Toronto & Region Remedial Action Plan

BUI Status Re-designation Document:
Fish Tumours and Other Deformities

December 2011

TORONTO & REGION
REMEDIAL ACTION PLAN

For additional copies of this report please contact:

Toronto and Region Conservation Authority
5 Shoreham Drive
Toronto, ON
M3N 1S4

phone: (416) 661-6600
fax: (416) 661-6898

The Toronto and Region Remedial Action Plan is managed by representatives from Environment Canada, Ontario Ministry of the Environment, Ontario Ministry of Natural Resources and Toronto and Region Conservation Authority.

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Summary

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 1980's the first liver cancer epizootic in brown bullhead from the Great Lakes drainage basin was reported in Black River, Ohio (Baumann *et al*., 1982). Research since that time has demonstrated elevated tumor prevalence in a variety of species across North America (Baumann, 1998), including brown bullhead and white sucker populations from a wide range 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) for Areas of Concern (AOC) in Annex 2 of the 1987 *Protocol Amending the Great Lakes Water Quality Agreement*.

In Toronto, the Stage 1 RAP report *Stage 1: Environmental Conditions and Problem Definition* classified the Fish Tumours or Other Deformities Beneficial Use as Requires Further Assessment. The 1994 the Toronto and Region RAP Stage 2 Report, *Clean Waters, Clear Choices: Recommendation for Action* used a precautionary approach and identified a delisting target for fish tumours and other deformities in the absence of tumour studies in Toronto. In 2001, the RAP Progress Report, *Clean Waters, Healthy Habitats* recommended a study of liver tumours and external deformities in fish as it was uncertain whether this BUI should be listed as impaired in Toronto. Studies of brown bullhead captured in the Toronto nearshore and marshes were conducted in 2003 and 2006.

This report summarizes the analyses of fish (brown bullhead) tumour data collected in the Toronto and Region Area of Concern. Based on the result that the liver tumour prevalence of 3.8% in Toronto and Region does not significantly differ from the 2% Impairment Criterion, as determined through comparison to the Impairment Criterion database of Great Lakes far field and reference sites (Baumann, 2010), it accordingly recommends that the classification of the Fish Tumours or Other Deformities Beneficial Use be changed from *Requires Further Assessment* to *Not Impaired*.

BUI Re-designation Criteria

BUI Status (Stage 1 Report; 1989):

Requires further assessment

Toronto and Region RAP Goals (Stage 2 Report; 1994):

The number of fish displaying diseases, tumours, ulcers or deformities associated with the presence of toxics are reduced or maintained at levels considered background for the community.

BUI Re-designation Goal (2011):

The number of fish displaying diseases, tumours, ulcers or deformities associated with the presence of toxics are reduced or maintained at levels considered background for the community.

Applied BUI Criterion (2011): Liver tumour prevalence in brown bullhead collected in the Toronto nearshore and marshes is not significantly different than the Impairment Criteria (IC) database of 2% liver tumour prevalence (Baumann, 2009).

Status of AOC Against BUI Criteria

Comparison of AOC conditions to Applied BUI Criterion

The results of 213 brown bullhead sampled from the Toronto and Region Area of Concern in 2003 and 2006 indicated an average tumour prevalence of 3.8%. The 3.8% prevalence was not significantly different (Fisher's exact test, P=0.14) than the 2% prevalence of the Impairment Criterion database (Baumann, 2010).

Has the BUI Criterion been met?

Yes. The liver tumour prevalence in Toronto and Region brown bullhead is not significantly different than that found at Great Lakes far field and reference sites.

Recommended Status of the Beneficial Use:

Not Impaired

Ongoing Actions

There are no actions underway for the express purpose of addressing the *Fish Tumours and Other Deformities* Beneficial Use.

Future Monitoring or Actions Required

There are no planned future activities, monitoring or otherwise, to expressly address the *Fish Tumours and Other Deformities* Beneficial Use.

Research Results

Background:

This report deals with the Toronto and Region Area of Concern Beneficial Use Impairment #7 "Fish Tumours or other Deformities"; the information is taken from the report Data Analysis and Fish Tumour BUI Assessment For the Lower Great Lakes and Interconnecting Waterways by Paul C. Baumann, PhD (March, 2010). The Baumann (2010) report assessed tumour prevalence in brown bullhead (Ameiurus nebulosus) in all the lower lakes AOCs and their associated far field and reference sites (Table 1). Sampling for the current monitoring project took place between 2001 and 2008, with many locations being sampled in several different years.

Tumour 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 elevated tumour prevalence in a variety of species across North America (Baumann, 1998), including brown bullhead and white sucker populations from a wide range 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 tumours 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 tumours or other deformities do not exceed rates at unimpacted control sites or when survey data confirm the absence of neoplastic or preneoplastic liver lesions in bullheads or suckers" (International Joint Commission, 1991). Details about the actual methodology used to establish this criterion were not provided, and as time has passed the understanding of what comprises accurate methodology in fish tumour surveys has changed (Blazer *et al*. 2006).

Table 1. AOC locations and their associated far field and reference locations with the years sampled for brown bullhead tumour studies (from west to east).

AOC Location	Far Field Site	Reference Site	Years Sampled
Detroit River	Grosse Isle	Peche Isle	2002
Wheatly Harbour	NA	Hillman March, Port Rowan	2002, 2006
Niagara River	Queenston	Point Abino	2004, 2008
Hamilton Harbour	NA	Jordan Harbour	2001, 2005, 2007
Toronto and Region	NA	Frenchman's Bay	2003, 2006
Bay of Quinte	Belleville	Prince Edward Bay, Deseronto	2004, 2005
St. Lawrence River (Cornwall)	Gray's Creek	Morrisberg	2004, 2005

A sample size of one hundred brown bullhead (*Ameiurus nebulosus*) was set for collection in each of the AOCs, far field sites and reference locations. In Toronto and Region, a total of 213 fish were collected for analysis using a Smith Root electrofishing boat. This sample, the one from which subsequent analyses and conclusions are derived, is independant from the 2003 collection of 254 brown bullhead along the Toronto waterfront by Toronto and Region Conservation (TRCA) that follows (p. 15-25). The data from the 2003 TRCA study indicated a liver neoplasm prevalence of 1.2%; these data were not incorporated into the present analyses.

