ST. CLAIR RIVER AREA OF CONCERN

Status Recommendation Report for Fish Tumours and other Deformities Beneficial Use Impairment

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St. Clair River Remedial Action Plan

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Executive Summary

The 1991 Stage 1 Report for the St. Clair River Area of Concern (AOC) identified the *Fish Tumours or Other Deformities* beneficial use impairment (BUI) as requiring further assessment (RFA) due to limited data on the prevalence of internal fish tumours other than one study conducted in 1989. In this study, one fish developed liver tissue changes after being held in a cage downstream from the industrial complex in Sarnia for two years. Around the same time, research on fish from urbanized areas around the Great Lakes suggested that chemicals in the aquatic environment were linked to the development of liver tumours in fish (Baumann et al. 1996). The 1991 Stage 1 Report also characterized the bottom sediment in the St. Clair River as having high concentrations of several chemicals resulting in the recommendation for additional scientific studies to determine the status of the *Fish Tumour or Other Deformities* beneficial use impairment (BUI) as either "impaired" or "not impaired". The BUI remained "requires further assessment" (RFA) in the 1995 Stage 2 and subsequent update reports.

Since publishing the 1995 Stage 2 Report, three scientific studies have been completed. The first study was conducted by the University of Guelph in 2002. This study collected sixty-three fish liver samples from seventeen species of fish representing different trophic levels. This study did not find liver tumours in any of the samples collected however the fish may have been too young to develop tumours and recommended older fish be examined in future studies (Hayes, 2002). In the two studies that followed, Environment and Climate Change Canada (ECCC) led the study design and sampling effort, targeting older, non-migratory, sediment dwelling, and benthivoric fish as they are good indicators of local aquatic conditions. These fish reside close to the sediments and feed on invertebrates that reside in the sediment, exposing them to sediment contaminants. The first study was conducted between 2002 and 2006 and evaluated 126 livers from shorthead redhorse suckers *(Moxostoma macrolepidotum),* a locally abundant sediment dwelling and bottom feeding fish. The second study was conducted between 2013 and 2014 and evaluated 60 livers from brown bullhead *(Ameiurus nebulosus)* collected from within the Walpole Island delta. Of the 186 livers evaluated between these two studies, no liver tumours were found.

Furthermore, in 2012, a sediment chemistry study was conducted by the University of Windsor's Great Lake Institute for Environment Research (GLIER) within the Walpole Island delta, where there was local concern for upstream contaminants settling in the bays and channels around the community. The study revealed low levels of contaminants, including polycyclic aromatic hydrocarbons (PAHs), a family of chemicals that are naturally found in coal tar, crude oil and are formed during the incomplete burning of these substances (Helmenstine, 2018). Research in the 1980's linked high concentrations of PAHs to the development of liver tumours in brown bullheads however the 2012 sediment study in the Walpole Island delta, revealed PAH concentrations were well below the provincial "lowest effect level" (LEL) threshold of 4ug/g.

Based on the scientific findings of the two above fish studies conducted by Environment and Climate Change Canada on the shorthead redhorse suckers and brown bullheads, as well as the 2012 sediment chemistry study of the Walpole Island delta by the Great Lakes Institute for Environmental Research, the Canadian Remedial Action Plan Implementation Committee (CRIC) recommends the status of *Fish tumours or other deformities* BUI be designated as "not impaired" for the Canadian portion of St. Clair River AOC.

1.0 Introduction

Tumour outbreaks in fish were first linked to environmental contaminants in the 1960s. In the 1970s, the first study was published implicating environmental carcinogens as part of the cause for papillomas (benign tumours) in white suckers in the Great Lakes. In the 1980s, the first liver cancer outbreak 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 rates in a variety of fish species in urbanized areas around the Great Lakes (Baumann et al., 1996).

Since the 1980's, extensive laboratory experiments and field studies linked a decline in the incidences of internal tumours with a decline in polynuclear aromatic hydrocarbons (PAHs) (Baumann and Harshbarger, 1995; Baumann and Okihiro, 2000), providing compelling evidence that liver neoplasms in fish are the most consistent markers of carcinogenic exposure. PAHs are a class of chemicals, some of which are carcinogenic, that are generated primarily during the incomplete combustion of fossil fuels and organic materials (e.g. coal, oil, gasoline, and wood) (Hussein I. Abdel-Shafy et al, 2015). Major man-made sources of PAHs include residential heating, coal-fired plants, gasification and liquefying plants, asphalt production, coke and aluminum production, petroleum refineries as well as and motor vehicle exhaust; making them ubiquitous environmental pollutants (Hussein I. Abdel-Shafy et al, 2015). Natural sources of PAHs include forest fires and volcanic activities.

The concern for elevated tumour rates in fish around the Great Lakes resulted in fish tumours being identified as one of fourteen beneficial use impairments (BUI) used to identify Areas of Concern (AOC) across the Great Lakes. The International Joint Commission's (IJC) guidance from 1991 stated that the beneficial use may be deemed "not impaired" only "*when the incidence rates of fish tumours or other deformities do not exceed rates at un-impacted control sites or when survey data confirm the absence of neoplastic (tumour) or pre-neoplastic liver lesions in bullheads or suckers*" (International Joint Commission, 1991). Unfortunately, pre-neoplastic lesions were not well defined nor was there definitive evidence that pre-neoplastic lesions progressed to liver tumours in either suckers or bullhead (Bunton, 1996). A similar challenge existed for external lesions, such as those found on lips and skin of suckers, as they are commonly caused by a retrovirus (Baumann and Okihiro, 2000). The inability to tease apart the interaction of contaminants and virus infection on *external* lesions is why *external* lesions are not used as a criterion to assess this BUI (Beaumann, 2010). Refer to Appendix A for more detail. The current IJC guidance focuses on internal tumours and states the impairment applies *when rates of fish tumours or other deformities exceed rates at unaffected comparison sites* (International Joint Commission, 2019).

Sediment dwelling fish, such as brown bullheads and shorthead redhorse suckers, feed on bottom sediment in waterways and therefore have higher exposure to sediment-binding contaminants, such as PAHs, than non-benthic fish. Brown bullheads tends to reside in specific areas and do not move far from their habitat. Because of their resident nature, they are a better reflection of the local aquatic environment than fish who are migratory or have large home ranges. Similar to mammals, the liver aids with digestion by secreting enzymes that break down foods, including contaminants. For this reason, the prevalence of liver neoplasms (tumours) in non-migratory, sediment dwelling and bottom-feeding fish, such as brown bullheads and suckers, was recommended as key criteria to assess this BUI.

There are two variables which might influence tumor prevalence in fish they are *age* and *gender*. Age has long been recognized as being positively correlated with tumor prevalence (Baumann, 1992) for two reasons; first, fish that have lived longer have generally been exposed to environmental contaminants longer and second, there is a latent period between induction and tumour development. Thus it is important to consider age when comparing liver neoplasm prevalence among populations (Baumann, 2010). 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) but a review of Great Lakes brown bullhead data taken at United States locations since 1991 indicated that that gender differences are not discernable but gender equivalency among samples collected for studies should be considered (Baumann, 2010).

