LIVER HISTOPATHOLOGY AND PAH BILE METABOLITES FOUND IN ENGLISH SOLE (*PLEURONECTES VETULUS*) COLLECTED FROM AN AREA ASSOCIATED WITH ALUMINUM SMELTING

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Abstract

Bioindicators of sublethal effects associated with aluminum smelting activities were examined in the bottom-feeding flatfish, English sole (*Pleuronectes vetulus*). Histopathological analysis of liver samples from fish collected near the aluminum smelter revealed neoplastic lesions. The liver tissues from this site also exhibited a pathological iron storage disorder, hepatocellular hemosiderosis. Synchronous scan fluorescence spectrometry was used to measure pyrenol conjugate concentrations in bile. The PAH bile metabolite concentrations reflected sedimentary PAH levels and were associated with the adverse histopathological conditions. The presence of pyrenol conjugates indicates biological activation of PAHs that are known to cause cancerous lesions. Another contributing factor towards neoplasia may arise from oxidative stress and DNA damage resulting from reactive oxygen species. These oxyradicals may have been generated from the Fenton reaction catalysed by excess iron. Additional research is underway to determine the nature of the above linkages and their roles in carcinogenesis.

Introduction

Marine environments near industrial and urban centers are contaminated with a wide range of chemicals that may be transformed, either biologically or chemically, into new potentially toxic compounds (Malins and Haimonot, 1981). Research has demonstrated a positive link (by association) between the presence of certain xenobiotic chemicals in sediments, seawater or food organisms and the onset of sublethal effects in demersal (resident) fish species. Sublethal toxicity tests may make it possible to detect incipient effects on fish and to estimate threshold concentrations. However, no single approach to the problem of biological effects monitoring can be fully satisfactory. Some methods seem more useful than others, but greatest insights have usually been obtained by multidisciplinary efforts, often those in which pathology has been allied with biochemistry, then augmented by chemical analyses of tissue and environmental samples (Adams et al., 1996).

This report examines a suite of selected stress responses on English sole (*Pleuronectes vetulus*) residing in area effected by aluminum smelting activity. A toxicological approach for early sublethal stress effects on this flatfish species was examined using the following levels of biological organization: histopathological, by diagnosis of fish liver for idiopathic lesions; histochemical, using Perls' method (Prussian blue) for ferric iron to demonstrate hemosiderosis: and bile metabolites, measured as 1-pyrenyl glucuronide (1-pyrenyl-β-D-glucopyranosiduronic acid) equivalents using synchronous scan fluorescence spectrometry (SFS).

Materials and Methods

Field collection

English sole, *Pleuronectes vetulus*, were collected on board the C.C.G.S. Vector using a beam trawl from Kitimat Harbor, the Kitamaat Village and the Kildala, areas, located on the north coast of British Columbia, Canada, in 1994 to 1997. Only female English sole greater than 26 cm and males greater than 24 cm were sampled to insure that all fish were 4 years of age or older (Garrison and Miller 1982, Harry 1959). Five to thirty English sole from each of the sampled populations were sacrificed, measured for condition factors, liver tissue excised and fixed in Dietrich's fixative for histopathology. Bile was collected and stored at -80^{o}C for measurement of polycyclic aromatic hydrocarbons (PAH) conjugated metabolites. The otoliths were removed and stored in glycerin/ethanol for subsequent determination of age (Chilton and Beamish 1982).

Liver histopathology

Fixed liver tissues were washed in water, dehydrated in an ethanol series, cleared in toluene and embedded in paraffin (Paraplast). Skip serial sections were cut at 5 µm, mounted on slides and stained with Gill's haematoxylin and eosin (Humason 1979) for general histology. Liver tissue was also stained with Perls' method (Prussian blue) for ferric iron (Pearce 1972). The slides produced were scored blind (without information of the particulars of the collection site), and liver abnormalities were classified using nomenclature consistent with the system of Myers et al., (1987).