As noted above, the IJC delisting guidelines from 1991 state that this Beneficial Use may be deemed to be Not Impaired "when the incidence rates of fish tumours or other deformities do not exceed rates at unimpacted control sites or when survey data confirm the absence of neoplastic or preneoplastic liver lesions in bullheads or suckers" (International Joint Commission, 1991). The use of other, external lesions including lip papillomas as criteria related to carcinogen exposure is no longer recommended. Certain types of papilloma have been demonstrated conclusively to be caused by a retrovirus (Baumann and Okihiro 2000), including one type of papilloma occurring on white sucker (Premdas and Metcalfe, 1996). Papilloma prevalence in brown bullhead does not correlate well with liver tumour prevalence either across locations or on individual fish within locations (Baumann *et al*. 1987). It is our current inability to tease apart the interaction of contaminants (both carcinogens and promoters) and virus infection that prevents us from confidently using external lesions as a criterion for BUI evaluation. On the other hand, liver lesions in wild fish including brown bullhead from the Great Lakes are caused by chemical contaminants (Baumann *et al*. 1996, Baumann and Okihiro 2000). In particular, polynuclear aromatic hydrocarbons (PAHs) have been proven by an extensive array of laboratory experiments to induce liver cancer in fish (Baumann and Okihiro 2000). Also no liver cancer in any species of fish has ever been diagnosed with a viral etiology (Dr. John Harshbarger, Director of the Tumour Registry in Lower Animals, Smithsonian Institution, Washington, DC). Furthermore, field studies have correlated a decline in tumour incidence with a decline in PAH contamination in sediment (Baumann and Harshbarger, 1995) and have shown that fish exposed to elevated PAH concentrations in the wild had significantly higher liver neoplasm prevalence than those that were not (Vogelbein *et al*. 1990; Baumann and Okihiro 2000). Thus liver neoplasms are the most consistent markers of carcinogen exposure.

The original wording of the "Fish Tumours or Other Deformities" BUI as described by the IJC included the occurrence of "neoplastic or preneoplastic liver tumours 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, Baumann and Okihiro 2000). 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 tumours in fish are, with rare stem cell exceptions, derived from either liver

cells (hepatocellular) or bile duct cells (cholangiocellular). A proliferation of bile duct cells has been demonstrated following laboratory carcinogen exposure in a number of species (Blazer *et al*. 2009). Similarly such lesions (bile duct hyperplasia and cholangiocellular fibrosus) have been reported along with tumours in wild populations from contaminated locations (Blazer *et al*. 2009). However none of these non-neoplastic cholangiocellular changes have been experimentally demonstrated as progressing to tumours. Also, at least in bullhead, a myxozoan parasite has 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 impairment criterion.

Age and Gender of Fish:

Two variables which might influence tumour prevalence are the age of the fish and fish gender. Age has long been recognized as being positively correlated with tumour 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 tumour 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 tumour 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 tumours 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 tumours 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 tumour 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 tumours 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 as 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 tumour 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 if tumour incidence is similar or different at two locations 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 tumour 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 tumour 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.

Determining Background Tumour Prevalence - Reference and Urban Non-Point Sites:

Theoretically one reference location should have the same tumour prevalence as any other reference location (given a certain variation around the true mean, and if age and gender are not badly skewed). In fact reference site liver neoplasm prevalence by location seems to be very consistent (Table 2). The only reference location not included was Hillman Marsh at Wheatley Harbour, since that location had 9% neoplasm prevalence which would exclude its use as a reference site. The eight remaining locations only varied from 0% to 2%. Median ages ranged from 5 to 7 except for Jordan Harbour (age 4), with an overall median age of 6. Females comprised 46% of the fish sampled across locations. Nothing prevented combining the reference locations into a single data set. Thus brown bullhead in the combined reference data base have a liver neoplasm prevalence of 1% ($n=701$). This is a considerably lower prevalence than the 5% figure from Baumann *et al*. (1996). However this change was not unexpected, given the much expanded data base of cancer surveys in Great Lakes fish in the last fifteen years.

The same sort of calculation was made using the Far Field locations sampled (Table 3). The number of bullhead with neoplasms in the four locations varied between 0% and 4% (Gross Isle with a very small sample size). The average median age was slightly younger (age 4) than at the reference locations, but the fish sampled for the group as a whole were evenly split between males and females. Again nothing prevented numbers from being combined, giving a combined prevalence of 2% (n=267). A data set of United States locations which correspond in some ways to the Canadian Far Field sites have a similar tumour prevalence (Table 4). The locations in this set include a "reference" location with modest PAH spikes near a railway bridge and a highway bridge (Old Woman Creek); two urbanized locations without a major point source (the Huron and Conneaut Rivers); and an AOC location that had undergone extensive remediation (the United States side of the Niagara River). The bullhead in this data set also have a liver neoplasm prevalence of 2% (n=204). This group from the United States combined with the Canadian Far Field locations would be best characterized as urban or having a low/moderate pollution level without a major point source. This combined group would have a liver tumour prevalence of 2% (n=471).

Criterion Selection:

The crux of criterion selection decision rests on being able to combine the reference location neoplasm data. With a data base of 700 reference fish, comparisons made with AOC locations are much more likely to be significant. The same is true of combining the Far Field and United States locations to achieve an *n* value of 471. The 1991 IJC guidelines state that locations determined to be impaired might be designated as restored when "tumours…do not exceed rates at unimpacted control sites". However in decades since then there has been much discussion of how bullhead from more urbanized areas might have an increased probability of tumours even if point sources had been eliminated and exposed contaminated sediments eliminated. In other words, holding urban areas to pristine standards might not be achievable.

The list of mixed far field and other lower level polluted locations suggests that even in urbanized areas without a major point source we could reasonably expect to have a liver neoplasm prevalence of 2% or less. This leads to the question of whether the 1% prevalence seen in true reference sites can really be distinguished from the 2% prevalence in the urbanized areas with realistic manpower (budget) restraints. To answer this question we need to choose a P value to indicate significance. The typical P value for biological studies is 0.05. Using this value, even if we combine the reference data sets and then combine the far field data and United States data sets, the difference between the 1% and 2% tumour frequencies of these two groups is not significant according to Fisher's exact test.

