Data Analysis and Fish Tumor BUI Assessment

For Lake Superior and the St. Clair River AOCs

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Introduction and History:

Tumor epizootics in fish were first linked to environmental contaminants in the sixties (Dawe et al., 1964). In the seventies the first study was published implicating environmental carcinogens as part of the etiology of papillomas in white suckers in the Great Lakes (Sonstegard, 1977). In the 1980s the first liver cancer epizootic in brown bullhead from the Great Lakes drainage basin was reported in the Black River, Ohio (Baumann, et al., 1982). Research since that time has demonstrated an elevated tumor prevalence in brown bullhead and white sucker populations from a variety of urbanized areas in bays and tributaries of the Great Lakes in both Canadian and United States waters (Baumann et al. 1996). Concern over these discoveries resulted in fish tumors being designated as a Beneficial Use Impairment (BUI) used to determine Areas of Concern (AOC) in Annex 2 of the 1987 Protocol Amending the Great Lakes Water Quality Agreement. The IJC delisting guidelines from 1991 state that this Beneficial Use may be deemed to be Not Impaired "when the incidence rates of fish tumors or other deformities do not exceed rates at unimpacted control sites or when survey data confirm the absence of neoplastic or preneoplastic liver tumors in bullheads or suckers" (International Joint Commission, 1991). Details about the actual methodology used to establish this criterion were not spelled out, and as time has passed the understanding of what comprises accurate methodology in fish tumor surveys has changed (Blazer et al. 2006).

This report deals with those Areas of Concern with fish tumor BUIs located in the Lake Superior basin (Thunder Bay AOC and Jackfish Bay AOC) and the St. Clair River. All three of these locations were far enough north to make collecting sufficient brown bullhead (*Ameiurus nebulosus*) to use as a sentinel species impractical. Thus all locations used a locally abundant catostomid (member of the sucker family) for determining tumor incidence. Fish surveys at both of the two Lake Superior locations used the white sucker (*Catostomus commersoni*) and the fish survey of the St. Clair River used the shorthead redhorse (*Moxostoma macrolepidotum)*. Two of these AOCs and the reference location of Mountain Bay (located on northern Lake Superior) had been documented with both external (lip and body combined) and liver tumors in white suckers during studies carried out by Ian Smith and others from 1985-90 (Table 1). The range of white sucker liver tumor neoplasm incidence from studies of that era indicated that the Thunder Bay and Jackfish Bay locations were elevated compared to the norm (Baumann et al. 1996). However external neoplasm percentages did not appear to be as elevated, since such neoplasms occurred in the early and mid 1980s in close to 40% of the white suckers sampled from Hamilton Harbour (Cairns and Fitzsimons 1988 and Smith et al. 1989).

Table 1. Neoplasm prevalence in white suckers documented from studies carried out from 1985-90 (Baumann et al. 1996), including sample size for external tumors (E) and for liver tumors (L).

Location	Thunder Bay	Jackfish Bay	Mountain Bay
Sample Size	**E=199; L=112**	**E=300; L=194**	**E=304; L=75**
External Neoplasm %	**2.5%**	**7.6%**	**3.6%**
Liver Neoplasm %	**7.1%**	**7.2%**	**2.6%**

Methodology:

A sample size of one hundred white sucker (Catostomus commersoni) from the Jackfish Bay and Thunder Bay Areas of Concern and the Mountain Bay reference location as well as 100 shorthead redhorse ((*Moxostoma macrolepidotum*) from the St. Clair River Area of Concern and the corresponding Lake Huron reference location at the head of the St. Clair River were collected. The Lake Superior sites were sampled by overnight gill net or hoop net sets, the St. Clair River exposed site was sampled using a Smith Root electrofishing boat and the Lake Huron reference site was sampled by overnight trap nets set by Purdy Fisheries. Following capture, fish were placed into a live well for transportation to the sampling site. Fish were anaesthetized in a clove oil bath (~0.05% + ~0.025% ethanol to aid emulsification), then were sacrificed using standard operating procedures, and their physical state was assessed using a visual examination of physical abnormalities. Fork length (mm) and weight (g) were measured and operculae were collected for aging. The liver was removed and separated into sections for histology (Blazer et al., 2007) and were stored in Davidson's Fixative and transferred to 70% ethanol 1-4 weeks after collection.

Histological Evaluation:

The tissues were processed at the Freshwater Institute, in Winnipeg, Manitoba. Prior to processing, the tissues were trimmed into an appropriate number of sub-samples (1 to 7) based on the original size of the sample. A small slice of tissue, between adjacent sub-samples, was removed and discarded. The sub-samples were processed in a routine ethanol/toluene series and individually embedded in paraffin blocks. The embedded tissues were sectioned at 4 – 6 microns and one slide, each with three tissue sections, was prepared from each block. The slides were stained with Harris hematoxylin and eosin.

