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Effect of Sediment from Two Sulphite-Bleaching Paper Mills, on Winter Flounder *(Pleuronectes americanus)* Following Chronic Exposure

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Effluent originating from pulp and paper mills have been reported to induce a variety of physiological changes in fish (Lindstrom-Seppa and Oikari 1990). Some of these culminate in impairment of growth and reproduction and ultimately in the disappearance of some important species such as salmonids and replacement by hardy fish, i.e., cyprinids (Neuman and Karas, 1988; Oikari and Kukkonen 1988; Munkittrick et al. 1991). Acute and chronic tests using dilutions of the effluent, as well as exposure of caged fish to the discharge, have verified these observations (Owens 1991). Other studies report that resin acids in the effluent, especially dehydroabietic acid, produced during thermomechanical pulping, might be responsible for the sublethal effects (Räbergh et al. 1992). Additionally, exposure of hatchery-reared rainbow trout *(Oncorhynchus mykiss)* to sediment taken from a site near a pulp mill discharging unbleached sediment resulted in elevation of monooxygenase activity (Otto et al. 1994).

Studies conducted on two benthic marine fish species living near two sulphite-bleaching paper mills in Newfoundland reported blood, tissue and reproductive anomalies in addition to changes in body condition and organismic indices compared to samples taken at reference sites (Khan et al. 1994a, 1996; Barker et al. 1994 a,b). Winter flounder *(Pleuronectes americanus)* showed evidence of external and tissue lesions, abnormal parasitic infections, anemia, lymphopenia and delayed spawning (Barker et al. 1994a). Another study has reported elevation of hepatic detoxifying enzyme ethoxyresorufin-O-deethylase (EROD) levels in liver was associated with a depression of muscle acetylcholinesterase values in winter flounder captured near one of the above-mentioned paper mills (Payne et al. 1996). Longhorn *(Myoxocephalus octodecemspinosus)* and shorthorn sculpins *(M. scorpius)* were less affected, but external and internal tissue lesions were apparent (Barker et al. 1994b). Sculpins exposed to sediment originating near one of the paper mills for periods of 5 to 13 mon harbored elevated levels of ciliated parasites that were attached to the gills which showed extensive histological alterations (Khan et

al. 1994b). The purpose of the present study is to provide further evidence of these changes in winter flounder following exposure to sediment originating from the same two sulphite-bleaching paper mills in Newfoundland.

MATERIALS AND METHODS

Sediment was collected at each site (depth 4-10m) by SCUBA divers within ~ 10cm of the benthic surface, placed in four 22 L buckets sealed in garbage bags and held after return to the laboratory at ~ O°C. Sediment from the first paper mill at Port Harmon (48°31'N, 58°33'W) was obtained about one km from the outfall and consisted of muddy sand with considerable quantities of bark, lignin and fiber and an odor of hydrogen sulphide in contrast to clear, odorless sand devoid of organic debris at St. George's located about 25 km up-current (Khan et al. 1994a; Barker et al. 1994a). Although the effluent from the mill is currently stored and aerated in holding ponds before discharge which occurs outside the inlet at Port Harmon, there is continual seepage and overflow into it. Sediment from the second paper mill at Corner Brook (49°32'N, 57°52'W) was also obtained at the benthic surface at Birchy Cove, about 2 km down-current where it consisted of a hydrogen sulphide-laden sludge with bark, wood chips and fiber in contrast to clear, odorless sand at the reference site, Meadows, situated about 12 km up-current (Khan et al. 1996). However, traces of bark, fiber and wood chips were also observed at the latter site. Both paper mills use mainly black spruce *(Picea mariana)* and balsam fir *(Abies balsamea)* and as a result of wet debarking in previous years, a considerable amount of wood derivatives have been deposited in both inlets. Analysis of the effluent from both mills revealed fluctuating levels of tannin and resin acids, especially dehydroabietic acid (Environment Canada, unpublished data). While no other industrial discharges occur at the inlet at Port Harmon, the Humber Arm, where the second paper mill is located, receives both industrial and urban effluent, especially untreated sewage from several communities nearby (Khan et al. 1996).