Table 2. List of reference locations including number of fish sampled and number of fish with neoplasms.

Location	Sample Size	Median Age	% Female	Neoplasms (#/%)
Peche Island	34	5	56	0/0%
Port Rowan	99	6.5	35	1/1%
Port Albino	40	5	50	0/0%
Jordan Harbour	193	4	53	3/1.6%
Frenchman's Bay	101	7	50	1/1%
Prince Edward Bay	38	No age data	38	0/0%
Desoronto	96	5	41	2/2.1%
Morrisberg	100	5	49	0/0%
Total/Average	**701**	**6**	**46**	**7/1%**

Table 3. List of far field locations including number of fish sampled and number of fish with neoplasms.

Location	Sample Size	Median Age	% Female	Neoplasms (#/%)
Gross Isle	25	5.5 (n=4)	56	1/4%
Queenston	43	4	48	0/0%
Belleville	99	4	51	2/2%
Gray's Creek	100	4	48	2/2%
Total/Average	**267**	**4**	**50**	**5/2%**

Table 4. Urbanized, reference (Old Woman Creek) and reclaimed AOC (Niagara) locations in the United States, including number of fish sampled and number of fish with neoplasms.

Location	Sample Size	Neoplasms (#/%)
Huron River	62	1
Old Woman Creek	59	1
Conneaut River	43	1
Niagara River (U.S. side)	40	1
Total/Average	**204**	**4/2%**

Based on these data, the best choice for a criterion would be the 2% prevalence level, which should be achievable even in more urbanized locations. Furthermore if we apply a bootstrapping technique to the reference site data base, the 95% confidence interval within which the true mean prevalence should exist, ranges from 0.73% to 1.5% (Figure 1). The 1.5% upper bound for the 95% confidence interval validates the choice of 2% as a delisting criterion. Since we have already sampled over 700 reference fish and over 450 far field and urban fish with a combined tumour prevalence of under 2%, we can use the combined sample size from these two groups (rounded to 1,150) as our "background prevalence" sample size for determining significant differences at the AOC locations. In order to clarify further fish tumour impairment discussions of the AOCs, this 1,150 fish data base with the assigned 2% tumour prevalence will be referred to in the rest of this report as the Impairment Criterion (IC) data base. In creating a contingency table for Fisher's exact test, the IC number for fish with neoplasms would be (2% x 1,150) or 23 and the IC number for normal fish would be (1,150 – 23) or 1,127. A hypothetical site having a hundred fish sample and a 5% liver tumour prevalence would have a P value of 0.066%, or just barely non-significant.

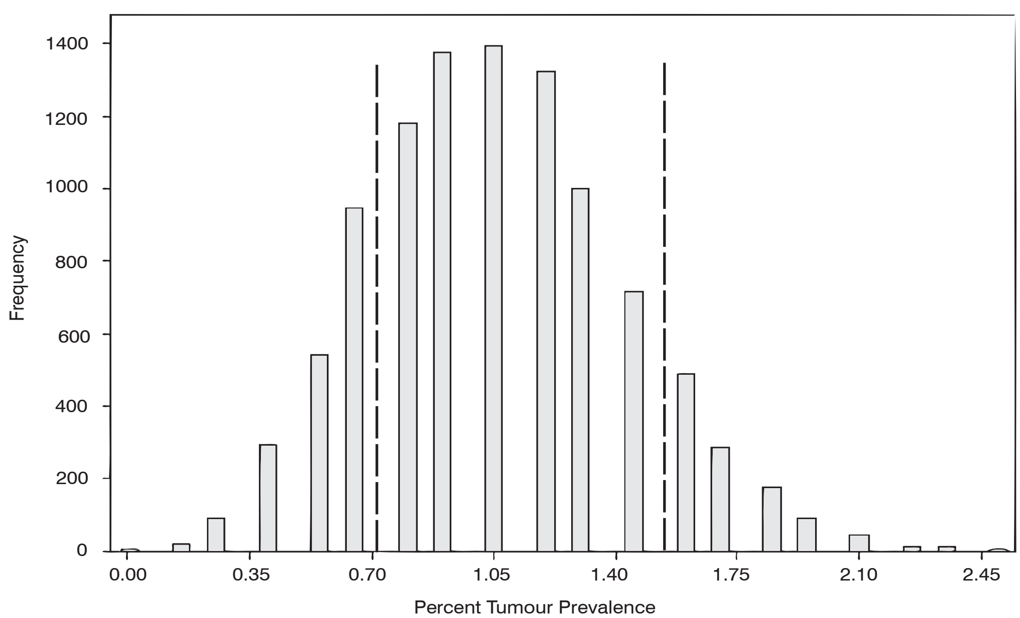


Figure 1. Bootstrapped 95% confidence interval (0.73 to 1.5% as indicated by the two dashed vertical lines) determined for the liver tumour prevalence of bullhead at reference locations. This confidence interval was estimated using 10,000 iterations.

Determination of BUI Status:

The Toronto and Region AOC had a total of 213 samples taken in 2003 and 2006, with a total bullhead liver tumour prevalence of 3.8%. There is not a significant difference between the liver neoplasm prevalence in Toronto and Region and that in the reference site/far field Impairment Criterion data base (Table 5). Thus the status of the fish tumours Beneficial Use in the Toronto and Region Area of Concern can be classified as Not Impaired.

Table 5. Canadian AOCs with completed surveys (sub-locations and multiple year samplings combined) with sample sizes, ages, gender percentage, neoplam numbers and prevalence, and difference (S) or not (N) from the impairment criterion.