To assess the status of this BUI in AOCs, Environment and Climate Change Canada (ECCC) conducted a comprehensive sampling effort to determine the average, or background, rate of liver neoplasms in fish from the lower and upper Great Lakes. For the lower Great Lakes, brown bullheads were collected from urbanized and non-urbanized sites between 2002 and 2006. Based on over 1100 fish collected, the prevalence of liver tumours in brown bullheads was found to be 2% (or 2 out of 100 fish). This occurrence became the accepted average rate for liver tumours in brown bullheads for the lower Great Lakes, allowing for Remedial Action Plan (RAP) Teams to compare liver tumour rates *within* the AOC to the accepted background rate *outside* the AOC. Through such a comparison, the status of this beneficial use as impaired or not impaired, could be determined. For example, if the tumour rate within the AOC was 2% or lower, the BUI could be considered “not impaired” and levels above 2% could be considered elevated and result in an “impaired” status.

For AOCs located in the upper Great Lakes, brown bullheads are less abundant so alternative indicator fish species from the sucker family were selected. Livers from the selected indicator fish were collected and compared to a suitable reference site located outside the AOC. If the tumour rate *in* the AOC was similar to the reference site located *outside* the AOC, the BUI could be considered “not impaired” as it suggests the aquatic conditions between the two sites are similar. In other words, the AOC is not worse with respect to tumour prevalence than the non-AOC site. Conversely, if tumour rates were significantly higher in the AOC compared to the reference site, the BUI could be considered “impaired”.

The St. Clair River is a connecting channel between the lower and upper Great Lakes and so the locally abundant shorthead redhorse sucker, was used in the first study designed specifically to assess the status of this beneficial use. This study was conducted from 2002 through to 2006 and used the mouth of Lake Huron, just above the Sarnia industrial complex, as the non-AOC reference site. Livers from one hundred and twenty-six shorthead redhorse suckers were collected from within the St. Clair River AOC and one hundred livers were collected from the reference location. Zero liver tumours (0%) were found in the fish collected from within the St. Clair River and one liver tumour (1%) was found in the fish collected from Lake Huron. In the second study, conducted between 2013 and 2014, in collaboration with Walpole Island First Nation, sixty brown bullhead livers were collected by community anglers from the Walpole Island delta and zero liver tumours were found. See Appendix B for the liver histopathology report on these fish.

In addition to the two specific fish studies, sediment samples were collected from the channels and bays of the Walpole Island delta which is the largest known habitat for brown bullheads. The sediment survey revealed low levels of contaminants, including PAHs. In fact, the PAH levels were well below the level associated with inducing liver neoplasms in benthic fish, such as brown bullhead (Drouillard, 2014).

The findings of the above studies are detailed below and provide compelling and sufficient scientific evidence for the Canadian RAP Implementation Committee (CRIC) to recommend the status of this BUI be designated as “not impaired”.

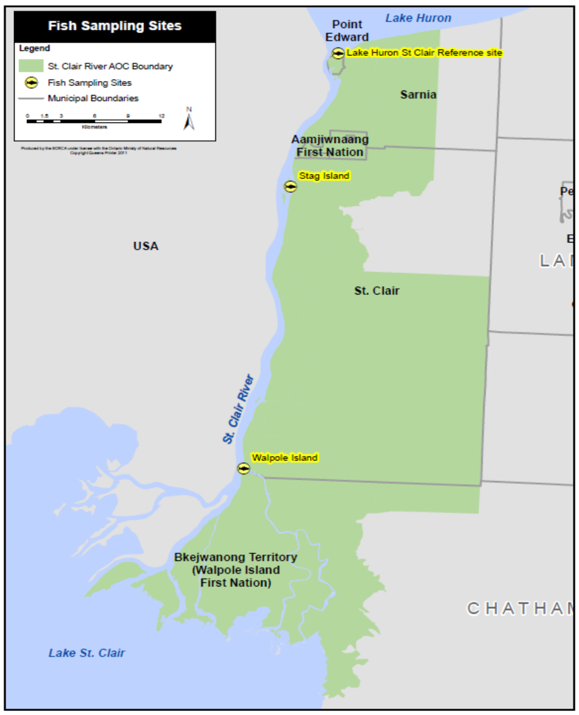
2.0 BUI Assessment Studies and Results

Study 1: 2002 - 2006 Shorthead Redhorse Sucker Study

Between 2002 and 2006, ECCC conducted a comprehensive study to evaluate the status of this BUI for the St. Clair River AOC using the locally abundant shorthead redhorse sucker, an accepted alternative indicator species to the common brown bullhead, for the evaluation of liver tumour rates.

A sample size of 126 shorthead redhorse suckers from sites along the St. Clair River and 100 from the reference site located at the mouth of the St. Clair River in Lake Huron, near the head of the St. Clair River, were collected.

Figure 1: Map of Sampling Locations



The St. Clair River was sampled using a Smith Root electrofishing boat and the Lake Huron reference site was sampled by overnight trap nets that were set by Purdey Fisheries. Following capture, fish were placed into a live well for transportation to the fish sampling site. Fish were anaesthetized in a clove oil bath (~0.05% + ~0.025% ethanol to aid emulsification), then were euthanized using standard operating procedures, and their physical state was assessed using a visual examination for physical abnormalities (Baumann, 2010). 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. The liver samples were then examined for neoplastic tumours at the Freshwater Institute in Winnipeg, Manitoba.

As per Table 2, the male/female ratio was similar at the AOC and reference location. The samples taken from the St. Clair River were markedly older (by 4 years) than those from the reference site and although this should imply a higher tumour rate because of the older age, the tumour prevalence was not higher (Baumann, 2010). The livers were divided into sections and the number of sections taken per liver were the same for both locations.

Table 2. Sample size, age, gender proportion and number of liver sections taken from the St. Clair River AOC and reference location between 2002 and 2006.

Location	Sample Size	Median Age	Percent Female	Sections/Liver
St Clair River	126	10	41%	2-4 (range)
Lake Huron	100	6	42%	2-4 (range)

As illustrated in Table 3, no liver neoplasms were found in the shorthead redhorse suckers sampled from within the St. Clair River however one was found in the reference site, located above the industrial complex on Lake Huron.

Table 3. Tumour prevalence in the St. Clair River and Lake Huron, the reference site**.**

Location	Sample Size	Neoplasm #	% Neoplasms
St Clair River	126	0	**0%**
Lake Huron	100	1	**1%**

Although the findings from this study supported a “not impaired” status recommendation, concern was expressed by Walpole Island First Nation that sampling within the delta was limited and that no other AOC had used the shorthead redhorse sucker as an indicator species for this BUI, limiting the ability to compare tumour prevalence spatially. The community was also concerned that upstream contaminates were accumulating in the sediment around their community and confirmed that brown bullheads resided in the channels and bays around Walpole Island. To address the local concerns and augment existing science on the BUI, a second study was planned and jointly executed with Walpole Island First Nation in 2013 and 2014.