PAH conjugates in bile

PAH metabolite conjugates in bile were measured by synchronous-scan fluorescence spectrometry (SFS) at Simon Fraser University following the method of Ariese et al., (1993). The samples of bile were diluted 1/500 with a 1:1 HPLC grade ethanol and HPLC water solution (Fisher Scientific Ltd.). The fluorescence response was measured on a Perkin Elmer Luminescence spectrophotometer LS-50 with FL data manager operating on an IBM-PC compatible computer. Both excitation and emission monochromators were scanned synchronously with a fixed wavelength difference of 37 nm. The area of the fluorescence emission response was measured from 335-356 nm on the emission monochromator. A six point linear external calibration curve of 1-hydroxypyrene was used to calibrate the fluorescence spectrophotometer. The fluorescent emission of the 1-hydroxypyrene standards was measured from 340-361 nm. A conversion factor of 2.2 was applied to account for the difference in fluorescence yield between 1-hydroxypyrene and its conjugate 1-pyrenyl glucuronide, which is the major metabolite conjugate of pyrene in the bile. Protein analyses of fish bile were done by the method of Lowry et al., (1951) using a Bausch and Lomb Spectronic 20 spectrometer.

Results

Catch size (or sample size) for English sole (*Pleuronectes vetulus*) were all well below optimum at each site due to limited availability and ship time. For both 1994 and 1997, catch sex ratios were not equal or consistent. Fish age differed between the collection sites and year, with a general trend of younger fish caught in 1994 compared to 1997 and an overall age range of 3 to 10 years (Table 1).

Table 1: Catch data and liver lesion prevalence for English sole (*Pleuronectes vetulus*) collected in 1994 and 1997 from areas influenced by aluminum smelting activity, Kitimat Harbor and Kitamaat Village, and a reference site, Kildala Arm.

Sample Site	Sample Size and Sex	Age (years) mean ± s.d.	idiopathic liver lesion (%)	hepatocellular hemosidersosis (%)
Kildala 1994	5 males 10 females	4 ± 1	7.2	33
Kildala 1997	1 male 4 females	7 ± 3	0	40
Kitimat 1994 Harbor	15 males 4 females	4 ± 1	21	87
Kitimat 1997 Harbor	1 male 9 females	6 ± 2	30	70
Kitamaat 1994 Village	11 males 19 females	5 ± 2	30	79
Kitamaat 1997 Village	4 males 6 females	7 ± 3	30	70

Liver histopathology

English sole with normal liver tissue were found at all sampling sites. Parasites were present in liver samples collected from all the study sites examined. The most common was the plasmodial form of the myxosporean genus Myxidium (Butschli), family Myxidiidae (Thelohan), found within the bile ducts and ductules. The helminths, acanthocephala or nematoda, were occasionally present either externally or internally. Associated with these infections were inflammatory responses, resulting in the formation of granulomas.

Five types of idiopathic lesions were observed in fish collected from the vicinity of the aluminum smelter. These lesions were: nonspecific necrotic lesions; two types of intracytoplasmic storage disorders, hepatocellular hemosiderosis (Fig. 1) and variable hydropic vacuolation; nuclear pleomorphism and megalocytic hepatosis (Fig. 2); foci of cellular alteration, clear cell, eosinophilic and basophilic foci (Fig. 3); and the neoplasms, liver cell adenomas and hepatocellular carcinomas (Fig. 4).