AOC Location	Sample Size	Median Age	Female (%)	Neoplasm #(%)	P Value	Significance
Wheatly Harbour	100	7	47	4/4%	0.27	N
Niagara River	101	5	50	3/3%	0.47	N
Hamilton Harbour	200	8	48	11/5.5%	0.013	S
Toronto and Region	213	7	45	8/3.8%	0.14	N
Bay of Quinte	100	5	42	4/4%	0.27	N
St. Lawrence River (Cornwall)	100	5	46	2/2%	1.0	N

2003/04 Tumour Survey (Toronto and Region Conservation)

Introduction

The 2001 Toronto and Region Remedial Action Plan (RAP) Progress Report *Clean Waters, Healthy Habitats* listed the *Fish Tumours or Other Deformities* BUI as "probably not impaired, but requires further assessment." Prior surveys of fish in Toronto and Region tributaries had revealed fairly high incidence rates of various types of tumours (Toronto and Region Remedial Action Plan, 2001), however anecdotal evidence from biologists working along the Toronto waterfront indicated that serious external deformities were rarely observed. *Clean Waters, Healthy Habitats* concluded that existing information suggested fish tumour and deformities were neither widespread nor severe in the AOC. Sufficient, unequivocal information on fish tumours and deformities from across the Toronto and Region, however, was not available.

To address this data gap, the 2001 RAP Progress Report identified the need for a survey of liver tumour and external deformity incidence in the Toronto and Region AOC. Detailed analysis of the existing data and assessment methods were required, along with discussions among the various stakeholders associated with the Toronto and Region RAP and experts in the field of fish tumours and deformities.

In January 2003, the Toronto and Region Conservation Authority (TRCA) retained Gartner Lee Limited to undertake a review and assessment of the status of Fish Tumours or Other Deformities within the Toronto and Region AOC. The objectives of the review were as follows:

- Review published and unpublished past and current data related to fish tumours and deformities within the Toronto AOC and compare the prevalence of fish tumours and deformities with the prevalence at other AOCs;
- Determine if the current data is sufficient to support a decision for an impaired or unimpaired beneficial use in Toronto AOC;
- Convene a workshop of experts in the field fish tumours and deformities to develop appropriate tumour/deformities targets for the Toronto RAP and feasible monitoring protocols;
- Review current methods and protocols for sampling and assessing the presence of fish tumours and deformities; and
- If necessary, develop a work plan to monitor tumours and deformities in the Toronto AOC, commencing in the spring of 2003.

The Gartner Lee report *Review and Assessment of the Status of Fish Tumours or Other Deformities as an Impaired Beneficial Use within the Toronto Area of Concern* (Gartner Lee Limited, 2003) concluded that the aquatic toxicology literature reported correlations between fish tumour incidence and causal factors including contaminants. The mechanism of fish tumour occurance, however, had not been fully elucidated and standardized methods to measure the incidence of fish tumours in a given population were not yet established (Gartner Lee Limited, 2003). Accordingly, expert consultation would be required to determine if the Toronto and Region AOC could be deemed "not impaired" for the *Fish Tumours or Other Deformities* BUI or if a study would be required. In the event of a study, the guidance of experts would be required to develop a defensible and repeatable study design that could be realistically implemented by the TRCA (Gartner Lee Limited, 2003).

A workshop of experts was convened in the spring of 2003 and participants agreed that the evidence available on the incidences of fish tumours in the Toronto and Region AOC was insufficient to determine the status of the BUI. The general consensus was that further research was required to arrive at a decision on the classification of the *Fish Tumours or Other Deformities BUI* in the Toronto and Region AOC. The methods and results of the study conducted by the TRCA in 2003 and 2004 to document the incidence of liver tumours in Toronto and Region fish, specifically brown bullhead, are reported below.

Study design

Fish Species

Brown bullhead (*Ameiurus nebulosus*) was selected as the study species. The bullhead lifecycle is lived in close association with sediment and occurs within a small home range, and brown bullhead have been examined in numerous comparable surveys examining the prevalence of internal and external tumours (Gartner Lee Limited, 2003).

As a result of previously identified correlations between age and tumour frequency (Baumann *et al*., 1990) brown bullhead assessed in the current study were restricted to those of length 250 mm or greater to ensure the sampling of mature (>3 year old) fish.

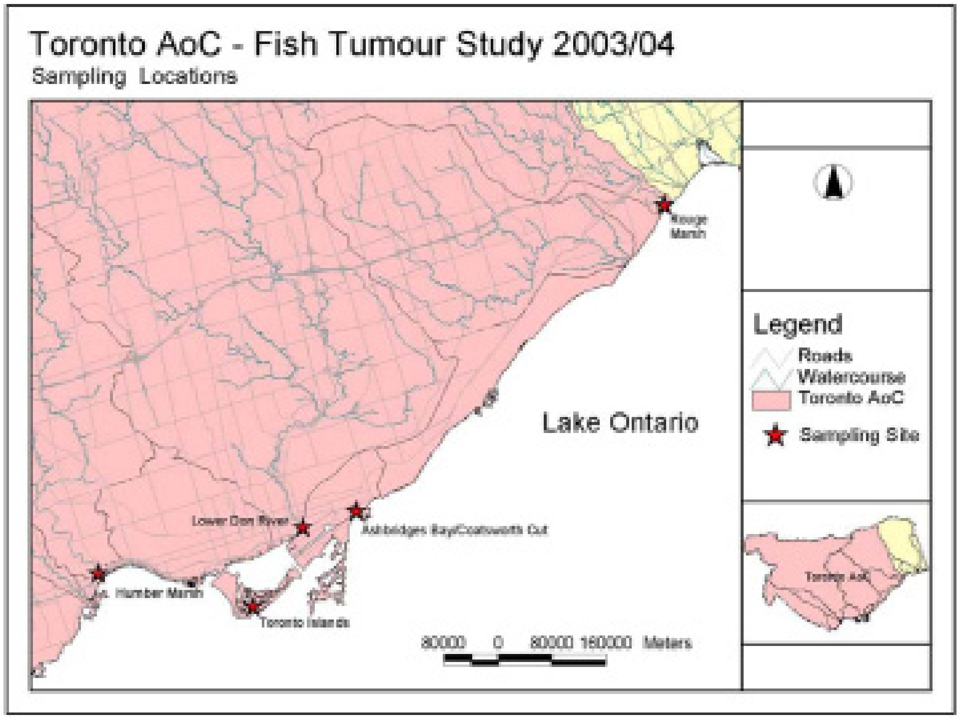
Sample size

Target sample size for the Toronto and Region AOC was established at a minimum of 250 individual fish, based on the collection of 50 individuals from five separate locations within the AOC. Previous studies (Baumann, 1992; USEPA, 1994) suggest minimum sample sizes of between 85 and 150 individuals to allow for confidence in the comparison of tumour frequencies between sites. The overall sample size of 250 individuals provided for comparison with other sites and studies, while the collection of 50 fish from each location allowed for the comparison of sites within the Toronto and Region AOC at reduced levels of confidence.