Study 2: 2013-2014 Brown Bullhead Study

In collaboration with the Walpole Island First Nation, a total of 60 brown bullhead livers were successfully collected over a two year period using fishermen from the Walpole Island community. The brown bullheads were caught in fyke nets that were set overnight in multiple locations along the Johnson and Chemetogan Channels, both of which are connected to the St. Clair River. The fyke nets were checked daily and captured fish were removed using hand nets and placed in oxygenated carrying containers while being transported to large near-shore holding pens immersed in the St. Clair River. From there, fish were transported by boat or truck in oxygenated coolers to an on-site laboratory set up by ECCC for the project.

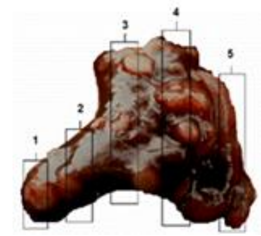
	
Walpole Island project fishermen (2014)	On site field lab at Walpole Island (2014)

At the on-site field laboratory, fish were removed from the carrying containers and were anaesthetized in a clove oil bath and then euthanized using Environment Canada's standard operating procedures (Environment Canada AU-0816). Each fish was visually examined for physical abnormalities, measured (fork length (mm)) and weighed (±0.1g). Pectoral spines were collected for aging and the liver was removed and sliced into three to five sections for histology (liver tumour analysis) (Blazer et al., 2007). The livers were stored in Davidson's Fixative then transferred to a 70% ethanol preservative at the ECCC lab 1-4 weeks after collection. The prepared livers were sent to the British Columbia Ministry of Agriculture (Dr. Gary D. Marty and Heindrich N. Snyman) for a histological evaluation.

Figure 2a: Field lab at WIFN to process fish.

Figure 2b: Fish processing.

Figure 2c: Sample histology slide (each liver is evenly divided into five samples if possible)



Of the 60 brown bullheads sampled, the majority of the fish were four to eight years old and the ratio of male to female was very close, as presented in Table 5.

Table 5. Walpole Island age range and gender (60 brown bullheads were caught; 31 were male, 28 female and 1 unknown)

Age Range	# Male	# Female	#Unknown Sex	Total # within age range	% Male within age range	% Female within age range
2-3	6	4	0	10	60%	40%
4-5	15	15	1	31	50%	50%
6-8	10	9	0	19	53%	47%
TOTAL	31	28	1	**= 60**		

The evaluation of the liver slides followed the *Blazer Protocol*, a process developed by experts with the U.S. Geological Survey and U.S. Environmental Protection Agency for the systematic identification of liver tumours in brown bullhead (Blazer et al., 2007). The *Blazer Protocol* has become the standard approach for identifying fish tumours and determining tumour rates in the Canadian Great Lakes Areas of Concern, including Jackfish Bay, St. Mary's River, Thunder Bay, St. Lawrence River, the Bay of Quinte, Hamilton Harbour, and Toronto Harbour.

To ensure the analysis and diagnosis of liver samples could produce a high-degree of confidence that detected tumours are linked to chemical contaminants in the environment, the 2013 and 2014 samples were examined for the following abnormalities:

- Neoplastic Hepatocellular Lesions - Hepatocellular Adenoma and Hepatocellular Carcinoma; which are cancers in the liver; and
- Neoplastic Biliary Lesions – Cholangioma and Cholangiocarcinoma; which are cancers in the bile duct.

For each liver sample, every section was systematically scanned using the 4× objective lens (low power), and then a single section was systematically scanned using the 10× objective lens (medium power). When needed, higher magnification (20× and 40×) was used.

Zero liver neoplasms were found in the 60 livers examined by experts in the Ministry of Agriculture, British Columbia. The histopathology report (Appendix B) notes:

"*The lack of neoplasia and the low prevalence of pre-neoplastic foci and lesions associated with toxin exposure provide evidence that chronic toxin exposure is not significantly affecting liver morphology in these fish populations. Most of the liver lesions in these bullheads are probably related to the presence of parasites, with 85% of the livers hosting at least one parasite. …"*

While 100 fish is the recommended sample size for this study, the results of the 2012 sediment chemistry study (described below) in the Walpole Island delta were published in 2014 and revealed low contaminant levels, including PAHs. Given the sediment chemistry results and lack of neoplastic lesions in the 60 brown bullhead livers analyzed, there was agreement between WIFN and ECCC that additional liver samples were not necessary.

3.0 Sediment Chemistry Survey Results of the Walpole Island delta

In 2004/05 and 2012, the University of Windsor Great Lakes Institute for Environmental Research conducted sediment chemistry surveys in partnership with the Walpole Island Heritage Centre of Walpole Island First Nation (WIFN) to collect and analyze sediment samples in the Walpole Island delta.

In the 2004/05 survey, sediment samples were collected and analyzed for priority contaminants from upstream and downstream sections of Chenal Ecarte, Johnston Channel, Chematogen Channel and Basset Channel (Figure 2a). These contaminants included organic pesticides (e.g., polychlorinated biphenyls (PCBs), octachlorostyrene (OCS) and trace elements (e.g., mercury). A total of 38 sediment samples were collected.

In the 2012 sediment survey (Figure 2b), the University of Windsor research team included all the sites examined during the 2004/05 survey as well as three additional locations within the Walpole Island delta in consultation with Walpole Island First Nation. Two of the additional sites were adjacent to Seaway Island in the South Channel, an area of interest for the community, and the third sampling station was located in Lake St. Clair. Researchers collected a total of 48 sediment samples during the 2012 survey.

As illustrated in Figure 3, sum PAH concentrations for individual sampling locations are presented for the survey conducted in 2004/2005 and 2012 (Drouillard, 2014). All sites were well below the provincial Lowest Effect Level (LEL) of 4 µg/g as shown by the dashed line. The Lowest Effect Level is a level of contamination that can be tolerated by the majority (95%) of sediment-dwelling organisms. Sediments meeting the LEL are considered clean to marginally polluted. The Severe Effect Level (SEL) is 10,000 µg/g which is the concentration where biological effects are expected to occur frequently. The bars represent the mean concentration at the site and the error bars represent standard error for sites where triplicate samples were taken.

