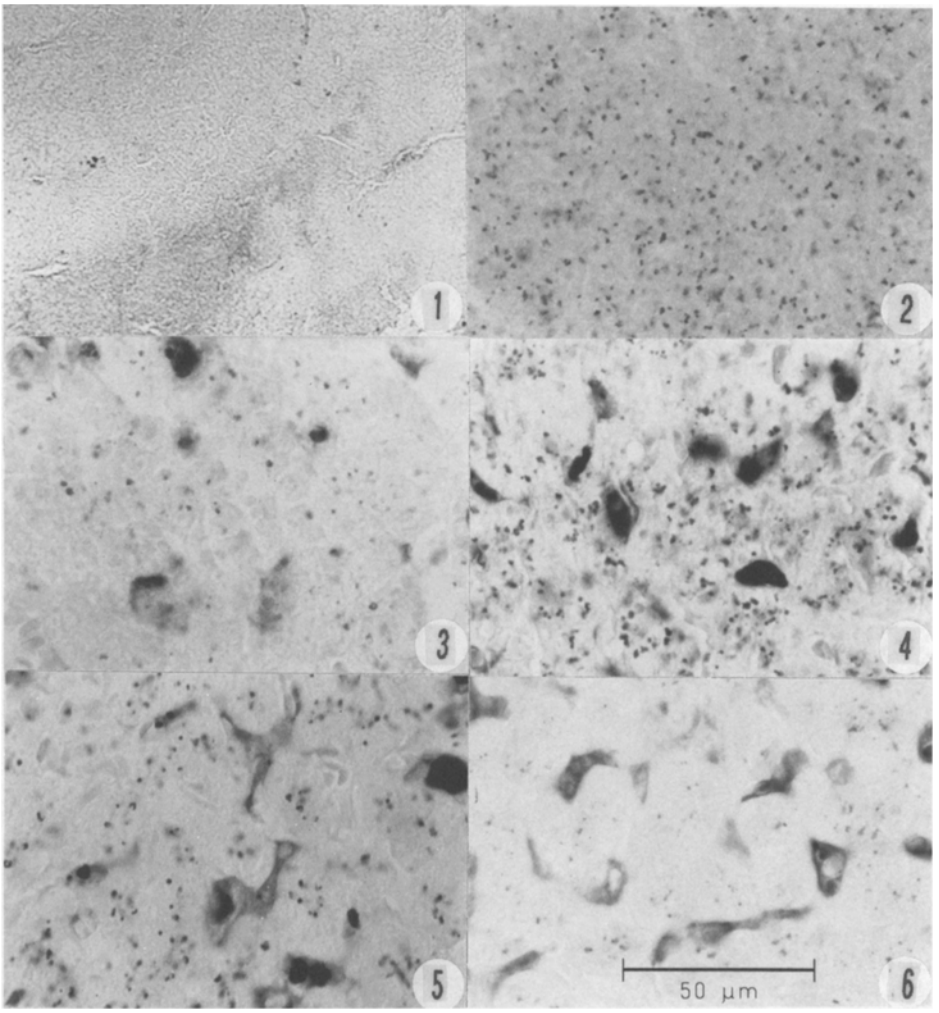
Common murres, which had been recovered in Newfoundland after contamination with crude oil, were rehabilitated for periods up to 42 d (Khan and Ryan 1991). A number of additional birds, including thick-billed murres (U. lomvia) and an oldsquaw (Clangula hyemalis) were also examined. Carcasses of four common murres that had been collected in February 1990 from the seashore were kept frozen until examination. At autopsy, the liver of each bird was fixed in 10% buffered formalin, processed by conventional histological methods reference and sections, 6μm in thickness, stained with Perl's Prussian blue (Drury et al 1967). Spleens from mature longhorn sculpins (26-42cm in length), exposed to oil-contaminated sediment from 3 to 12 mon and controls, were also processed and stained as mentioned previously. Spleens were also removed from mature yellowfin sole (Limanda aspersa, 27-36cm), quillback rockfish (Sebastes maliger, 40-48cm), and kelp greenling (Hexagrammos decagrammus, 41-52cm), and fixed shortly after capture by hook and line from depths of 60-100 m in July 1990 in Wildcat Cove, Alaska. This area had received a considerable amount of crude oil following the Exxon Valdez oil spill in March 1989 (Khan 1990). Reference fish of comparable sizes were captured by hook and line at similar depths 3 d later at Seward, Alaska.