Sampling Locations

Five discrete sampling sites were selected within the Toronto and Region AOC; these sites included the Toronto Islands, Lower Don River, Humber River Marshes, Ashbridges Bay, and the Rouge Marsh (Figure 2A). Collectively, the selected sites represented areas influenced by urban, agricultural, and industrial inputs, and were distributed throughout the geographic extent of the AOC boundary at Lake Ontario.

A



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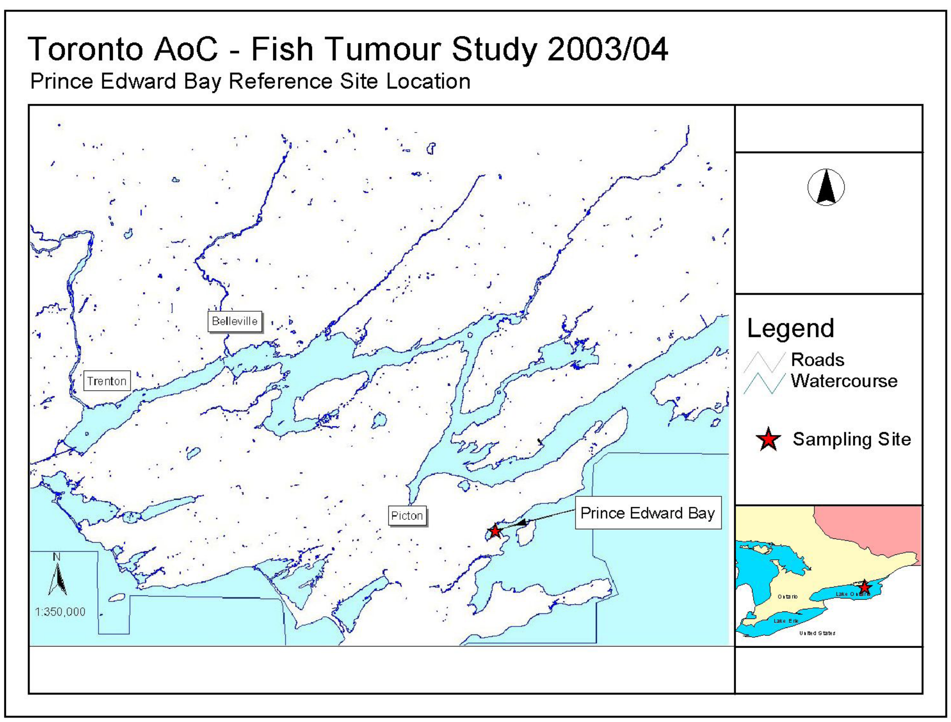

Figure 2. Brown bullhead sampling sites within the Toronto and Region Area of Concern (A) and Prince Edward Bay reference site (B)

Methods

Fish Collection

Brown bullhead were collected using a combination of electrofishing and trap netting techniques from May 26 to July 28, 2003 in the Toronto and Region AOC, and on October 6, 2004 from the Prince Edward Bay reference site (Figure 2B). The collection at Prince Edward Bay was conducted in conjunction with Environment Canada.

Bullhead were collected from the Toronto Islands, Ashbridges Bay/Coatsworth Cut, Lower Don River and Humber River Estuary using a Smith Root SR18 electrofishing vessel equipped with a 7.5kw pulsed DC electrofishing unit that allowed for the optimization of operational settings by the operator. Additional samples were collected from the Rouge River Marsh using a Smith Root SR12 electrofishing vessel. Fish collected in this manner were netted and placed in aerated live-wells prior to processing.

A limited number of bullhead were captured using trap nets deployed in Humber Marsh and Rouge Marsh for several nights. This technique was abandoned due to poor catch rates and the extra resources associated with setting and retrieving the nets.

On-site Fish Processing

Bullhead were measured and weighed immediately following capture. Captured fish were screened for size, and individuals < 250mm were released at the point of capture. Following euthanasia, the internal organs were examined and any abnormalities were noted. The gonads were also examined and the sex of the fish was recorded. The liver was then removed, and slices of liver tissue (5 mm max thickness) were sampled from four discrete quadrants of the liver. Slices were placed in plastic tissue cassettes and preserved in 10% buffered formalin. This process was standardized such that samples from each quadrant were similarly labeled in the tissue cassettes. All liver samples were preserved in formalin within 5 minutes of euthanasia. As a final measure, the right pectoral spine was removed for aging by the Ontario Federation of Angers and Hunters' Fisheries Research Unit.

Histology

Formalin-fixed liver samples were submitted to the histology lab, Animal Health Laboratories - University of Guelph, where they were processed by routine paraffin embedding followed by staining with hematoxylin and esoin for examination by light microscopy. All sections were initially examined by eye and then scanned at 16x magnification to identify altered foci or inflammatory lesions. Confirmation of lesion classification was made at 40 and 160x magnification. Sub-classifications from these, for example altered foci as acidophilic, basophilic or 'clear cell', was not performed as there was limited functional evidence to warrant sub-classification. Counts of all altered foci or neoplasms within a section were taken. Lesion size was estimated by comparison with the calibrated microscopic field of vision. All sections were examined by J.S. Lumsden, Associate Professor, Department of Pathobiology, University of Guelph. A second independent pathologist confirmed the classification of liver lesions identified by J.S. Lumsden.