Figure 2a: 2005 sampling locations

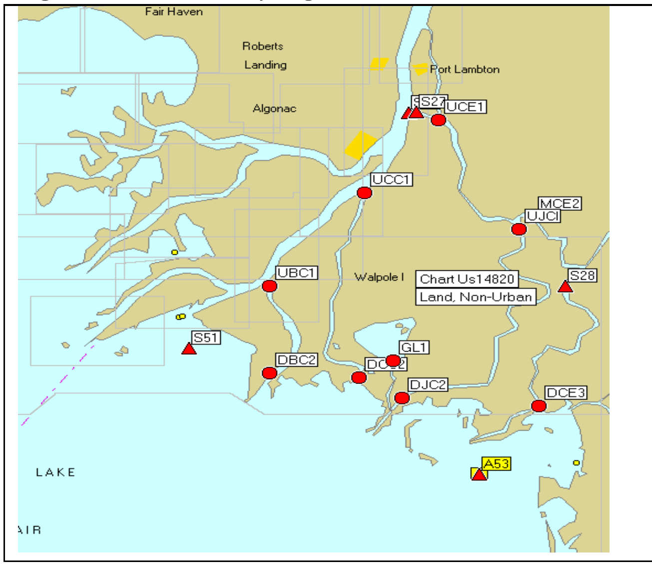
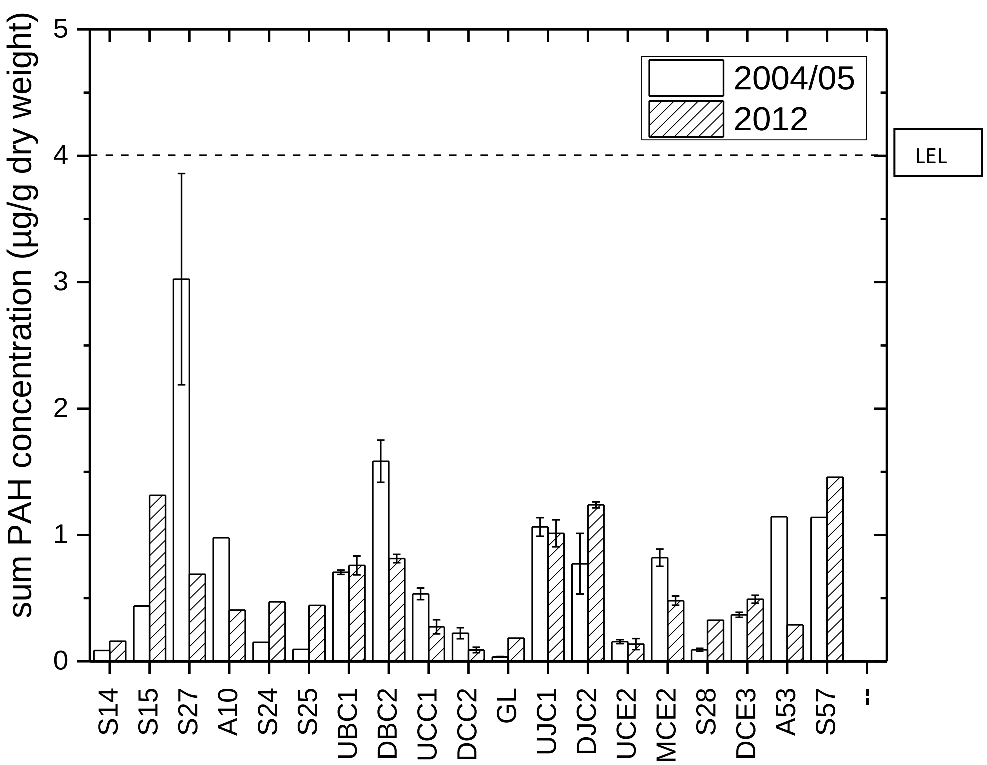


Figure 2b: 2012 sampling locations

Figure 3: A comparison of sum PAH concentrations for individual sampling locations between the two sampling efforts (2004/5 and 2012).



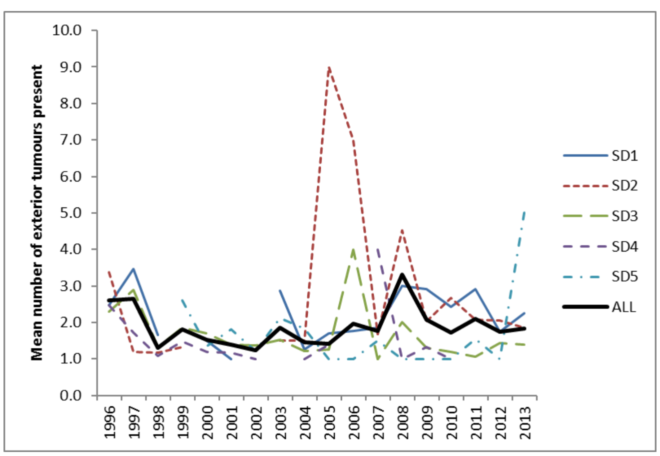
4. Walleye Monitoring for External Lesions and Internal Tumours

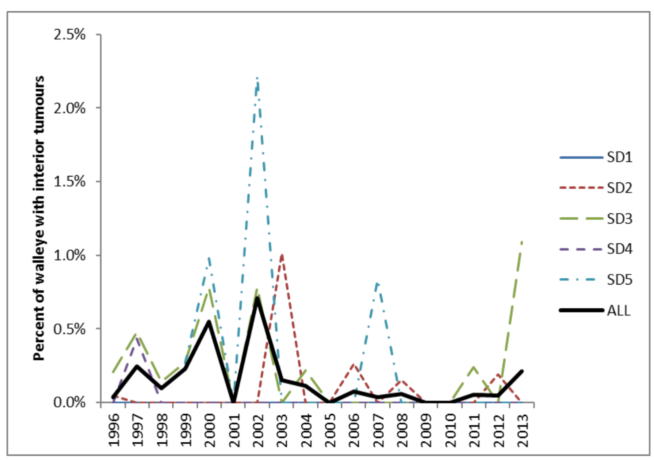
While there is no relevance of external walleye lesions to the status of this BUI, local concern for external lesions on walleye was first raised in the 1980's and persists today. The purpose of this brief section is to summarize the study conducted to address the local concerns on external walleye lesions and to highlight the continued monitoring of walleye internal and external lesions by the Ontario Ministry of Natural Resources.

In 1987, the Ontario Ministry of the Environment conducted a study on the spawning population of walleye (*Stizostedion vitreum*) in the Thames River, which includes walleye from the Detroit River, Lake St. Clair, the St. Clair River and lower Lake Huron (OMOE, 1991). The study examined five hundred and ninety fish and concluded that the 9% incidence of external lesions was not unusually high compared to other walleye populations suggesting contaminants in Lakes St. Clair, Erie and Huron, and the St. Clair and Detroit Rivers, are not inducing an abnormal incidence of external lesions (OMOE, 1991). Histological examinations were made on 72% (79/109) of the individual lesions and confirmed that the skin lesions were predominantly lymphocystis (68%), a cauliflower-like growth on the skin or fins, or dermal sarcoma (30%) which are dome or spherical shaped pale coloured nodules (OMOE, 1991). Both of these external skin lesions are caused by viral infections (OMOE, 1991), not contaminant exposure.

The Ontario Ministry of Natural Resources and Forestry (MNRF) monitors walleye populations around the Great Lakes to support annual harvest quotas. Part of the population monitoring includes observing internal and external tumours and while internal tumours are *not* scientifically analyzed, they are *assumed* to be cancerous (Drouin, R. personal communication). As indicated in Figure 4, the *internal* tumour rate for walleye in the west basin of Lake Erie (SD1), is 0% which is why the blue line is hardly visible. This is the population that transits the Huron-Erie corridor and includes the St. Clair River, Lake St. Clair and the Detroit River. The internal tumour rate of Lake Erie, as a whole, is approximately 1% (1 out of 100 fish). This rate of internal tumours is considered to be very low (Drouin, R. personal communication). Exterior lesions are also low.