Adult male winter flounder (length 23-37 cm, weight 155-526 g) were captured by SCUBA divers in Conception Bay, Newfoundland (47°39'N, 52°55'W) in an area where no industrial or urban effluent occur. The fish were held, prior to exposure, in a flow-through sea water aquarium (300 L) and fed chopped capelin *(Mallotus villosus)* two to three times weekly for 4-12 weeks. The fish, devoid of external lesions or parasites, were weighed, measured and placed subsequently in flow-through aquaria (300L) into which sediment (about 10 cm in depth) from either the impacted or reference site had been introduced previously. The exposure period, which varied from either 6 or 12 mon, began in autumn to simulate the time when winter flounder cease feeding and submerge themselves in sediment until spring (Van Guelpen and Davis 1979). Ambient water temperature varied from 0-14°C as areas adjacent to the island are under the influence of the cold Labrador Current (Drinkwater 1994). A record was made of the time and location of external

Table 1. Comparison of variables in male winter flounder (P. americanus) following exposure to sediment from a sulphite-bleaching paper mill at Port Harmon (PH) and a reference site, St. George's (SG), Newfoundland for either 6 or 12 mon.

Variable	Fish groups			
Exposure period (mon)	6		12	
Collection site	PH	SG	PH	SG
No. Exposed (% mortality)	23 (52)	23 (16)*	24 (54)	24 (8)*
External lesions (%)[+]	64	5*	91	4*
Length (cm)	28±0.6	29±0.2	29±0.3	31±0.2
Weight (g)	224±19	304±28*	202±14	411±22*
K-factor ($x10^{-3}$)	0.98±0.03	1.12±0.02*	0.80±0.02	1.00±0.03*
Hepatic s.i. ($x10^{-2}$)	1.13±0.03	0.98±0.01*	1.98±0.04	1.38±.04
Gonad s.i. ($x10^{-2}$)	4.5±0.4	4.7±0.3	3.4±0.4	3.1±0.5
Gill hyperplasia (%)	100	8	100	0
Pericholangiolar fibrosis (liver %)	100	0	100	0
Splenic hemosiderin (%/mm^2)	16	1*	27	3*

[+]Based on surviving fish.
[*]Significantly ($P<0.05$) different from PH group.

lesions on the body of flounder. Fish which succumbed during the experimental period were autopsied. Following the completion of each trial, the length, eviscerated weight and weight of the liver and gonad of the fish were determined subsequently. Tissues including gill, liver, kidney and spleen were fixed in 10% buffered pH 7.6) formalin and processed by conventional methods for histological examination after staining with hematoxylin and eosin and/or periodic acid schiff (PAS) for mucopolysaccharides in the gills. Sections of spleen and kidney were stained also with Perl's Prussian blue for hemosiderin deposits which were estimated by digital image analysis and expressed as a percentage of the area scanned (Khan and Nag 1993). EROD was assayed fluorometrically in only one group of flounder as reported by Porter et al. (1989) using a Perkin-Elmer LS-5 fluorescence spectrophotometer.

Data, which included K-factor (w/l^3), organ somatic indices (s.i.[s]), EROD activity and hemosiderin concentration were compared between the groups by the one way analysis of variance and Duncan's multiple range test (SPSS™ Software Package) for differences which were considered significant when $p \leq 10.05$.

Table 2. Comparison of variables in male winter flounder *(P. americanus)* following exposure to sediment from a sulphite-bleaching paper mill at Birchy Cove (BC) and a reference site at Meadows (M), Newfoundland for either 6 or 12 mon.

Variable	Fish groups			
Exposure period (mon)	6		12	
Collection Site	BC	M	BC	M
No. Exposed (% mortality)	12 (50)	12 (8)	15 (33)	15 (7)
External lesions (%)[+]	67	9*	90	7*
Length (cm)	32 ±0.3	30 ±0.2	30 ±0.2	26 ±0.1
Weight (g)	222 ±12	253 ±16	172 ±10	183 ±9
K-factor ($x10^{-3}$)	0.79±0.11	1.08±0.07*	0.86±0.05	0.95±0.02
Hepatic s.i. ($x10^{-2}$)	1.61±0.05	1.10±0.04*	1.92±0.02	1.40±0.06*
Gonad s.i. ($x10^{-2}$)	1.3 ±0.3	1.7 ±0.7	4.0 ±0.5	3.9 ±0.5
Gill hyperplasia (%)	100	0	100	0
Pericholangiolar fibrosis (liver-%)	100	0	100	0
Splenic hemosiderin ($\%/mm^2$)	16	1*	34	2*

[+]Based on surviving fish.
*significantly ($P \leq 10.05$) different from BC group.