Slides were examined with a Zeiss Photomicroscope III with Plan lenses and an Olympus Q-Color 3 digital camera. Images were captured from the system using QCapture Suite (Q-Imaging Corp.) software (Version 2.70.0 for Windows) at 2082 x 1542 pixel resolution. Brightness/contrast adjustments were performed in Adobe Photoshop 6.0 for Windows (Adobe Systems, Inc., San Jose, CA).

The data was presented with a 1 indicating the presence of a particular lesion and 0 indicating the absence of the indicated lesion. The proliferative lesions of the white sucker and the shorthead redhorse liver were categorized as non-neoplastic or neoplastic as described by Blazer et al., (2007).

The non-neoplastic hepatocellular lesions included the 4 types of foci of cellular alteration based on tinctorial characteristics of the hepatocyte cytoplasm. The non-neoplastic biliary lesion included only bile duct hyperplasia.

The neo-plastic hepatocellular lesions included hepatocellular adenoma and hepatocellular carcinoma. The neoplastic biliary lesions included cholangioma and cholangiocarcinoma.

In addition, the presence of non-proliferative liver lesions was noted. Small accumulations of lymphocytes/leucocytes were recorded as “Inflammation”, melanomacrophage aggregates in numbers in excess of the norm were recorded as “Excess MA’s”, and focal areas (minor) of necrosis were noted as “Necrosis”.

The visible presence of any parasite(s) in each liver was noted (“Parasites”), as were granulomata (”Granuloma”), which were generally associated with parasites. Although many livers had minor areas of blood congestion (increased blood vessel size and blood flow to an area), those with excessive areas were noted (“Congestion”). Instances of cholangiofibrosis (Baumann et al. 1990) were reported (“Cholangiofibrosis”). This was largely composed of large encapsulating masses that encircled six or more normal-appearing bile ductules. Under “Other Lesions” minor biliary fibrosis and other anomalies were reported.

Types of Lesions:

The use of external lesions including lip papillomas as a criteria related to carcinogen exposure is not recommended. Epidermal papillomas affecting white suckers come in several morphologically distinct varieties, some of which are known to regress under laboratory conditions (Smith and Zajdlik, 1987). Subsequently certain types of papilloma were demonstrated conclusively to be caused by a retrovirus using cell-free transmission experiments (Premdas and Metcalfe, 1996). These same authors believed that the etiologies of such tumors in wild fish would be multifactoral, with induction and progression of a virally induced lesion being influenced by environmental factors. It is our current inability to tease apart the interaction of contaminants and virus infection that prevents us from confidently using external lesions as BUI evaluation criteria. However, it is highly probable that liver lesions in white suckers from the Great Lakes are caused by chemical contaminants (Baumann et al. 1996). In particular polynuclear aromatic hydrocarbons (PAHs) have been proven to induce liver cancer in fish, and other compounds may also be carcinogenic to this species (Balch et al. 1995). Also no liver cancer in any species of fish has ever been diagnosed with a viral etiology (Dr. John Harshbarger, Director of the Tumor Registry in Lower Animals, Smithsonian Institution, Washington, DC, personal communication).

The original wording of the 'Fish Tumors or Other Deformities' BUI included the occurrence of "neoplastic or preneoplastic liver tumors in brown bullhead or suckers". However, no specifics were given for the definition of preneoplastic lesions. Foci of cellular alteration, depending upon morphological and staining characteristics, can be classified as basophilic, eosinophilic, vacuolated, and clear cell. Basophilic foci have been reported to advance to hepatocellular carcinoma in several species of fish (Blazer et al. 2006). However not all basophilic foci advance (Hinton et al. 1988), and the number of fish with basophilic foci from the two Lake Superior AOCs and one reference site only varied from 2% to 4%. There is no definitive evidence that other types of altered foci progress to neoplasia (Bunton, 1996). No studies on progression of any foci of cellular alteration have been performed on suckers or bullhead. Liver tumors in fish are, with rare stem cell exceptions, derived from either liver cells (hepatocellular) or bile duct cells (cholangiocellular). No non-neoplastic cholangiocellular changes, such as bile duct hyperplasia and cholangiocellular fibrosus, have been experimentally demonstrated as progressing to tumors. Such proliferation of bile duct epithelial cells has been demonstrated following laboratory carcinogen exposure in a number of species (Blazer et al. 2009). Similarly, such lesions have been reported along with tumors in wild populations from contaminated locations (Blazer et al. 2009). However, at least in bullhead, a myxozoan parasite has also been implicated in bile duct proliferation and fibrosus (Baumann et al. 2008). Because of the uncertainties concerning progression of both foci of cellular alteration (hepatic) and cholangiocellular proliferation and fibrosus (biliary), it is best that none of these preneoplastic lesions be used as an actual delisting criterion.