Figure 4: Mean number of external lesions and percent of walleye with internal tumours collected from Lake Erie. The west basin population (SD1) is most relevant to the AOC.





Legend: SD1 = West Basin (the population that transits the Huron-Erie corridor, including the St. Clair River). The SD1 line for *internal* tumours (right chart) is hardly visible as it is 0%.
SD2 = West Central Basin
SD3 = East Central Basin
SD4 and 5 = East Basin

The fact that walleye are transitory and are not sediment dwelling fish, feeding out in the open water makes them poor indicators of the local aquatic environment and therefore not a good indicator for the assessment of this beneficial use, which quite simply, aims to determine if sediment contamination in AOCs is inducing liver tumours in fish.

5. Conclusion and Status Recommendation

In the 1991 Stage 1 Report and subsequent reports, the *Fish tumours or other deformities* beneficial use was deemed as "requiring further assessment" (RFA). Since 2000, two fish studies were specifically designed and conducted to assess the status of this BUI as well as a sediment survey in the Walpole Island delta to assess contaminant levels in the bays and channels of Walpole Island, an area where sedimentation can occur due to slower moving water and significant fish habitat exists.

To determine the status of this BUI, the CRIC used the most current assessment guidance for this BUI which states *the BUI could be considered Not Impaired when the rates of fish tumours or other deformities (in the AOC) do not exceed rates at un-impacted control sites* (IJC, 2019*).*

The most recent fish studies conducted in 2006 through to 2006 and 2013 through to 2014 and the sediment chemistry study conducted in 2012, revealed:

a) The rates of fish tumours in the St. Clair River AOC do not exceed the rates at an un-impacted control site. In the study conducted in 2002 – 2006 on the shorthead redhorse sucker, no (0%) liver tumours were found in the fish collected from St. Clair River.
b) Of the sixty (60) brown bullhead livers collected in the 2013-14 study from the waters around Walpole Island, no (zero) liver tumours were found; and
c) The mean total PAH levels in the bays and channels of Walpole Island were well below 4 ug/g, the provincial lowest effects level (LEL) and more than one thousand times lower than the severe effects level (SEL).

Based on the findings of the above studies, the Canadian RAP Implementation Committee recommends the status of the *Fish Tumours or Other Deformities* BUI be designated as **Not Impaired** for the Canadian portion of St. Clair River Area of Concern.

6. Glossary:

Basophilic: Relating to tissue components that stain readily with basic dyes used for microscopic examination.

Cholangiocellular: Pertaining to the bile ducts.

Clear cell: A type of cell, especially a neoplastic one, which does not take on a color with the ordinary tissue stains used for microscopic examination.

Eosinophilic: Containing the white blood cells eosinophils. The cytoplasm (the fluid environment inside the cell) of eosinophils is filled with coarse, refractile granules that stain intensely with acid dyes.

Epithelia: Membranous tissue covering internal organs and other internal surfaces of the body.

Epizootics: An epidemic outbreak of disease in an animal population.

Etiology: The cause or origin of a disease.

Far field site: Far field site is generally further downstream of the exposed site and is often used to look for improved fish performance with dilution of the contaminant source.

Fibrosus: A proliferation of fibrous connective tissue that occurs normally in the formation of scar tissue to replace tissue lost through injury or infection.

Foci: Plural of focus. The origin or centre of a disseminated disease.

Hepatic: Having to do with the liver.

Hepatocellular: Of or pertaining to the cells of the liver.

Indicator species: An organism that serves as a measure of the environmental conditions that exist in a given locale.

Medaka: A small Japanese fish (Oryzias latipes) commonly found in rice fields and often used in biological research.

Myxozoan parasite: The Myxozoa are a group of parasitic animals of aquatic environments.

Necropsied: Autopsy: An examination and dissection of a dead body to determine cause of death or the changes produced by disease.

Neoplasia: The process of abnormal and uncontrolled growth of cells.

Neoplastic: An abnormal new growth of tissue in animals or plants; a tumour.

Operculum: Is the hard bony flap covering and protecting the gills. In most fish, the rear edge of the operculum roughly marks the division between the head and the body.

Papillomas: A benign tumour growing exophytically (outwardly projecting) in finger-like fronds.

Preneoplastic: Before the formation of a tumor.

Reference Site: A sampling site selected for its relatively undisturbed conditions, generally upstream of the exposed zone in a river system. It is a specific locality which is unimpaired or minimally impaired and has habitat similar to that of the exposed zone. At sites within a lake, a Reference site is a site as similar as possible to the Exposure Zone except for the presence of the contamination source.

Retrovirus: Retroviruses belong to the Retroviridae family of viruses. The genetic material of retroviruses consists of ribonucleic acid (RNA), instead of deoxyribonucleic acid (DNA). Retroviruses are known to lead to certain types of cancers.

Vacuolated: Formed into or containing one or more small membrane-bound cavities within a cell.

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Appendix A:

Data Analysis and Fish Tumor BUI Assessment For Lake Superior and the St. Clair River AOCs

Paul C. Baumann, PhD.

Submitted to: Environment Canada

March 31, 2010

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 un-impacted 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 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 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 <u>tumor prevalence of less than 5%,</u> 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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Appendix B

FINAL HISTOPATHOLOGY REPORT
Case #: 2013/2014 WI Brown Bullhead
Sample year = 2013 and 2014
Species = brown bullhead (BrB)
Pathologists: Gary D. Marty, DVM, Ph.D., Diplomate, A.C.V.P.
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Report Date: March 16, 2016

Diagnoses: For details of microscopic findings in the liver, see the e-mailed spreadsheet <2013-2014WIBullhead_LiverHistopathology-Final.xlsx>. In the spreadsheet, the same data are sorted by age groups (worksheet "Data by age") and by sex (worksheet "Data by sex"). Diagnoses related to the lip samples from fish #41 follow:

1. Lip: papillary hyperplasia, regionally diffuse, moderate (slide 41-1 Upper Lip Lesion), severe (slide 41-1 Lower Lip Lesion)

Final Comment: The lack of neoplasia and the low prevalence of preneoplastic foci and lesions associated with toxin exposure provide evidence that chronic toxin exposure is not significantly affecting liver morphology in these fish populations. Most of the liver lesions in these bullheads are probably related to the presence of parasites, with 85% of the livers hosting at least one parasite, and three livers each hosting four different parasite species. Lesions more likely to be associated with contaminant exposure are uncommon: biliary ductular cell hyperplasia (12%), eosinophilic foci of cellular alteration (5%), spongiosis hepatis (3.3%), basophilic foci of cellular alteration (1.7%), and hepatocellular megalocytosis/ karyomegaly (1.7%).

The livers have other lesions that are not clearly related to parasite infestation, but most of these are probably within the range of background lesions that affect populations of brown bullhead, regardless of toxin exposure.

