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Assessment of Stress-Related Bioindicators in Winter Flounder (*Pleuronectes americanus*) Exposed to Discharges from a Pulp and Paper Mill in Newfoundland: A 5-Year Field Study

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Abstract. This study assessed the effects of discharges from a sulphite-bleaching paper mill on winter flounder (*Pleuronectes americanus*) sampled each spring over a 5-year period in St. George's Bay, Newfoundland, prior to foraging activity. The fish were captured by SCUBA divers near the mill and at a reference site 10 km up current. Several bioindicators were used to assess fish health. Larger and older flounder exhibiting gross and microscopic tissue lesions, lower condition factor, and elevated hepatosomatic index but lower gonadosomatic index that was associated with delayed development, were observed in samples caught near the mill compared to those at the reference site. Additionally, fish examined near the mill were infested with an ectoparasite, *Cryptocotyle lingua*, that causes black spot disease but harbored fewer numbers of a digene, *Steringophorus furciger*, in the digestive tract than samples taken at the reference site. These results suggest that abnormal size distribution, interruption of growth, high prevalence of lesions, lower condition factor, enlarged liver, delayed gonadal development, and differences in parasitic levels were indicative of stress in winter flounder caused by discharges from the mill compared to samples from a reference site. There was no evidence of a population decline in the inlet because of annual recruitment possibly from St. George's Bay into which it opens.

Several studies have reported that sulphite-bleaching effluents discharged by pulp and paper mills induced toxic stress–related changes in wild fish living downstream in contrast to nutrient enrichment (Lehtinen *et al.* 1999; Culp *et al.* 2003). Compounds in the effluent such as resin acids and other derivatives bind to particulate material and can accumulate in sediments, eventually entering the food chain and becoming concentrated at higher trophic levels (Lappanen and Oikari 1999). There are reports that two resin acids, dehydroabietic and abietic acids, present in softwoods and released during the pulping process induced detoxificating enzyme activity in the eel, *Anguilla anguilla* L., while wood-related sterols (phytosterols) affected both eggs and larvae of female brown trout, *Salmo trutta lacustris* L. (Lehtinen *et al.* 1999; Pacheco and Santos 1999). As a result, resident bottom-dwelling species of fish were used to assess the impact of the discharge. A plethora of bioindicators have been proposed to assess the effects of the discharged waste in fish (Adams 1990). Several studies have reported a variety of biological responses to the effluent such as impaired growth, enlarged liver, elevated detoxificating enzymes in the liver, delayed gonadal development, skeletal abnormalities, histopathological changes, and differences in the levels of parasitism in contrast to reference fish (Lehtinen *et al.* 1984; Bengtsson *et al.* 1988; Lehtinen 1990a; Axelsson and Norrgren 1991; McMaster *et al*. 1991; Munkittrick *et al*. 1991, 1992, 1994, 1997; Adams *et al.* 1992; Khan *et al*. 1994a; Barker *et al*. 1994; Otto *et al.* 1994; Tierney *et al*. 2004).

Winter flounder (*Pleuronectes americanus*) is a sediment-inhabiting fish species that remains burrowed when not foraging for food in coastal areas of Newfoundland. There is evidence from short-term studies that flounder living in the vicinity of discharges from sulphite-bleaching pulp and paper mills exhibited external lesions, depressed condition (k) factor, enlarged livers, elevated levels of mixed function oxygenase (MFO), histopathological alterations in the gills, liver, and spleen, and differences in the levels of parasites when compared to fish living up current (Khan *et al.* 1994a; Barker *et al*. 1994; Khan and Payne 1997). Moreover, it was observed that exposure of winter flounder to sediment originating from the vicinity of the mill caused gross lesions, tissue pathology, reduction of k-factor, and elevated hepatosomatic index after 6 months (Khan 1997). These anomalies increased in a second group of flounder that were exposed to the sediment over 12 months (Khan 1997). Since previous reports were based on short-term results, a long-term study, over a 5-year period, was performed in the spring of each year to assess further the health of winter flounder living in the vicinity of the sulphite-bleaching pulp and paper mill by comparing several biological variables with reference samples living upstream in St. George's Bay, Newfoundland.