RESULTS AND DISCUSSION

Winter flounder died at intervals following exposure to the paper mill sediment. External lesions first appeared as ulcers on various sites of the body within two mon in all fish which succumbed. Ultimately the lesions increased in size and became hemorrhagic especially on the left (ventral) side. Winter flounder also suffered from fin necrosis which progressed to a stage, primarily in the tail region, when only the fin rays remained. Mortality was high in flounder exposed to contaminated sediment originating from both Port Harmon (52-54%) Table 1) and Birchy Cove (33-50%) Table 2) in contrast to control fish. In those which survived, the prevalence of external lesions were 64 or 91% after 6 mon and 67 or 90% after 12 mon in contaminated sediment from Port Harmon and Birchy Cove respectively, when compared to substantially lower levels in the control groups (Tables 1 and 2).

There were also a number of biological variables that were significantly different between fish that were exposed to sediment taken from the vicinity of the pulp mills and the reference sites. The condition (K) factor was

significantly lower in fish exposed to sediment taken from the paper mills than in the control groups (Tables 1 and 2). Hepatic s.i.^{s} in the treated groups were also significantly elevated in contrast to the controls but the gonad s.i.^{s} appeared not to be affected.

Histopathological changes also occurred in winter flounder exposed to the pulp mill sediment. All flounder exposed to the contaminated sediment showed moderate to extensive hyperplasia of the secondary gill lamellae, especially in the distal one-third in contrast to the controls which had mainly none to slight hyperplasia (Tables 1 and 2). There was also evidence of hyperplasia in the lamellar troughs culminating in a thickening of the region and ultimately in lamellar fusion. An increase of mucus-secretion was also apparent in the treated fish after determination by PAS technique. Histopathological changes in the liver of sediment-contaminated fish included bile duct hyperplasia (pericholangiolar fibrosis) and multifocal hemosiderosis whereas none was observed in control fish (Tables 1 and 2). Additional anomalies such as depletion of storage products i.e., lipid/glycogen, were also apparent in flounder exposed to the contaminated sediment. Occurrence of depleted storage products was rarely seen in the controls. Tissue changes, especially multifocal hemosiderosis, were also observed in the spleen and kidney of fish exposed to paper mill-contaminated sediment (Tables 1 and 2). Estimation by digital image analysis indicated a significantly greater concentration of hemosiderin in the experimental than in control fish. Moreover, the concentration was greater in both species of fish exposed for the longer rather than the shorter periods.

Examination of MFO activity in winter flounder exposed for 12 mon to the pulp mill sediment originating from Birchy Cove indicated a significantly elevated level ($\bar{x}$, 520 ±49 pmol/mg/min) compared to controls that were submerged in sediment from Meadows ($\bar{x}$, 303 + 77 pmol/mg/min). These results are consistent with reports that exposure of fish to chlorine - or sulphite - bleaching effluent from pulp mills induces MFO (=EROD) activity (Servizi et al. 1993; Munkittrick et al. 1991; Soimasuo et al. 1995) and is associated with increased liver size (McMaster et al. 1991). Although the liver of the experimental flounder showed evidence of pericholangiolar fibrosis, multifocal hemosiderosis and depletion of storage products (see Tables 1 and 2), these changes did not appear to impair the MFO activity. Kohler and Pluta (1995) reported that MFO activity decreased in liver with degenerative, preneoplastic and neoplastic lesions.

The results from the present study have provided evidence that sediment originating from the vicinity of two paper mills can induce a number of biological changes including mortality in winter flounder following chronic exposure. These observations are consistent, moreover, with those seen in flounder originating from sites where the sediment was obtained (Khan et al. 1994a, 1996; Barker et al. 1994b). Enlargement of the liver of winter

flounder and induction of elevated levels of MFO in one trial are also consistent with numerous reports that these variables are important biomarkers in pollution studies (Payne et al. 1996). It is likely that the significantly higher hepatic s.i.'s in other trials in the present study are associated with a marked increase of MFO activity as shown in numerous studies (Munkittrick et al. 1991). Additionally, the high prevalence of external lesions, organ and histopathological changes observed in the groups of flounder exposed to the contaminated sediment corroborate our field studies and support the view that xenobiotics discharged from the paper mills occur in the sediment and are eliciting toxic effects.

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