For the lip samples, the diagnosis of proliferative lesions as "papillary hyperplasia" rather than "papilloma" is based on the relatively normal progression of maturation in both lesions. Papillary hyperplasia would be expected to regress if the inciting stimulus was removed, whereas papillomas would not be expected to regress after removal of the inciting stimulus. We are not aware that research has been done to support predictions about the outcome of proliferative lip lesions in brown bullhead.

Age-related comparisons: Liver histopathology results were combined into three groups: 2 and 3 years old (n = 10); 4 and 5 years old (n = 31); and, 6 – 8 years old (n = 19). These groupings revealed trends in several microscopic features:

1. Lesions that **occurred in older groups but did not affect any fish in the age 2 – 3 group** – biliary ductular cell hyperplasia, cholangitis/pericholangial leukocytes, and clear cell foci;
2. Lesions that **occurred in all groups, but were less common or severe in the age 2 – 3 group than in the older groups** – pigmented macrophage aggregates, granulomatous inflammation, ascarid parasites, and acanthocephalan parasites;
3. Lesions that **occurred in all groups, but were more common in the age 2 – 3 group than in the older groups** – sinusoidal congestion, non-ascarid nematode parasites;

4. Lesions that **occurred in younger groups but did not affect any fish in the age 6 – 8 groups** – focal/multifocal necrosis, and trematode parasites;
5. Lesions that **occurred in all groups, but were less common in the age 6 – 8 group than in the younger groups** – fibrous capsule granuloma, and peritonitis;
6. Lesion that **occurred in the age 6 – 8 group but did not affect any fish in the younger groups** – eosinophilic cell foci.

Sex-related comparisons: The prevalence or severity of several microscopic features tended to be different based on sex:

1. **More common in females** – pigmented macrophage aggregates, granulomatous hepatitis, intraluminal myxosporean parasites, cestode parasites, ascarid parasites, and non-ascarid nematode parasites;
2. **More common in males** – hepatocellular glycogen, trematode parasites, and acanthocephalan parasites.

Lip Histopathology: The sections of the lip/oral mucosa from fish# 41 have similar features that are consistent with a proliferative response, but the lesions are probably not cancerous. The "Upper Tissue Lesion" is composed of lip mucosa and submucosa; the thickness of the epithelium varies from 80 – 300 µm, and one margin of hyperplastic mucosa and submucosa is about 1 mm in diameter. The "Lower Tissue Lesion" is composed of lip mucosa and submucosa; the thickness of the epithelium varies from 60 – 500 µm, and one margin of hyperplastic mucosa and submucosa is about 2 mm in diameter. This lesion has narrow rete epithelial pegs that extend another 500 µm into the submucosa. Some of these pegs lack a connection with the overlying epithelium in the plane of section, but this is probably a function of the cut rather than evidence of invasion through the basement membrane. Differentiation of these proliferative foci is in ordered progression and seems to be normal.

General comments on microscopic liver feature that were scored for this group (in alphabetical order):

ACN = acanthocephalan parasites. Acanthocephalans are common in the abdominal cavity and viscera (e.g., liver) of fish captured from the wild. They are differentiated from other helminthes based on a hypodermis thicker than muscle layers, lacunar channels, embryonated eggs, a spiny proboscis, and lack of a digestive tract.

AIB = amphophilic/eosinophilic inclusion bodies. Amphophilic inclusion bodies in the liver might be remnants of ingested erythrocytes (this type of inclusion has not been described with any fish virus). They are nearly always associated with multifocal sinusoidal congestion (SSC), but many cases of SSC do not have AIB.

ASC = ascarid nematode parasites. Ascarids are a type of nematode parasite that in the liver are usually coiled and have distinctive cuticular longitudinal lateral alae. The parasites are usually surrounded by granulomatous inflammation.

BCF = basophilic foci of cellular alteration. Foci of cellular alteration are identified by subtle changes in staining pattern and morphology that differentiate a group of hepatocytes from the bulk of hepatocytes in the liver. Basophilic cell foci are composed of a well-delineated group of hepatocytes with cytoplasm that stains more basophilic than surrounding hepatocytes. Basophilic cell foci are considered to be preneoplastic.

BDH = biliary ductular cell hyperplasia. Hyperplasia of biliary ductular epithelial cells is evidence of exposure to toxins. The toxins could be produced inside the fish (e.g., bacterial toxins) or come from outside the fish (e.g., from the water or food). As a differential, ductular cell hyperplasia might be secondary to biliary obstruction (e.g., from parasites or inflammation).

CAF = cholangiofibrosis. Fibrosis of the biliary tract is most commonly a result of repair following ulceration or necrosis. Fibrosis that accompanies biliary hyperplasia is not scored here; instead, it is scored as part of either BDH or CPL. None of the bullhead livers were scored with cholangiofibrosis.

CCF = clear cellular foci. Foci of cellular alteration are identified by subtle changes in staining pattern and morphology that differentiate a group of hepatocytes from the bulk of hepatocytes in the liver. Clear cell foci are composed of a well-delineated group of hepatocytes with clear wispy to angular cytoplasmic vacuoles (probably glycogen). Clear cell foci are considered to be preneoplastic in rats, but we are not aware of studies that have made this link in fish.

CES = Cestode parasites. Cestode are common in the abdominal cavity and viscera (e.g., liver) of fish captured from the wild. They are differentiated from other helminths based on a parenchymatous body with an internal ring of smooth muscle and no digestive tract; adult cestodes usually have calcareous corpuscles. Because of the relative large size of the species affecting these bullheads (up to 4 mm long and 0.3 mm in diameter), even single organisms might adversely affect fish health. Hepatic cestodes have previously been identified in brown bullhead by histopathology (Figure 6, "helminth parasites", Blazer et al. 2006).

CPL = cholangitis/pericholangial leukocytes (liver). Inflammation in and around bile ductules (mostly lymphocytes) is evidence of chronic immune stimulation. This type of inflammation can result from bacteria ascending from the intestine to the liver through the biliary system; the cause is usually not determined.

ECF = eosinophilic foci of cellular alteration. Foci of cellular alteration are identified by subtle changes in staining pattern and morphology that differentiate a group of hepatocytes from the bulk of hepatocytes in the liver. Eosinophilic cell foci are composed of a well-delineated group of hepatocytes with cytoplasm that stains more eosinophilic than surrounding hepatocytes. Eosinophilic cell foci are considered to be preneoplastic in rats, but we are not aware of studies that have clearly made this link in fish.

EGF = eosinophilic granular cell foci. Foci of cellular alteration are identified by subtle changes in staining pattern and morphology that differentiate a group of hepatocytes from the bulk of hepatocytes in the liver. Eosinophilic granular cell foci are composed of a well-delineated group of hepatocytes with distinctly granular eosinophilic cytoplasm (e.g., fish #42, slide 3-1). Because descriptions of ECF in fish livers include the presence of eosinophilic granules (Blazer et al. 2006; Boorman et al. 1997), EGF might be a type of ECF. However, available photomicrographs do not match the features of the EGF in the current study, so we diagnose them separately.