Lesion Classification
Histopathology was undertaken following standard criteria for the identification of internal (liver) neoplasms. The criteria used to identify and classify phenotypically altered foci and hepatocellularneoplasms from bullheads are summarized in Table 6. These criteria have been applied in a number of published studies on environmental neoplasia rates in bullheads and were based primarily on those of Baumann *et al*. (1990) and those of M.A. Hayes, Department of Pathobiology, Ontario Veterinary College, University of Guelph.

Results

A total of 257 fish were collected from five sites within the Toronto and Region AOC (Table 7). Fifty (50) fish were collected from the Prince Edward Bay reference site. Although the study design called for the collection of 50 individuals from each of five discrete locations within the AOC, the fish captured at the mouth of the Don River and along the Toronto harbourfront were of insufficient size and number to constitute discrete sampling sites. As a result, additional fish were collected in the Toronto Islands in order to maintain an overall sample size of at least 250 fish for the Toronto and Region AOC. Fish collected from the Don River and along the harbourfront were not included in subsequent analyses.

Liver Lesions
Altered foci were identified in 20.4% of Toronto and Region AOC brown bullhead liver samples, and ranged from 17% to 26% within the four AOC sites (Table 8), with the highest incidence (26%) occurring in the Humber Marsh. Altered foci were also present in 8% of the livers taken from reference fish.

The incidence of liver neoplasms at sample locations within the Toronto and Region AOC ranged from zero to two individuals per site (Table 8), with an overall neoplasm frequency of 1.2% for the AOC. Of the three neoplasms identified, two were cholangiomas and one was a cholangiocarcinoma. Other forms of liver neoplasia including hepatoma, hepatoclelular carcinoma, and adenocarcinomas were not found. Two of the three neoplasms detected were found in fish from the Ashbridges Bay site, including the only carcinoma identified during the survey. The third neoplastic lesion (cholangioma) was found in the Rouge Marsh sample. No neoplasms were identified in any of the fish sampled from the Prince Edward Bay reference site (Table 8).

Age
The age distribution and size-at-age of the bullheads collected from the Toronto AOC sites and Prince Edward Bay reference sites are shown in Figures 3 and 4, respectively. Bullhead collected in the Toronto and Region AOC ranged from 300 mm to 390 mm, while those collected from Prince Edward Bay ranged from 250 mm to 374 mm. One fish of 265 mm collected from the Toronto Harbour was sampled. Based on aging techniques, fish collected within the 250 mm – 390 mm size range varied in age from three to 10 years of age in theToronto and Region AOC, with a predominant age group of five to seven years (Figure 3). Fish aged between three and eight years were found at the Prince Edward bay reference site, with a predominant age between three and five years (Figure 3).

Table 6. Diagnostic criteria used to classify brown bullhead hepatocellular alterations and neoplasms of the liver (Baumann *et al*. 1990)

<table>
<tr><th>Criteria</th><th>Description</th><th>Notes</th></tr>
<tr><td>Altered hepatocellular foci</td><td>Microscopic populations of discrete, distinctly phenotypically altered hepatocytes usually less than 1mm in diameter</td><td>Altered foci were not ennumerated as tumours in the present study. Altered hepatocellular foci are considered to be abnormal, and can be taken as evidence as of exposure to mutagens. Some authors have classified them as “tumours” in fish surveys becasue they are focally proliferating phenotypically altered hepatocytes considered to be the first in a multi-stage sequence of development leading to maignant neoplasms. Altered foci are not neoplasms, hawever, and many of them will eventually regress. A small number of altered foci are to be expected in the livers of aged fish.</td></tr>
<tr><td>Hepatocellular nodules (Hepatoma)</td><td>Expansive (compress surrounding tissue) but non-invasive populations of phenotypically altered (e.g. trabecular pattern with small alterations in cytology) hepatocytes in which exocrine pancreas or macrophage aggregates are reduced or absent and that are usually 1-4mm in diameter</td><td>A conservative approach was used in the classification of altered foci and neoplastic lesions. Altered foci that did not meet all the criteria of Baumann et al. (1990) were not classified as nodules. Lesion size was the least strictly applied criterion in the differentiation of an altered focus from a nodule, as differentiation on this basis is inconclusive. the most important criterion applied in the differentiation of an altered focus from a nodule was the compression of surrounding tissue (implying growth) and the reduction or absence of macrophyte aggregates and/or exocrine pancreas (Baumann et al., 1990)</td></tr>
<tr><td>Hepatocellular carcinoma</td><td>Invasive and pleomorphic populations of proliferating hepatocytes</td><td rowspan="4">These were all ennumerated as tumours. A tumour is an abmormal mass of tissue that results from excessive cell devision and perform no bodily function.</td></tr>
<tr><td>Cholangioma</td><td>Expansive but non-invasive populations of phenotypically altered biliary epithelial cells</td></tr>
<tr><td>Cholangiocarcinoma</td><td>Invasive and pleomorphic populations of proliferating bile duct epithelial cells</td></tr>
<tr><td>Pancreatic adenomas or adenocarcinomas</td><td>Expansive but non-invasive (adenoma) or invasive and pleomorphic populations of pancreatic epithelial cells</td></tr>
</table>

Table 7. Survey collection data from the Toronto and Region AOC and Prince Edward Bay reference site

Sample Site	Date	Total Sample (n)	Male (n)	Female (n)	Sex Undetermined
Toronto and Region AOC					
Humber Marsh	13/6/03 - 28/7/03	58	38	19	1
Don River/Toronto Harbour*	3/6/03	3	2	1	0
Toronto Islands	26/5/03 - 28/7/03	90	56	34	0
Ashbridges Bay	29/5/03 - 14/7/03	53	36	17	0
Rouge Marsh	25/6/03 - 10/7/03	53	32	20	1
AOC TOTAL		**257**	**164**	**91**	**2**
Reference Site					
Prince Edward Bay	6/10/04	50	31	19	0

* Samples not carried forward for further analysis

Table 8. Frequency of liver lesions observed in brown bullhead collected from the Toronto and Region AOC and Prince Edward Bay reference site