Age and Gender:

Two variables which might influence tumor prevalence are the age of the fish and fish gender. Age has long been recognized as being positively correlated with tumor prevalence (Baumann, 1992). This is not only because fish that have lived longer have usually been exposed to environmental contaminants longer, but also because there is a latent period between induction and tumor development. For instance the prevalence of spontaneous neoplasms in medaka (*Oryzias latipes*) of ages 1 through 5 was greatest in females of age 4 and 5 and males of age 5

(Masahito et al. 1989). This same positive correlation between age and tumor prevalence has also been noted in wild populations of several species exposed to contaminants. English sole from contaminated locations in Puget Sound had a nearly 40% increased probability for having a hepatic neoplasm with each additional year lived (Rhodes et al. 1987). Similarly bullhead from the Potomac River also had an increased risk of hepatic carcinomas with age (3.5 times greater per year) (Pinkney et al. 2001). Brown bullhead from the Black River, Ohio were found to have a significantly ($p<0.05$) higher prevalence of biliary liver cancers at ages 4 and 5 (35.5%) than at ages 2 and 3 (18.4%) (Baumann et al. 1990). Blazer (2009) also reported an increasing prevalence of liver tumors with age in bullhead from Presque Isle Bay, particularly at ages 8 and older. Furthermore Slooff (1983) found that of 7,209 bream necropsied in Europe, all fish with grossly visible tumors were age 7 or older. White sucker have also shown this age and neoplasm link. In samples from five locations in the St. Lawrence Basin lip neoplasms occurred almost exclusively in fish >350mm (length being an age surrogate) (Mikaelian et al. 2000). Thus it is important to consider age when comparing neoplasm prevalence among populations.

Gender related differences in tumor prevalence have been less consistently reported than age related differences, particularly in wild exposed populations. Several species of laboratory fish have been reported to have a higher prevalence of spontaneous tumors in females (Baumann 1992). However gender was not a significant factor in the prevalence of hepatic lesions in English sole from Puget Sound (Rhodes et al. 1987). Female brown bullhead from the Black River, Ohio had a significantly higher ($P<0.05$) incidence of hepatocellular carcinoma only, but not of any other neoplasms. A review of Great Lakes brown bullhead data taken at United States locations since 1991 reinforces the view that gender differences are not discernable. However, an analysis of the brown bullhead data base for Chesapeake Bay found that being female was a significant ($P<0.001$) positive co-variant for liver neoplasms (Pinkney et al. 2009). Gender equivalency among samples should be considered for comparative purposes.

Variability and Statistics:

Determining whether a fish has a tumor provides a “yes” or “no” answer (binary response) rather than a number. Thus contingency table analysis is required for statistical differentiation of population values. Such statistics will test whether two locations have meaningfully different results at some level of confidence. The level of confidence is determined by selecting a P value to indicate significance. The typical P value for biological studies is 0.05 (a 5% or one in twenty random chance of being wrong). Thus P values less than or equal to 0.05 would indicate a real difference between the tumor prevalence at the sites being compared.

There are two methods which are commonly used to compute a P value from a contingency table: Chi-square and Fisher’s exact test. Fisher's exact test gives the exact P value, while the Chi-square test calculates an approximate P value (Graphpad Software 2009). Chi-square often works better with multiple rows and columns, but the data here only has two of each. Additionally, Fisher's exact test is supposed to perform better when the expected values are small, which is the case here. Thus Fisher's exact test was used to determine the P values when comparing tumor prevalence at AOC locations and reference sites. Statistical calculations were done using a QuickCalcs online calculator by GraphPad Software (Graphpad Software 2009). This software includes a statement acknowledging that the Fisher's test actually has three methods that can be used to compute the two-sided (two-tailed) P value. The software used here incorporated the method of summing small P values.

Results:

White sucker were collected at two AOCs on Lake Superior: Thunder Bay and Jackfish Bay. The same species was collected at Mountain Bay on Lake Superior, as a reference location. Shorthead redhorse were collected at the St. Clair AOC, and Lake Huron at the head of the St. Clair River was used as a reference site. Fish were captured using electrofishing, gill nets, and trap and hoop nets, some run by commercial fishermen. All locations had at least one hundred fish collected (Table 2). Liver sections from the Lake Superior fish averaged around four per individual, while sections from both redhorse populations averaged less (Table 2). Females comprised 48% to 55% of the Lake Superior white sucker collections and 41% to 42% of the St. Clair River and Lake Huron redhorse collections, making each group of reference and AOC locations comparable in gender. Ages varied from a median of 6 to a median of 11, and will be discussed within the individual AOC impairment conclusions sections. Neoplasms were rare at both AOC and reference locations (Table 3). None of the five locations sampled had a neoplasm prevalence that exceeded 2%. All three locations in Lake Superior had a smaller percentage of fish with neoplasms than they had in the late 1980s (Table 1). White suckers from Thunder Bay and Jackfish Bay had declined in liver neoplasm prevalence by over 5% and 7% respectively. This decrease at Jackfish Bay was statistically significant. None of the AOCs differed significantly from their respective reference locations in the proportion of the population found to have liver neoplasms.