FCG = Fibrous capsule granulomas. Fibrous capsule granulomas are fairly common in fish that are captured from the wild and/or reared outdoors. Contents of fibrous capsule granulomas vary from viable parasites (e.g., metacercariae in fish #46, slide 2-1), degenerating parasites, other foreign bodies (e.g., fish #52, slide 2-1), to amorphous eosinophilic debris, which might be indigestible remnants of foreign bodies or long-dead parasites. Mild cases are probably of little significance for fish survival.

FPL = focal/multifocal parenchymal leukocytes. This category is used for foci of leukocytes that are not obviously associated with bile ductules, veins, or arteries. In some cases, foci of parenchymal leukocytes might include increased numbers of biliary preductular epithelial cells. In other cases, many of the cells are hematopoietic (blood forming) cells. The foci probably develop in response to chronic immune stimulation (e.g., focal bacteria, parasites, or other antigens), but the exact cause is rarely determined.

GLY = hepatocellular glycogen. Glycogen is a common form of readily available energy that is stored by many fish species in their liver cells (hepatocytes). Lack of hepatocellular glycogen is sometimes a result of food assimilation that is not adequate to supply energy needs; it occurs most commonly in fish that go off feed, but it can also be an indicator of inadequate nutrition. In general, glycogen will be depleted within about 2 days in healthy fish that stop feeding. In many fish during their spawning season, hepatocellular glycogen depletion is common and probably normal.

HCH = Hepatocyte hyalinization. Affected hepatocytes contain homogeneous cytoplasmic inclusions, probably as a result of cellular degeneration. The inclusions might be phagocytosed cellular debris or plasma protein, or accumulation of protein synthesized in hepatocytes. Features of the inclusions are not consistent with viral inclusions. The inclusions are not associated with inflammation.

IMP = intraluminal myxosporean plasmodium parasites. Plasmodia of myxosporeans are common in the biliary system of wild fish. As in this case, they are generally well adapted to their host and cause minimal lesions. Myxosporean plasmodia have been identified in brown bullhead by histopathology (Figure 6 in Blazer et al. 2006), but further work (e.g., special stains, genetic analysis) is needed to speciate the plasmodia.

LFN = focal/multifocal necrosis. Hepatocellular necrosis can be caused by inadequate vascular perfusion (e.g., as occurs with harmful algal blooms or hypoxia), direct cytotoxicity from viral or bacterial infections, or other toxin exposure; the cause is not determined in most cases. In this case, the necrosis is most commonly associated with what seem like parasite migration tracks. Lack of proliferative lesions in the biliary system is evidence against a chronic toxic cause for the hepatic necrosis.

LGR = granulomatous inflammation. Foci of granulomatous inflammation in the liver are evidence of persistent foreign material. In wild fish, this occurs in response to persistent bacteria, fungi, or parasites. Sometimes the foreign material is surrounded by multinucleate giant cell macrophages. When the inflammatory response is mostly a fibrous capsule surrounding a central core with few inflammatory cells, it is not scored here; instead, it is scored as part of FCG above.

MEG = hepatocellular karyomegaly and megalocytosis. Hepatic megalocytosis and karyomegaly can result from exposure to several types of toxins, including aflatoxins, pyrrolizidine alkaloids, complex chemical mixtures from marine sediment extracts, and the algal toxin microcystin-LR.

NEM = nematodes (not obviously ascarids) parasites. Section of nematodes are differentiated from other helminthes based on the presence of a body cavity (= pseudocoelom). Lateral alae on ascarid nematodes are sufficient to score them in their own category (ASC); therefore, the NEM category is used only for nematodes that do not have obvious features of ascarids.

PER = peritonitis/coelomitis. Peritonitis is consistent with a reaction to foreign material; it is common in fish with parasites or inflammatory mediators in the visceral cavity. The plane of section might not include the inciting parasite.

PMA = Pigmented macrophage aggregates. Pigmented macrophage aggregates are irregular spherical structures that are a normal component of parenchymatous organs (liver, kidney, and spleen) of fish, amphibians, and some reptiles. Lipofuscin and iron are the most common nonmelanin pigments, and three main functions have been identified (Wolke 1992): 1) immunity, including humoral and cellular responses; 2) storage, destruction, or detoxification of exogenous and endogenous substances; and 3) iron recycling. Accumulation of lipofuscin in the liver is a nonspecific change that can result from a variety of insults, including rancid feed, low levels of antioxidants in the feed, chronic infections, and exposure to organic contaminants; it is more common in older fish. Conditions that lead to moderate to abundant hepatic lipofuscin have been associated with decreased growth and survival in several studies. Accumulation of hemosiderin might result from increased turnover of red blood cells.

PVL = perivascular leukocytes. Leukocytic inflammation around vessels in the liver is evidence of chronic immune stimulation. Differentials include a low-grade bacterial infection, but the cause is rarely determined. The lesions in this case have no obvious infectious agents.

SPH = spongiosis hepatis. Affected foci are composed of irregularly spherical or angular spaces surrounded by narrow strands of net-like connective tissue. The spaces are often filled with pale proteinaceous fluid and small numbers of lymphocytes. Foci of SPH are often associated with bile ductules. Cystic degeneration is an alternative term for this lesion (Wolf and Wolfe 2005).

SSC = sinusoidal congestion. Sinusoidal congestion in the liver is either a nonspecific vascular lesion or a result of pooling of blood after death (i.e., passive postmortem congestion). Sometimes sinusoidal congestion results from intrahepatic occlusive thrombi; here, three of the livers contain thrombi, but the thrombi are not clearly associated with foci of congestion. Differentials include algal toxins, other toxins, substances released from inflammatory cells or bacteria, and infection with a virus; the cause is usually not determined.

TMT = trematode parasites. Digenetic trematodes have several life stages, but only metacercariae occur in these samples. Metacercariae tend to be roughly spherical and surrounded by a fibrous capsule produced by the fish. They are differentiated from other helminthes based on a parenchymatous body with a digestive tract, an oral sucker, and an acetabulum.

VAC = vacuoles in hepatocellular cytoplasm. Hepatocellular vacuoles might be a result of accumulation of cytoplasmic lipid, glycogen, air, or isotonic fluid. The contents of these vacuoles cannot be differentiated with confidence based on standard H&E staining. Vacuoles in this category tend to be angular, and affected cells have no more than a few of these vacuoles per cell. When vacuole morphology is characteristic of lipid, glycogen, or cell swelling, then the specific diagnoses are used (LIP, GLY, or HHD, respectively).

VCF = vacuolated cellular foci. Foci of cellular alteration are identified by subtle changes in staining pattern and morphology that differentiate a group of hepatocytes from the bulk of hepatocytes in the liver. A variety of staining/morphology patterns have been described. Among those types, vacuolated cell foci are composed of a well-delineated group of hepatocytes with clear round cytoplasmic vacuoles (probably lipid). VCF are not considered to be preneoplastic. The U.S. National Toxicology program classifies these lesions as “focal fatty change” rather than VCF (Maronpot 2014).