Estimation of hemosiderin in tissues of birds and fish was accomplished by image analysis after a method of Nag et al. (1990). Image processing and analysis is controlled by a computer (PC3865X) with menu driven software (JAVA, Jandel Scientific) connected to an image capture board (TARGA-M8), a digital VHS video cassette recorder (JVC, HR-D700V) and a monochrome monitor. Spatial resolution of the image-capture board is up to 512 by 482 pixels (Nag et al. 1990). Images originating from stained slides were visualized by a Zeiss photomicroscope, captured and stored through the TARGA board and recorded on a video tape (VHS). Stored images were then retrieved and image analysis performed (Nag et al. 1991). This method can measure areas of irregular shapes by enumerating the total number of pixels in the objects and their location coordinates in space. The area scanned varied from 0.96 x 0.81 to 1.02 x 0.88 mm. The estimated area with hemosiderin was expressed as a percentage of the total area examined. Means and standard errors were calculated for the different groups. A one-way ANOVA was used to compare oil-treated with control/reference animals.

RESULTS AND DISCUSSION

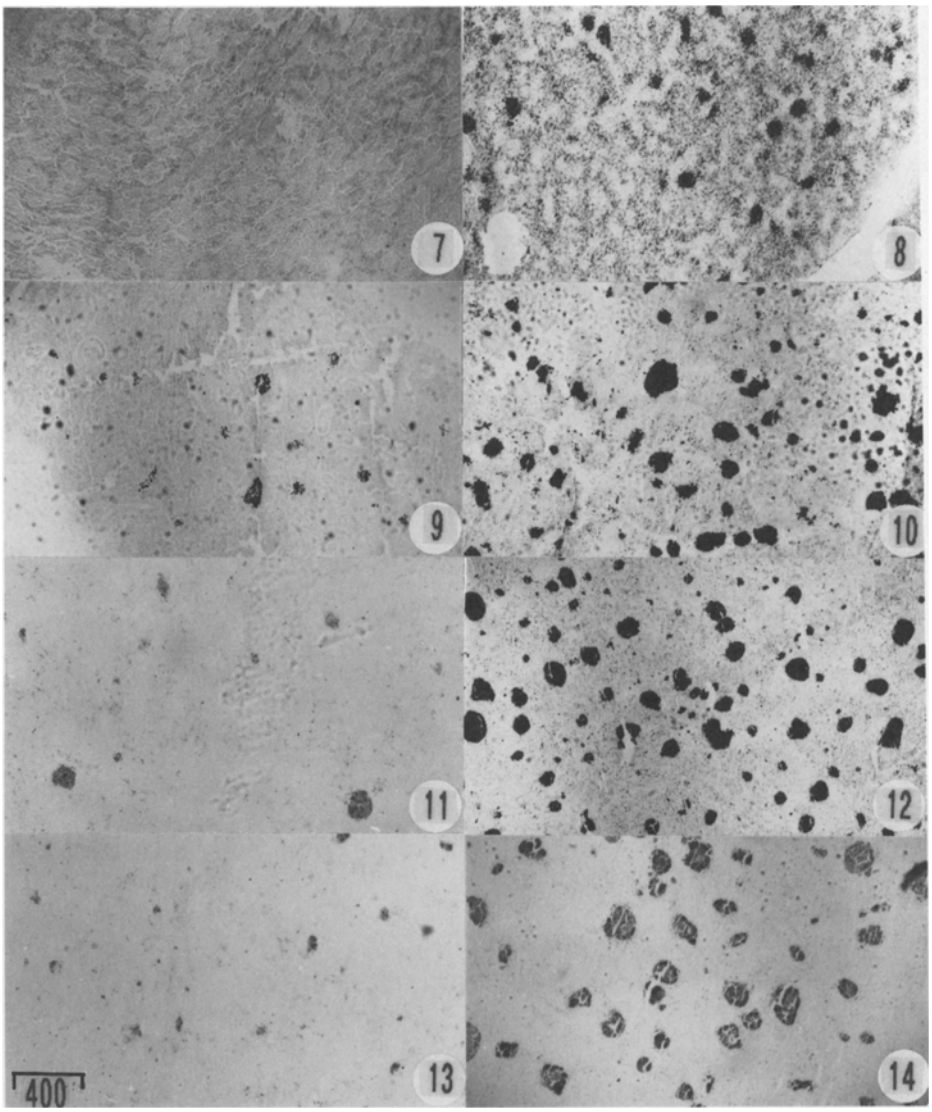
The amount of hemosiderin in the liver of common murres varied according to the length of time when seabirds became contaminated with crude oil. Its concentration was negligible in uncontaminated birds (Fig. 1, Table I). However, it increased in common murres from 0-18 d after retrieval and rehabilitation (Figs. 2-3) reaching a maximum of 21% of the liver at 26-30 d and decreasing thereafter (Figs. 4-6). The level present in live contaminated birds that were collected from oiled seashores was also variable (Table 1). These values, ($\bar{x}$ 4.1 $\pm$ 0.2 to 26.1%), were also greater in 10 oil-contaminated common murres than



Figures 1-6. Liver sections from common murres stained with Perl's Prussian blue. Note absence of hemosiderin pigment in control murre (fig.1) and its presence in others, naturally contaminated with crude petroleum shortly after capture (fig. 2) and at 4,15,27 and 36 days after rehabilitation (figs. 3-6 respectively).

in corresponding uncontaminated specimens. Similar results ($\bar{x}$, 5.3 $\pm$ 0.3% hemosiderin) were obtained from the livers of 4 common murres that were held frozen until examination. Generally, hemosiderosis appeared to be greater in birds that were heavily oiled and emaciated than in lightly oiled birds that showed evidence of slight weight loss.