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Table 1. Number of winter flounder sampled for biological variables near a pulp and paper mill at Port Harmon (PH) and a reference site at St. George's (SG) in St. George's Bay, Newfoundland, 1993–1997 inclusive

Year	Site	Epidermal lesions/[a] liver discoloration	K-f/HSI/GSI[b] Male	Female	Histology:[a] gills, gonads[c], liver, spleen	*S. furciger*[a]
1993	PH	44	20	20	30	20
	SG	40	20	20	30	20
1994	PH	163	30	40	40	20
	SG	158	30	30	40	20
1995	PH	57	25	25	50	20
	SG	54	25	25	50	20
1996	PH	59	25	25	35	20
	SG	57	25	25	35	20
1997	PH	74	25	25	20	20
	SG	89	25	25	20	20

[a] pooled sexes of total collection.
[b] K-f = condition (K) factor; HSI = hepatosomatic index; GSI = gonadosomatic index.
[c] 15–20/sex.

Materials and Methods

The pulp and paper mill is located at Port Harmon (48E 31′N, 58E 33′W) in western Newfoundland adjacent to St. George's Bay that connects to the Gulf of St. Lawrence and the reference site about 10 km up current. Black spruce (*Picea mariana*) and balsam fir (*Abies balsamea*) are used for pulping. The effluent, which received primary treatment prior to discharge, contained fluctuating levels of resin acids (<10 × 10/3 mg/L) at the surface (Environment Canada, unpublished data). Sediment at the site where the winter flounder were captured consisted of dark mud mixed with bark and fiber and emitted an odour of hydrogen sulphide. The site was devoid of invertebrates with the exception of the periwinkle (*Littorina littorea*), which was numerous near the shoreline. In contrast, a variety of macro-invertebrates including molluscs, polychetes, and echinoderms were located on a white, sandy bottom at the reference site. A more detailed description of the two study sites was provided by Barker *et al.* (1994).

Winter flounder were captured over a 3-day period by SCUBA divers using a dipnet at 5 to 10 m deep where the water temperature was 0–1°C between the end of April and early May prior to foraging activity. However, both sites were covered with ice, 5–10 cm in thickness, in 1997. The flounder were necropsied on location and a record made of external lesions, black spots of encysted metacercaicae of a digenetic trematode, C*ryptocotyle lingua*, total body length, eviscerated body mass, and that of the liver, spleen, and gonad. The digestive tract was removed, examined for metazoan parasites following conventional methods, and subsequently identified and enumerated after staining. The stomachs of all samples were empty since the flounder do not feed shortly before and after winter (Scott and Scott 1988). Samples of tissues, including the gills, liver, spleen, and gonad were fixed on site in 10% buffered formalin and later processed for histological examination by conventional methods, using hematoxylin and eosin as a standard stain. Gonadal development was assessed by macroscopic and microscopic examination of histological sections. Sections of spleen were stained with Prussian blue for hemosiderin, which was estimated by digital image analysis and expressed as a percentage of the area scanned (Khan and Nag 1993). Hyperplasia of the secondary gill lamellae was classified as slight, moderate, or excessive as noted in a previous report (Khan *et al.* 1994a). Mean intensity of metacercariae of *C. lingua* was estimated from 10 secondary lamellae of the gills (Khan 2004a). The mean number of clear cell foci in stained sections of the liver was estimated/mm^2. Variables such as k-factor (body mass/$length^3$), hepatosomatic index (liver mass/body mass), and gonadosomatic index (gonad mass/body mass) were compared between sexes and site using a one-way ANOVA and ANCOVA (using site as a fixed factor) and Tukey's HSD test. The non-parametric test of Kruskal-Wallis was used to compare parasitic mean abundance in fish from the two sites. Differences were considered significant when $p < 0.05$. Means and standard errors were calculated for each fish group. Data from the year 1994 compared fish length (cm) distribution between the two sites by means of a t-test. Chi-square and Fisher's exact probability tests were used to compare prevalence (%) between sampling sites. Oogenesis and spermatogenesis were determined following the methods of Janssen *et al.* (1995) and Grier (1981). Mean abundance, intensity and prevalence follow the terminology proposed by Bush *et al.* (1997).