Table 2. Sample size, age, gender proportion and number of liver sections taken from three northern AOCs and two reference locations in 2006.

Location	Sample Size	Median Age	Percent Female	Sections/Liver
Thunder Bay	**100**	**6**	**48.5%**	**4.6 (average)**
Jackfish Bay	**100**	**9**	**50%**	**3.75 (average)**
Mountain Bay	**100**	**11**	**55%**	**3.9 (average)**
St Clair River	**126**	**10**	**41%**	**2-4 (range)**
Lake Huron	**100**	**6**	**42%**	**2-4 (range)**

Table 3. Upper Great Lake AOCs (Thunder and Jackfish Bays and the St. Clair River) and reference locations (Mountain Bay (Lake Superior) and Lake Huron) tumor prevalence, and the significance of differences between AOCs and reference sites (2006).

Location	Sample Size	Neoplasm #	% Neoplasms	Significance
Thunder Bay	**100**	**2**	**2%**	**None**
Mountain Bay	**100**	**0**	**0%**	
Jackfish Bay	**100**	**0**	**0%**	**None**
Mountain Bay	**100**	**0**	**0%**	
St Clair River	**126**	**0**	**0%**	**None**
Lake Huron	**100**	**1**	**1%**	

Conclusions by AOC:

Thunder Bay AOC:

In the late 1980s Thunder Bay was determined to have a liver neoplasm prevalence of 7.1% (Table 1). That frequency of liver tumor would have been viewed as elevated, and helped to assign a fish tumor BUI to the AOC. However a sample of 100 white suckers revealed that in the 2006 population of white sucker in Thunder Bay the tumor prevalence had declined to 2%. This neoplasm occurrence is not significantly different from the white sucker reference location rate (Table 3), nor would it be significantly different from the brown bullhead reference neoplasm prevalence. The 2% prevalence is also not significantly different from the 1980's 7.1% prevalence at the P= 0.05 level usually accepted. However this may well be due to relatively low sample sizes, as the P level was 0.1. In other words, even with relatively limited data there is only a one in ten chance that the actual population neoplasm prevalence has not declined. However the median age of these fish is 5 years younger than the reference location and three years younger than Jackfish Bay. This is partially compensated for by the more numerous liver sections examined. An additional survey of 100 fish is recommended, using a length cut-off to reduce younger age groups. Such a survey emphasizing older fish would add certainty to the decision on the status of this Beneficial Use. If the results of the additional fish survey indicate a tumor prevalence of less than 5%, then the status of this Beneficial Use should be changed to Not Impaired.

Jackfish Bay AOC:

In the late 1980s Jackfish Bay had a liver neoplasm prevalence of 7.2% (Table 1). That frequency of liver tumor would have been viewed as elevated, and helped to assign a fish tumor BUI to the AOC. However a sample of 100 white suckers taken in 2006 did not reveal any liver neoplasms. This is, of course, not statistically different from the neoplasm prevalence at the Mountain Bay reference location. Furthermore Fisher's exact test demonstrates that the liver neoplasm prevalence in the 2006 sample was significantly lower ($p<0.01$) than in the sample from the 1980s. This verification of a lower tumor prevalence was helped by the robust size of the 1980s sample taken for liver pathology (n=194). Although the median age is two years younger than the Mountain Bay reference location, at 9 years of age this is not a deterrent to delisting. The status of this Beneficial Use can now be considered to be Not Impaired. No further monitoring specifically for tumors is needed.

St. Clair River AOC:

This location was not listed among the older (1980s and early 1990s) studies demonstrating tumor epizootics (Baumann et al. 1996). Concerns for fish tumors might have been raised by the perception that external walleye lesions, probably with a viral etiology, seemed more common in the AOC population (Myllyoja and Johnson, 1995). However no tumors were seen in the shorthead redhorse samples taken in 2002, 2003, and 2006. Reference samples from Lake Huron had a 1% prevalence of tumors, which matches the prevalence in the brown bullhead reference data base. The male/female ratio was similar at the AOC and reference location, as were the number of sections taken per liver. The sample population from the St. Clair River was markedly older (4 years) than that from the reference site (Table 2). Although this should imply that the tumor prevalence would also be greater because of the older age (as discussed previously), in actuality, the tumor prevalence was not greater. The status of this Beneficial Use can now be considered to be Not Impaired. No further monitoring specifically for tumors is needed.

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