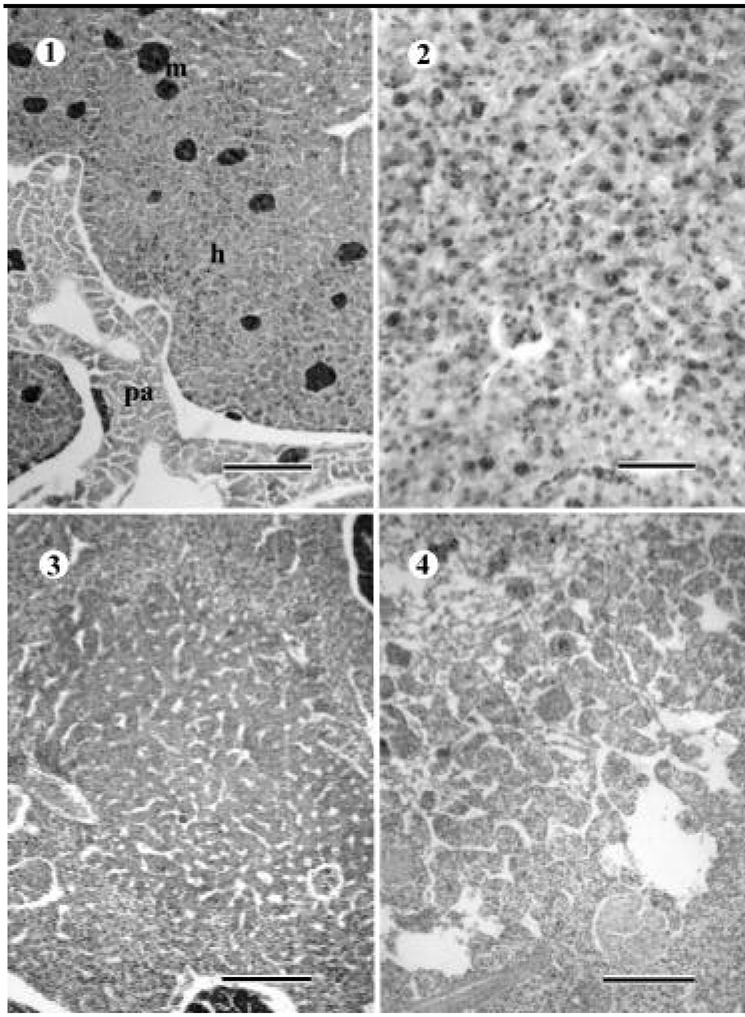

Fig. 1: Hepatocellular hemosiderosis shown as dark pigments (h) within the hepatocytes. m = melanomacrophages, pa = pancreatic acini. Perls' iron. Bar = 100 µm.
Fig. 2: Megalocydtic hepatosis. Note the enlarged nuclei scattered throughout the parenchyma. H&E. Bar = 50 µm.
Fig. 3: Basophilic foci. Note the discrete nodular center with minimal compression of the surrounding parenchyma. H&E. Bar = 100 µm.
Fig. 4: Hepatocellular carcinoma. Note the atypical cellular morphology with significant compression of the adjacent tissue. H&E. Bar = 100 µm.

In 1994, idiopathic liver lesions were observed in English sole collected from all three sites. Prevalences of lesions, however, were found to be higher in fish collected from the Kitamaat Village (30%) compared to Kitimat Harbor (21%) and Kildala Arm (7.2%) (Table 1). In 1997, both the Kitimat Harbor and Kitamaat Village population revealed a 30% liver lesion incidence, whereas all reference population liver samples were normal. When samples were tested for the iron storage disorder, hepatocellular hemosiderosis, percent differences between the reference and study populations were observed. In 1994, 79 and 87% of the Kitamaat Village and Kitimat Harbor population exhibited this condition compared to 33% of the Kildala population. The 1997 results were similar, with 70% of the fish from Kitimat Harbor and Kitmaat Village affected compared to 40% from Kildala Arm.

PAH conjugates in bile

Lowest levels of pyrenol conjugate concentrations in English sole bile were found in fish collected from the Kildala site, ranging from 13.9 $\pm$ 2.8 to 15.8 $\pm$ 5.4 µg 1-pyrenyl glucuronide/mg bile protein for samples collected in 1994 and 1997 respectively. These values were found to be on or slightly below detection limits. Higher levels were observed in the fish bile samples obtained from the Kitimat Harbor and Kitamaat Village ranging from 38.1 $\pm$ 18.7 to 210.8 $\pm$ 164.4 µg 1-pyrenyl glucuronide/mg bile protein, depending on the site and year of collection. In general, bile pyrenol conjugate concentrations showed a constant and low trend for the fish collected from the reference site, whereas fish collected near and adjacent to the aluminum smelter, particularly Kitamaat Village populations, showed high, but declining levels (Table 2).

Table 2: PAH metabolite conjugates in English sole (*Pleuronectes vetulus*) bile expressed as µg 1-pyrenyl glucuronide equivalents / mg protein, measured by synchronous-scan fluorescence spectrometry (SFS) (Ariese et al., 1993), for fish collected near an aluminum smelter (Kitimat Harbor and Kitamaat Village) and a reference site (Kildala) in 1994 and 1997.