Results

A number of significant differences were observed between winter flounder sampled at Port Harmon near the pulp and paper mill and the reference site at St. George's. A greater percentage (74%) of the fish sample ($n = 101$) captured near the mill were large (>30 cm in length) and limited in its range of sizes whereas those taken at the reference site ($n = 102$) were smaller (73% <29 cm in length) with a larger range of sizes (Fig. 1). Flounder smaller than 20 cm in length were absent in collections near the mill whereas at St. George's, many were observed but evaded capture. Although sample sizes were smaller, this trend consisting of larger fish at Port Harmon than St. George's was observed during all other years. Epidermal (skin) lesions, primarily ulcers on the body and fin necrosis, were seen only in samples from Port Harmon (Fig. 2a). Macroscopic discoloration of the liver, which varied from 44 to 76% over the 5-year period, was observed in fish captured near the mill and was rare (0 to 2%) in samples from St. George's. Numerous black spots of metacercariae of *C. lingua* occurred in the skin of flounder near Port Harmon where the prevalence varied from 80 to 100% and was rarely seen on fish at the reference site. Condition (k) factor in both males and females was significantly greater in the reference fish than at the mill site in 3 or 4 years, respectively, from 1993 to 1997 (Fig. 2b,c). However, hepatosomatic index (HSI) was more significantly elevated in males in 1993, 1996, and 1997

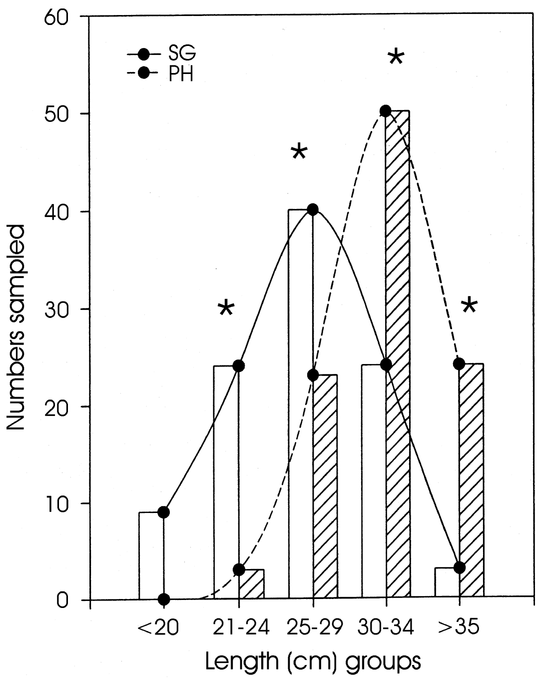

Fig. 1. Comparison of length (cm) groups of winter flounder sampled near a pulp and paper mill at Port Harmon (PH) and a reference site, St. George's (SG), in spring 1994. Asterisk indicates a significant difference between comparison groups.

and in 1993, 1994, and 1996 in females sampled near the mill (Fig. 2d,e) than at the reference site. Although no differences in the gonadosomatic index (GSI) of male flounder were observed between the two sites, significant differences were noted between the GSI of female fish during 3 of the 5 years (Fig. 2f,g). These values were greater in samples taken at Port Harmon than at S. George's. A greater percentage of male flounder was spermiating at the reference site while samples at the site near the mill displayed evidence of delayed gonadal development coinciding with greater GSI in all 5 years (Fig. 2h). Similarly, a greater percentage of female fish had been spawning or was in spawning condition at St. George's in contrast to samples in which gonadal development was delayed (Figs. 2i, 3). Examination of stained sections of the testes of samples taken near the mill revealed that most were at the spermatid stage of development while others failed to develop beyond the spermatocyte stage. In females captured near the mill, the ovaries were either in previtellogenesis (33–45%) or in early vitellogenesis (55–67%), identified as oocytes in the yolk granule stage.

Pathological conditions in the gills, liver, and spleen and the abundance of a digenetic trematode of the digestive system were also different between the groups of flounder captured at the two sites. The most commonly occurring lesion in the gills was epithelial hyperplasia of the secondary lamellae although lamellar fusion and telangiectasis were also observed infrequently. While slight hyperplasia, characterised by minimal thickening of the lamellae, occurred in the reference fish, it was considerably greater (80–100%) and classified as moderate to excessive in samples taken near the mill in the 5-year period. Multifocal hemosiderosis, monocytic infiltration near bile ductules, and bile ductile hyperplasia occurred in all (100%) flounder sampled during the 5-year period near the mill whereas it was considerably less (0–8%) in fish caught at the reference site. Clear cell foci in the liver, identified as vacuolated cells arrange in clumps and in radiating rows, were observed only in fish taken near the mill (Fig. 2j). Moreover, there was a greater concentration of hemosiderin deposits in the spleen of fish taken near the mill than at the reference site over the 5-year period (Fig. 2k). The mean abundance and prevalence of a parasite, an acanthocephalan, *Echinorhynchus gadi*, in the digestive tract was significantly greater in flounder from St. George's (0 ∀ s.e., 1.6 ∀ 0.3–3.1 ∀ 0.5; %, 62–91) than from Port Harmon (0.0– 0.4 ∀ 0.1, %, 5–18) during the same period. Additionally, the mean abundance of the digene, *S. furciger*, was also significantly greater in flounder examined at the reference site than near the mill in the years 1993–1997 (Fig. 2l).