Sample Site	Year	n	Lesion Free (%)	Hepatocellular Alterations (Not Tumours)		Neoplasms (Tumours)		
				Altered Foci (%)	Hepa-toma (%)	Benign (Cholan-gioma) (%)	Malignant (Cholangio-carcinoma) (%)	Total Liver Neoplasms (%)
Toronto and Region AOC								
Humber Marsh	2003	58	72.4	25.9	0	0	0	0
Toronto Islands	2003	90	78.9	17.8	0	0	0	0
Ashbridges Bay	2003	53	75.5	17	0	1.9	1.9	3.8
Rouge Marsh	2003	53	73.6	20.8	0	1.9	0	1.9
AOC TOTAL		**254**	**75.6**	**20.4**	**0**	**0.8**	**0.4**	**1.2**
Reference Site								
Prince Edward Bay	2004	50	86	8	0	0	0	0

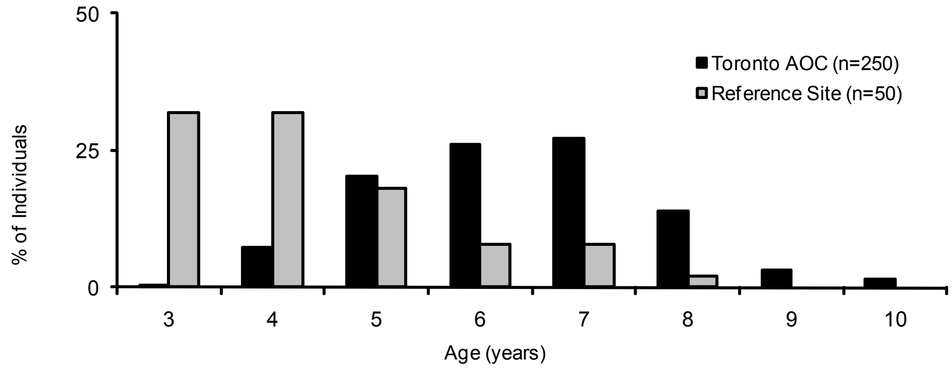

Figure 3. Age distribution of brown bullhead (>250 mm) sampled at Toronto and Region AOC and Prince Edward Bay reference site

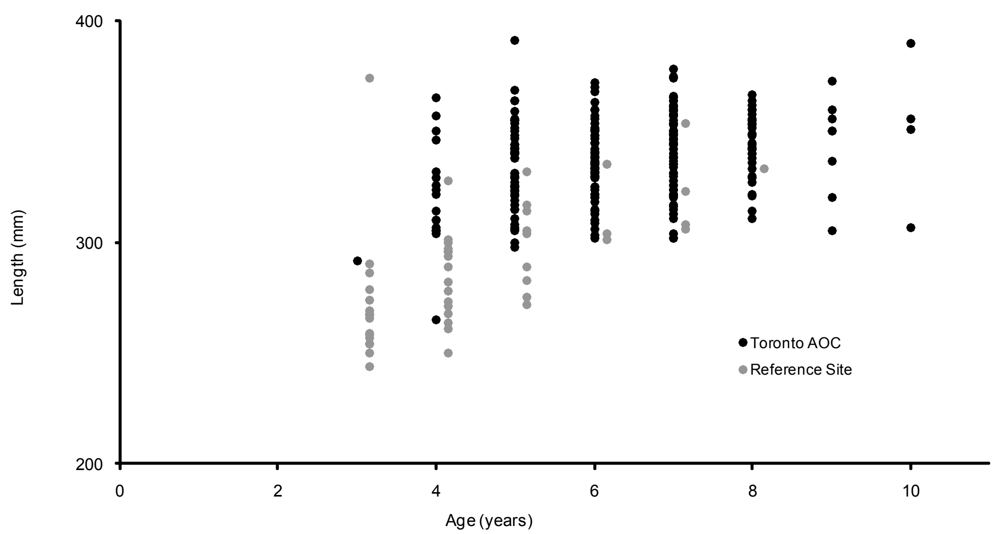

Figure 4. Size-at-age of brown bullhead from Toronto and Region AOC and Prince Edward Bay reference site

Discussion

Mature brown bullhead were found and sampled from all sub-sites examined within the Toronto and Region AOC. There were, however, poor catch rates of fish of sufficient size at the mouth of the Don River and along the Toronto Harbourfront. It is suspected that the poor catch rate at these two sites was a function of the lack of suitable spawning habitat at the sites, as high numbers (thousands) of mature spawning bullhead were concurrently observed at the nearby Toronto Island sampling site which contained suitable spawning habitat.

Although the study design targeted mature fish (> 3 years old), the histogram of the bullhead sampled in the Toronto AOC (Figure 3) suggests a well balanced age distribution within the size range sampled. There did not appear to be a significant impact on any age classes that would indicate a previous acute or chronic effect related to contaminants or contaminated sediments in the last decade as has been reported at sites with contaminated sediment (Baumann *et al*. 1998).

Liver Lesions

A low incidence of liver neoplasia was found during the 2003-04 survey. In the Toronto and Region AOC only 1.2% of the 254 fish sampled had a liver neoplasm (Table 8). Although no fish from the Prince Edward Bay reference site contained liver neoplasms, the smaller sample size (n=50) at this site may have been insufficient to detect liver neoplasms at the low incidence rate (0.6 neoplasms per 50 fish) detected in the Toronto and Region AOC.

There was little variation in liver neoplasm frequency between the four sites sampled within the AOC. As two of the three liver neoplasms were observed in fish sampled at the Ashbridges Bay site, however, this site may be considered the most highly impacted of those sampled. The potential for negative impact at this site may be a function of its location adjacent to Toronto's main sewage treatment plant and a combined sewer outfall.

The frequency of liver neoplasia observed in the present survey was lower than those reported in previous surveys within the Toronto and Region AOC. Total neoplasm rates of between 3.5% and 12.5% were reported in white suckers (*Catostomus commersoni*) from the lower reaches of AOC watersheds between 1987 to 1996 (Table 7) (V. Cairns in Toronto and Region Remedial Action Plan, 2001). These data are provided below with the strong caveat that white sucker histopathology data may not be directly comparable with those obtained from brown bullhead (V. Cairns, pers. comm.).