Sample site	µg 1-pyrenyl glucuronide equivalents / mg protein*	
	1994	1997
Kildala	13.9 ± 11.1 (15)	15.8 ± 5.4 (5)
Kitimat Harbor	54.9 ± 19.9 (19)	39.5 ± 16.9 (10)
Kitamaat Village	210.8 ± 164.4 (30)	38.1 ± 18.7 (10)

*values expressed as averages ± standard deviation (n=sample size)

Discussion

English sole collected from Kitimat Harbor and Kitamaat Village had higher incidences of preneoplastic and neoplastic liver lesions compared to sole collected from the Kildala. Studies of flatfish populations from non-urban reference sites in Puget Sound, Washington have shown no evidence of idiopathic liver lesions (Tetra Tech, 1987). In contrast, studies of wild fish populations from polluted environments have shown a higher prevalence of hepatic lesions (Harshbarger and Clark 1990). For a number of areas in Puget Sound (eg. Eagle Harbor and Duwamish Waterway) (Malins et al., 1984 and 1988) and Vancouver Harbor (Goyette et al., 1988) a positive correlation has been shown between sediment-associated polycyclic aromatic hydrocarbons (PAHs) and prevalences of several categories of idiopathic liver lesions in English sole. Similar to the these studies, prevalences of English sole with idiopathic liver lesions appear to be dependent on location of capture with high prevalences (~30%) of preneoplastic and neoplastic lesions found in English sole collected from both Kitimat Harbor and Kitamaat Village. A possible positive correlation may also exist with the observed PAH metabolite levels found in their bile samples and the observed incidences of liver lesions. Only preneoplastic (megalocytic hepatosis and all foci of cellular alterations) and neoplastic (liver cell adenomas and carcinomas) lesions were considered in calculating these frequencies. Other idiopathic lesions were omitted due to the uncertainty in their relationship to exposure to xenobiotic, carcinogenic chemicals.

The intercytoplasmic storage disorder, hepatocellular hemosiderosis, was prevalent in a high percentage of sole collected from Kitimat Harbor and Kitamaat Village compared to those collected from the reference site, Kildala. Hemosiderosis suggests an underlying metabolic disorder characterized by excessive accumulation of intracytoplasmic iron within the hepatocytes (Myers et al., 1987). When this condition is experimentally induced in rats the frequency of developing hepatocellular carcinomas with exposure to xenobiotics increases. DNA damage and mutagenesis brought about by iron are likely to occur by a Fenton-type mechanism that involves the generation of (i) hydrogen peroxide by the autooxidation of iron and (ii) hydroxyl radicals by the interaction of hydrogen peroxide with iron (Loeb et al., 1988). Recently Payne et al., (1998) has reported levels of DNA oxidative damage in trout collected from lakes receiving effluents from iron-ore mines and these authors suspect that the high environmental iron loading may catalyze reactions generating free radicals and DNA damage.

Recent studies in the Kitimat marine environment have attempted to establish a link between environmental concentration of contaminants and biological effects. The surprising result so far has been that the predicted acute toxicities of the sediments, based on their PAH concentrations, have not been observed in sensitive short term bioassays and benthic infaunal surveys. It has been proposed that the PAHs, which are produced predominantly by the aluminum smelter, are not readily bioavailable (Paine et al., 1996, Naes et al., 1998). The PAHs appear to be associated with soot particles released from the electrodes in the smelter (Naes et al., 1998, Cretney, unpublished). The partitioning of PAHs from soot particles to the water may be up to a 1000-fold less that that from natural organic carbon (Gustaffson et al., 1997). Our research has revealed PAH metabolites in bile from English sole. Thus, PAHs are bioavailable in Kitimat Arm, though at concentrations much less than would be expected from their concentration in the sediments (Paine et al., 1996). More study is required to determine whether the main source of the PAHs metabolites in the bile is soot carbon from the smelter or other sources, such as petroleum from marine traffic.

Conclusion

Due to the environmental importance of epizootics of neoplasia in benthic animals and their apparent association with mammalian carcinogens, considerable interest has arisen in studying genotoxic and carcinogenic responses in these organisms. There are numerous examples of hotspots, where tumour incidences in fish have been correlated with raised concentrations of anthropogenic chemicals, but causal mechanisms are seldom established. This study, plus other available information from recent research in aquatic and medical toxicology, suggests that responses associated with xenobiotic metabolism, oxidative stress, and DNA damage may be mechanistically linked. Additional research is currently underway to determine the nature of these linkages, their roles in carcinogenesis, and the causative factors for the associated responses in aquatic organisms exposed to sediments of complex natures. Results from this study may also provide sufficient information towards the potential use of marine organisms as sentinels, that can provide early warning signals of potential threats to man.

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