Discussion

The current study has presented evidence that the health of winter flounder sampled near a sulphite-bleaching pulp and paper mill was compromised based on the prevalence of gross lesions. The latter included epidermal ulcers and fin necrosis that occurred only in fish sampled near the mill. Similar observations were reported in flounder examined near the same mill previously (Khan *et al.* 1992, 1994a; Barker *et al.* 1994). Fin necrosis has been noted in fish living downstream of chlorine-bleaching kraft paper mills (BKME) (Lindesjoo and Thulin 1990). The presence of toxic compounds in the sediment originating from the mill was confirmed in the laboratory following exposure of winter flounder. The fish, burrowed in the sediment for 6 to 12 months exhibited a high prevalence of external lesions, including fin necrosis and epidermal ulcers that varied from 67 to 90%, respectively. In addition, 52 and 54% succumbed at 6 and 12 months, respectively, with severe epidermal erosion in which the peduncle and tailfin rays were exposed (Khan 1997). Epidermal lesions and hemorrhaging were also observed in longhorn sculpins (*Myoxocephalus octodecemspinosus*) following exposure to the sediment (Khan *et al.* 1994b). Similar external lesions were also noted in winter flounder sampled near an oil refinery terminal and a PCB-contaminated dockyard in Placentia Bay, Newfoundland (Khan 2003b). The presence of lesions in flatfish taken from other contaminated sites supports the view that these abnormalities are useful bioindicators of environmental degradation (Murchelano and Ziskowski 1976; Sindermann 1982; Lehtinen 1990b; Khan and Payne 1997).

There was a greater percentage of larger and older winter flounder living near the pulp and paper mill than at the reference site in the present study. It is likely that a paucity of small flounder in samples taken from the vicinity of the pulp and paper mill was associated not with migration from the inlet but with mortality caused by the toxic sediment since 52 to 54% of mature fish died following exposure to it for 6 to 12 months under laboratory conditions (Khan 1997). Moreover, K-factor was significantly lower in these fish than samples living up current. K-factor was also significantly greater in controls than in fish experimentally exposed to sediment taken near the mill (Khan 1997). Growth disparity has also been