Hemosiderosis was more pronounced in the spleen of fish exposed to crude oil. It was significantly greater in the longhorn sculpin experimentally exposed for



Figures 7-8. Spleen sections from control (fig. 7) and oil-contaminated longhorn sculpins at 3 mon (fig. 8) after exposure.
Figures 9,11 and 13. Spleen sections from reference yellowfin flounder, rock greenling and quillback rockfish collected at Seward, Alaska. Note low concentration of pigment. Figures 10,12 and 14. Spleen sections from the same fish species taken from oil-contaminated Wildcat Cove, the Pye Islands, Alaska about 16 mon after the Exxon Valdez spill. Scale bar in μm.

periods from 3 mon to greater than 1 yr than in corresponding control fish (Figs. 7-8, Table 2). No differences were apparent in fish exposed between periods of 3 to 12 months. In the three species of benthic/demersal fish examined after

Table 1.Hemosiderin concentration (% of area, $\bar{x} \pm$ SE) in liver sections of seabirds at various intervals (days) after exposure to petroleum and rehabilitation.

Bird Species	n	Exposure period (days)	% Hemosiderin
Common murre (uncontaminated)	10	0	0.4 ± 0.1
Common murre	5	3-10	2.0 ± 0.2
Common murre	2	12-18	10.1 ± 0.5*
Common murre	2	26-30	21.4 ± 1.1*
Common murre	3	36	13.2 ± 0.7*
Common murre	2	42	7.3 ± 0.4*
Old squaw Φ	1	?	6.1
Common murre Φ	10	?	4.1 ± 0.2*
Thick-billed murre Φ	1	?	26.1

* significantly different ($P \leq 0.01$) from uncontaminated birds.
Φ oiled but not rehabilitated

capture in Alaska, values of 11 to 14% were significantly greater than those in the reference groups (Figs. 9-14, Table 2).

Results from the present study confirm that image analysis can be used for estimating hemosiderosis in tissues. Additionally, the presence of hemosiderin in tissues appears to be a useful indicator of hemorrhage/anemia following exposure of animals to crude oil and, to some extent, the severity of the exposure. This was apparent in common murres from 4 d after retrieval and in groundfish that inhabited an area that became contaminated more than 1 yr after the Exxon Valdez oil spill. Since hemosiderin in tissues of fish tends to be transient, degenerating 5 d after their appearance (Herraez and Zapata 1986), then the high levels recorded in demersal/sedentary species from Alaska suggests continuous exposure to PAH which presumably still persists in bottom sediment. In view of these findings, this technique can be used as an additional tool to determine whether or not birds/fish have been exposed to hydrocarbons, especially near drilling rigs and oil refineries when there is little or no evidence of additional contaminants. This approach also circumvents the difficulty encountered with tissues of fish in estimating the extent of hemosiderosis especially when the melanomacrophage centers are of irregular shapes and sizes. It would be impossible at this time to compare the usefulness of hemosiderin determination by image analysis to other monospecific methods for assessing exposure to crude oil such as tissue levels of petroleum aromatic hydrocarbons (PAH), assay of bile metabolites and liver aryl hydrocarbon hydroxylase (AHH). All of these require liquid nitrogen for rapid freezing of tissue/fluid samples which is not always available in the field. Additionally, AHH activity has

Table 2.Hemosiderin concentration (% of area, $\bar{x} \pm$ SE) in spleen sections of fish (n = 5/group) after exposure (in months) to petroleum.

Fish Species	Exposure period (mon)	% Hemosiderin in fish groups Control/ reference	Oil-exposed
Longhorn sculpin	3	1.0 ± 0.10	8.8 ± 0.3*
Longhorn sculpin	6	0.9 ± 0.07	8.6 ± 0.4*
Longhorn sculpin	12	0.8 ± 0.06	9.8 ± 0.6*
Yellowfin sole	>12ξ	0.7 ± 0.04	12.9 ± 0.5*
Quillback rockfish	>12ξ	0.5 ± 0.02	11.6 ± 0.4*
Kelp greenling	>12ξ	0.5 ± 0.04	13.5 ± 0.7*

* significantly different ($P < 0.01$) from controls/reference group
ξ samples from Alaska

been reported to be affected during gonadal maturation and spawning (Walton et al. 1983). Our method requires a less-demanding protocol for tissue preservation especially in field studies and is not affected by an animal's reproductive cycle. Moreover, it could aid in ascertaining the cause of death of seabirds held frozen, after major oil spills i.e. Exxon Valdez (vide Piatt and Lensink 1990).

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