The low occurrence of liver tumours in fish sampled in the Toronto and Region AOC is generally congruent with reported AOC sediment contaminant concentrations. Findings of a 1995 Toronto Harbour Sediment Survey indicated that Polycyclic Aromatic Hydrocarbon (PAH) concentrations ranged approximately an order of magnitude from below the lowest effect level guideline of 4 ug/g south of Mugg's Island (Toronto Islands) to 42.9 ug/g in the vicinity of the Bathurst and Portland combined

Table 9. Liver neoplasm frequencies in white sucker (*Catostomus commersoni*) within the Toronto and Region AOC (V. Cairns in Toronto and Region Remedial Action Plan, 2001)

Sample Site	Year	n	Hepatoma (%)	Hepatocellular Carcinoma (%)	Cholangioma (%)	Cholangiocarcinoma (%)	Total Liver Neoplasms	Total Carcinomas (%)
Toronto and Region AOC								
Humber River	1987	192	0.5	0.5	1.6	1.6	4.7	1.3
Rouge River	1987	199	0	0	2.5	2.5	3.5	0.5
Don River	1994	64	1.6	1.6	3.1	3.1	12.5	8.7
Rouge River	1994	121	0	0	3.3	3.3	4.1	0.8
Humber River	1996	200	1	1	1.5	1.5	5.5	1.5

sewer overflow discharges (Figure 6). These PAH concentrations suggested moderate levels of sediment contamination but that, overall, Toronto Harbour PAH concentrations were well below the severe effect level guideline of 200-800 ug/g (OMOE, 1993; Boyd *et al*. 2001).

Two sampling factors bear examination as potential mitigating factors on the liver neoplasia rates observed in the Toronto and Region AOC. Ninety fish, or 35% of the total AOC sample, were collected from an embayment just south of the relatively uncontaminated Mugg's Island location (Figure 6). The brown bullhead collected at this location had gathered on the basis of suitable spawning habitat, however it is anticipated that these fish would likely have inhabited other locations within the Toronto Islands and Toronto Harbourfront complex over the course of the year. It is therefore not anticipated that the collection of greater numbers of fish from this area compromised the representativeness of the AOC data.

A second mitigating factor with respect to liver neoplasia rates may have been sampling of Toronto and Region AOC fish during spawning. Tumour prevalence is usually lower in the spring than in the fall, as many of the older fish (particularly those with tumours) die over the winter (Baumann, unpublished). Additionally, tumours tend to develop more quickly and become more noticeable in histopathological analysis during the summer months when fish are more metabolically active. The low rate of liver neoplasms detected, in combination with the lack of a discernable correlation between age and liver lesion prevalence, however, suggest that this phenomenon was not a substantial confounding factor in the present survey.

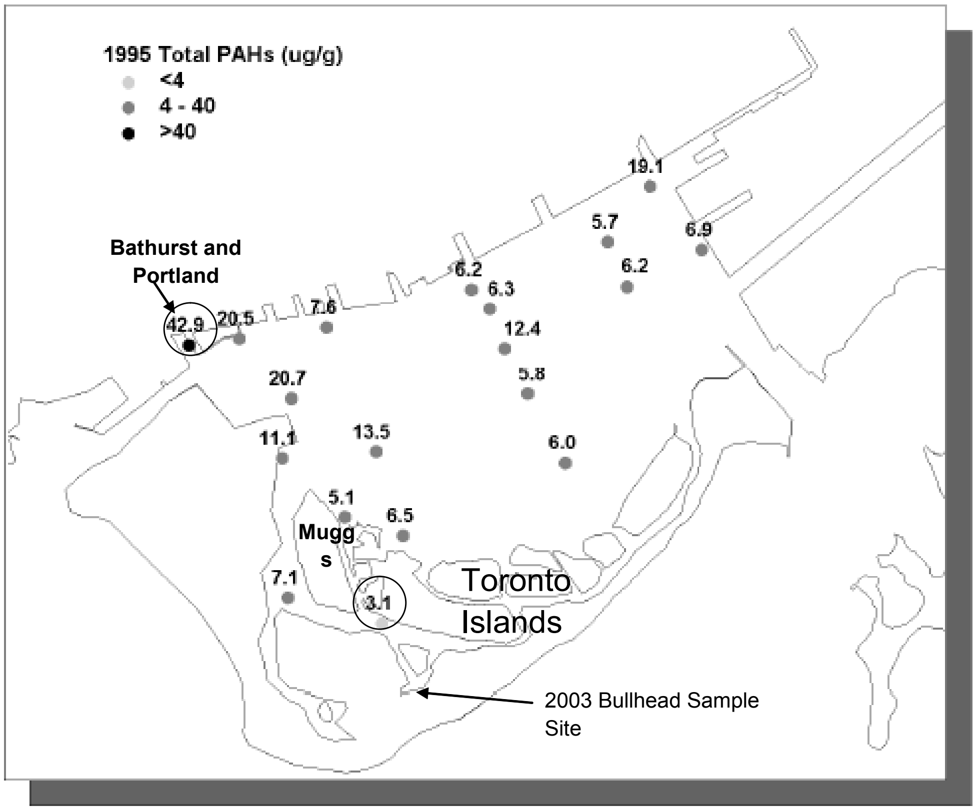

Figure 5. Toronto harbour sediment total PAH concentrations in 1995 (Boyd *et al*., 2001)

Conclusions

The 2003 Toronto and Region AOC survey data yielded a brown bullhead liver tumour incidence rate of 1.2%. This rate did not substantively differ from the liver tumour incidence observed at the Prince Edward Bay reference site (0%) and was well below the 3.8% tumour rate observed in other surveys and that did not significantly differ from the reference site/far field Impairment Criterion (Table 5). Accordingly, the liver tumour incidence rate in the Toronto and Region AOC fails to meet the first IJC impairment criterion "[t]he incidence of fish tumours or other deformities exceed rates at unimpacted control sites…" (IJC, 1991) while complying with the Toronto and Region RAP Stage II restoration target that "[t]he numbers of fish displaying diseases, tumours, ulcers, or deformities associated with the presence of toxics are reduced or maintained at levels considered background for the (fish) community" (Toronto and Region Remedial Action Plan,1994).

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