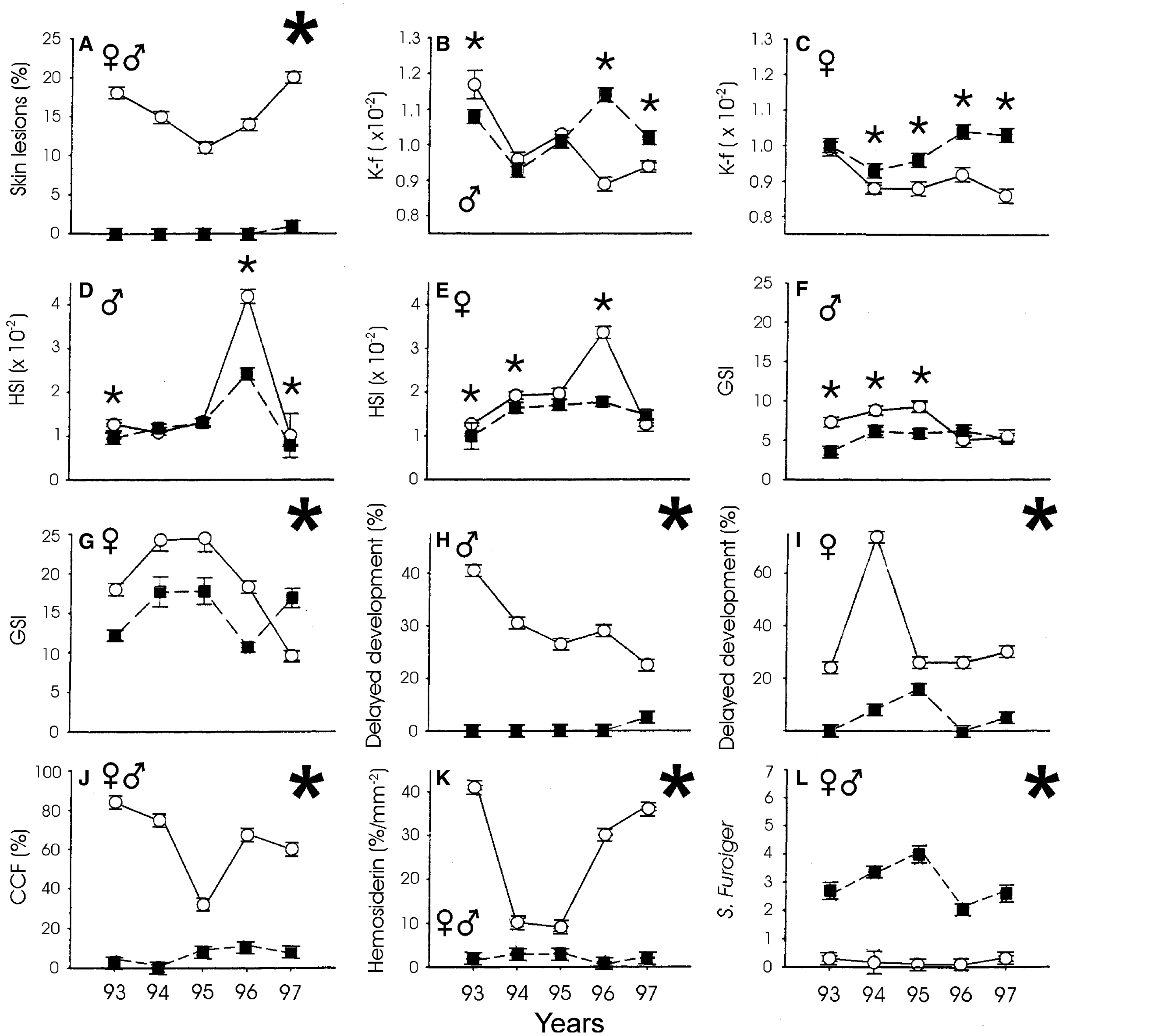

Fig. 2. Comparison of biological variables (x ± s.e) of winter flounder sampled near a pulp and paper mill near Port Harmon (open circles) and a reference site at St. George's (squares) from 1993 to 1997. Asterisks indicate significant differences, smaller for individual years and larger for all years (1993 to 1997 inclusive). Roman numerals indicate (a) skin lesions, (b) K-factor-male, (c) K-factor-female, (d) hepatosomatic index (HSI)-male, (e) HSI-female, (f) gonadosomatic index (GSI)-male, (g) GSI-female, (h) delayed gonadad development-male, (i) delayed gonadal development-female, (j) clear cell foci (CCF) in the liver, (k) hemosiderin in the spleen, and (l) *S. furciger* in the digestive tract.

Fig. 3. Samples of ovaries taken from winter flounder captured at St. George's (SG) a reference site located up current from Port Harmon (PH), near a pulp and paper mill in 1977. All fish were mature, 29.2 ± 3.1 cm in total length and 6.1 ± 0.8 years. Development of about 47% of 17 ovaries was disrupted at PH in contrast to none at SG.

reported previously in fish living downstream from BKME (Munkittrick *et al*. 1991, 1992, 1994). Both lake whitefish (*Coregonus clupeaformis*) and white sucker (*Catostomus commersoni*) exhibited evidence of delayed age to maturity. Although spoonhead sculpin (*Cottus ricei*) exposed to BKME were older and larger than samples at a reference site, their K-factor was greater (Gibbons *et al*. 1998). Another study reported that the slimy sculpin (*Cottus cognatus*) exhibited greater growth and K-factor captured downstream where discharges of sewage and pulp and paper mill effluent occurred than in samples taken from a reference site whereas white sucker from both sites were similar (Galloway *et al*. 2003). It was suggested that the difference in response to chronic exposure was related to the fishes' different life histories.

Hepatosomatic index (HSI) was significantly greater in winter flounder living near the pulp and paper mill than at the reference site in the current study. Additionally, exposure of flounder to sediment taken near the mill resulted in significantly greater HSI values than controls (Khan 1997). Previous studies have noted also that exposure of fish to certain contaminants caused an induction of detoxificating enzymes such as ethoxyresorufin-o-deethylase (EROD) by the liver that enlarged subsequently (Jimenez and Stegeman 1990). EROD activity was significantly elevated in fish sampled near the mill (Khan and Payne 2002a). Several observers have also reported that exposure of fish to certain toxic compounds, including BKME, caused elevated EROD activity that was associated with an enlarged liver (Munkittrick *et al*. 1991, 1992; Otto *et al*. 1994; Gibbons *et al*. 1998). However, detoxificating enzyme activity has been used more often than HSI in biological monitoring studies (Jimenez and Stegeman 1990). There are limitations in the use of EROD activity in biomonitoring studies since temperature and sex steroids have been reported to affect its activity in female fish (Gagnon *et al*. 1994; Khan and Payne 2002b).

The GSI and gonadal development were significantly different in female winter flounder sampled near the pulp and paper mill near Port Harmon than samples taken up current at St. George's in the present study. Gonadal development was delayed in both sexes of fish living near the mill during spring sampling over the 5-year period. Differences in GSI and gonadal development have been reported previously in fish captured near the mill (Barker *et al*. 1994). During 1993 through 1996, the GSI was significantly greater in females from the reference site as most were in the prespawning condition, whereas in 1997 some were spent and fewer were ovulating. Female flounder sampled near the mill were either at the preovulatory stage or the ovaries failed to develop. Consequently, no difference in the GSI was observed between spent flounder taken at the reference site and samples near the mill in which gonadal development was disrupted. During the sampling period in most years, male flounder taken at St. George's were spermiating whereas fish captured near the mill showed evidence of delayed development of the testes. Examination of histological sections revealed spermatids within the seminiferous tubules in contrast to sperm in fish from the reference site noted in the years 1993–1995. Consequently, testicular regression at the reference site but delayed or retarded development near the mill were factors contributing to the similarity. Several studies have reported delayed gonadal development in fish living downstream from pulp and paper mills (Munkittrick *et al*. 1991, 1992, 1994; McMaster *et al*. 1991; Parrot *et al*. 2003). In addition, failure of secondary male characters to develop was associated with lower than normal levels of sex steroids (Munkittrick *et al*. 1991, 1994). Prolonged chronic exposure to pulp and paper mill effluent containing resin acids can disrupt sex steroids and eventually result in reproductive impairment (Munkittrick *et al*. 1994). Moreover, exposure of brown trout (*Salmo trutta*) to untreated effluent discharged by a magazine paper mill using hydrogen peroxide as a bleaching agent, affected egg quality and milt and had a negative impact on egg hatching and growth of fry (Johnsen *et al*. 2000). It is likely, then, that the scarcity of small flounder near the paper mill at Port Harmon was connected with low survival of offspring from the parental fish that were exposed to the toxic effluent containing resin acids. It is less likely that the paucity of small fish was associated with avoidance of effluent toxicity, habitat preference, or size-related migration.

Histopathological lesions were also observed primarily in the gills, liver, and spleen of winter flounder sampled near the mill and were rare or absent in fish up current from the reference site over the 5-year period. Lesions in the gills, including hyperplasia of the epithelium of the secondary lamellae, culminated in increased thickness that probably affected gaseous exchange and ultimately foraging and escape from predation. A high prevalence of hepatic lesions, especially clear cell foci that exceeded more than 50% of the fish sampled, was probably the underlying cause of hepatic discoloration noted in the samples near the mill. This abnormality, in addition to bile duct hyperplasia and hemosiderosis, probably altered hepatic function. Accumulation of hemosiderin deposits in the spleen was likely the result of excessive erythrocyte destruction as noted in other studies (Khan *et al*. 1994a). All of the above-mentioned lesions have been reported in winter flounder taken from the same site previously and also in samples taken near an oil refinery terminal and a PCB-contaminated dockyard in Newfoundland (Khan *et al*. 1994a; Khan 2003b). The occurrence of similar hepatic lesions in flatfish taken from sites contaminated with petroleum hydrocarbons and other industrial waste on both the east and west coasts of North America suggests a multifactorial origin (Moore and Stegeman 1994, Myers *et al*. 1987). Consequently, histopathological methods in fishery research are useful also as indicators of environmental degradation (Myers *et al.* 1987; Couillard *et al.* 1988).

The present and a previous study have revealed that the prevalence and/or abundance of parasites differed between flounder sampled near the pulp and paper mill and the reference site over a 5-year period (Khan 2004a,b). Prevalence and abundance of the ectoparasitic digene, *C. lingua*, in the skin and gills were significantly greater in samples near the mill than at the reference location as reported in earlier studies (Khan *et al*. 1992; Barker *et al*. 1994). This observation is in agreement with previous reports that fish living in habitats degraded by pollutants tend to be more heavily parasitised with external parasites than samples from uncontaminated habitats (Khan 2003a). Since lymphocytic values declined in flounder living near the paper mill, it is probable that the impairment of the immune system resulted in increased susceptibility (Barker *et al*. 1994). Exposure of fish to wood derivatives such as resin acids have been reported to impair the immune response and increase susceptibility to pathogens (Tierney *et al*. 2004). This might have been the underlying cause for the wide distribution of xenomas of a parasitic microsporan, *Glugea stephani*, in several internal organs of winter flounder sampled near the same mill whereas the infection was restricted to the wall of the digestive system at the reference site (Khan 2004b). In contrast, prevalence and/or abundance of parasites in the digestive tract, namely, *S. furciger* and *E. gadi*, were significantly greater in reference samples than in fish taken near the paper mill. This would suggest that either parasitic transmission was interrupted or the parasites were voided after acquisition. Some contaminants might be toxic to the intermediate hosts and/or the larval stages of parasites following release into the environment (Marcogliese and Cone 1997). Steyermark *et al.* (1999) reported that the prevalence of a larval cestode, *Proteocephalus* sp., infecting the viscera of the brown bullhead, *Amieurus nebulosus*, from a river receiving urban and industrial waste, was unusually low (2%) in contrast to reference samples (100%). The authors attributed this difference to sensitivity of the intermediate host to contamination. A decline in food intake, based on K-factor values, occurred in fish following exposure to toxicants and culminated in voiding of enteric parasites (Khan and Kiceniuk 1983). Toxicity of the resin acids could have been responsible for voiding *S. furciger* following ingestion of sea water for osmoregulation or prevented the parasite from becoming established in the winter flounder's digestive system (Khan and Payne 1997). However, infestation of winter flounder at Port Harmon with metacercariae of *C. lingua* suggests that its free-swimming larval stages were not affected by the concentration of resin acids in the water column. Consequently, it is likely that the transmission of *S. furciger* was also not interrupted and flounder that acquired the infection voided the parasites subsequently. Previous experimental studies have reported that the parasite is voided following exposure to pollutants (Khan and Kiceniuk 1983; Khan and Payne 2004). These observations that enteric parasites tend to be fewer in abundance in fish living in contaminated than pristine habitats were also reported recently in both field and laboratory studies (Khan 2003b; Khan and Payne 2004). Consequently, there is a preponderance of evidence that suggests that some fish parasites can be useful as indicators of environmental change in feral species (Overstreet 1993; Khan and Thulin 1991; Broeg *et al*. 1999; MacKenzie 1999).

In conclusion, an integrated study using several bioindicators has revealed adverse effects in winter flounder living in sediment adjacent to a sulphite-bleaching pulp and paper mill over a 5-year period. Abnormal size distribution, external and histopathological lesions, lower body condition factor, elevated hepatosomatic indices, disruption of gonadal development, and differences in parasitic levels were observed in fish sampled near the mill compared to those at the reference site. Although a cause-and-effect relationship was not established, the evidence suggests that chemical stress adversely affected fish health. This study also provides further evidence that the currently used biological variables, including some parasites of fish, can be useful as bioindicators of environmental degradation. Sampling of winter flounder was conducted in spring and summer between 1990 and 1998 but no evidence of a population decline was apparent despite evidence of reproductive disruption (Khan *et al*. 1992; Barker *et al*. 1994; Khan and Payne 1997; present study). Previous studies have reported that egg size and number were affected in fish living downstream from BKME (Munkittrick *et al*. 1991). Future studies should focus on egg size, number of eggs produced, and survival of larvae in order to assess the impact of chronic exposure to wood derivatives discharged by the pulp and paper mill on the population of winter flounder at Port Harmon.

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