Methods

Liver sections stained with H&E from 60 brown bullhead (*Ameiurus nebulosus*) were received for histopathology in two shipments: 1) slides 28 – 60 (plus four “pathos” slides of what is probably oral mucosa or lip) received 2015-11-17; and 2) slides 1 – 27 received 2015-12-11. Sections of five liver samples from each fish are distributed on 10 slides, two slides per sample. Most slides contain 3 – 5 serial- or step-sections from the same paraffin block. For each of the five liver samples per fish, one slide (usually #1) was selected for examination; every section from that slide was systematically scanned using the 4× objective lens (low power), and then a single section on each selected slide was systematically scanned using the 10× objective lens (medium power). Higher magnification (20× and 40× objective lenses) was used as needed. The second slide from each liver sample was examined as needed (e.g., a large focus of granulomatous inflammation from fish #52 spans slides 1-1 and 1-2, but sections of an ascarid parasite are limited to the sections in slide 1-2). Published guidelines were used as references for lesion classification (e.g., Smith et al. 1989; Wolf and Wolfe 2005).

For foci of cellular alteration and neoplasia, the number of foci per liver is recorded. Parasites are scored as absent (0) or present (1) in the sections. For other lesions, findings are scored on a relative scale as none (0), mild/small amounts (1), moderate (2), or severe/abundant (3). The location of uncommon focal lesions is often identified by a comment added to the spreadsheet cell (e.g., “slide 5-1” designates the slide and “22 x 111” designates the coordinates on the lead pathologist's microscope). As needed, additional features of the sections are described in the Comments column.

The four slides of oral mucosa/lip are all labelled W1 2014; they include:

- “Upper Tissue Lesion” – 41-1 and 41-2; four step sections per slide
- “Lower Tissue Lesion” – 41-1 and 41-2; four or five step sections per slide

When histopathology was conducted, the pathologists (GDM and HNS) knew the name of the project (Walpole Island), but we had not been provided with any information about the fish other than the species and year when the fish were sampled. Histopathology results from this blinded analysis were e-mailed to Mark McMaster and Jim Bennett (Thu 2016-02-04 2:30 PM). We received information about sample location, sex, age, and weight in an e-mail from Jim Bennett (Fri 2016-03-11 11:54 AM); the e-mail includes instructions to guide further analysis: “These fish were all caught on and surrounding Walpole Island. No comparison between the locations [is] necessary.”

Histopathology data were rearranged and basic summary statistics were calculated based on sex and age groups. This final report presents and discusses the history, lesions, and potential significance on population health from a medical perspective.

Quality control

Erythrocyte cytoplasm consistently stains poorly (clear instead of orange); this often occurs when tissues are immersed in alcohol (this includes alcohol-based fixatives like Bouin's and Davidson's) and then transferred to water or a water-based fixative (e.g., 10% formalin) before being processed into paraffin. [We usually do not see the problem of lost erythrocyte staining when formalin-fixed tissues are transferred to water or physiologic saline before processing; however, even erythrocytes in organs not transferred to water from alcohol sometimes lose their eosinophilic staining after exposure to bile.] Sectioning artifact is consistently mild, staining variability is minimal, and thickness of sections and coverslip media are within slide preparation standards used at the BC Animal Health Centre.

Liver preservation is excellent for most of each section, but tissues adjacent to major bile ductules and blood vessels often have mild decomposition (autolysis), probably due to bile digestion before formalin fixation. This level of autolysis does not seem to obscure parasites or foci of cellular alteration. [Bile digestion can be minimized by aspirating bile from the gallbladder before handling the remainder of the liver.]

In 75% of the fish, some foci of erythrocytes have precipitates of acid hematin. Acid hematin accumulates as brown birefringent deposits when tissues are not fixed in neutral buffered formalin and when tissues become acidic before or during fixation (as can happen with thick bloody pieces of tissue or with acid decalcification).

One liver section (slide 25-5-1) has a single focus on the capsule, 2 × 0.4 mm, that stains darker than the remainder of the section, and part of this piece is dehydrated. This focus might be a “float on” artifact from another liver. Because of the uncertain origin of this small piece, it was not used for scoring any lesions.

In addition to blinded examination, we used two methods to minimize pathologist bias in assigning scores:

1) To minimize bias in scoring that can occur over time (e.g., the same lesion assigned a score of “1” at the beginning of the study might be assigned a score of “2” at the end of the study, no more than four livers from a given slide box were sequentially examined by a pathologist at one time; once analysis of two to four livers per box was complete, the next slides to be examined were selected from a different box. In this way, the 5th liver to be examined from a given box was not examined until at least one liver from every box had been examined.

2) To minimize bias in scoring between pathologists, after each lead pathologist had completed analysis of 10 livers, the reviewing pathologist randomly selected one of the 10 livers for independent analysis; scores were then compared. Reported scores are agreed on by both pathologists; for minor differences in scoring (e.g., 1 vs. 2 severity), the lead pathologist's scores are retained.

Minor differences in pathologist scoring are not likely to have any impact on conclusions about the health status of these fish. Among the 37 microscopic features scored, for only a few do the pathologists’ scores seem to be different. Inflammatory cells are fairly common near vessels and bile ductules; these cells were scored more commonly near vessels by GDM (26 vs. 4%), whereas they were scored more commonly near bile ductules by HNS (30 vs. 15%). GDM scored amphophilic/eosinophilic inclusion bodies in 37%, whereas HNS scored them in only 7.4%. Some of these differences might be a result of true differences in the livers.

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Appendix C: 2012 PAH Concentrations in sediment samples collected from the Walpole Delta PAH concentrations (ug/g dry wt).

Site	NA	AL	AE	FL	PHE	AN	FLT	PY
A004	0.001	0.000	0.000	0.001	0.005	0.001	0.002	0.003
A010	0.007	0.007	0.004	0.014	0.075	0.012	0.043	0.074
S14	0.004	0.002	0.002	0.007	0.039	0.006	0.023	0.025
S15	0.026	0.006	0.022	0.030	0.205	0.040	0.198	0.178
S24	0.010	0.005	0.004	0.012	0.078	0.015	0.066	0.071
S25	0.012	0.005	0.003	0.011	0.068	0.010	0.069	0.062
S27	0.013	0.004	0.003	0.009	0.059	0.016	0.096	0.112
UBC1-1	0.012	0.008	0.006	0.023	0.108	0.027	0.082	0.141
UBC1-2	0.009	0.009	0.005	0.020	0.096	0.023	0.076	0.127
UBC1-3	0.018	0.014	0.008	0.031	0.130	0.034	0.103	0.181
DBC2-1	0.020	0.010	0.005	0.016	0.103	0.019	0.107	0.111
DBC2-2	0.016	0.010	0.005	0.016	0.097	0.017	0.096	0.117
DBC2-3	0.025	0.014	0.006	0.019	0.115	0.021	0.105	0.126
UCC1-1	0.008	0.004	0.003	0.010	0.063	0.008	0.058	0.061
UCC1-2	0.006	0.002	0.002	0.008	0.044	0.008	0.040	0.042
UCC1-3	0.005	0.001	0.002	0.007	0.037	0.007	0.029	0.033
A009	0.016	0.008	0.003	0.014	0.085	0.014	0.100	0.145
DCC2-1	0.002	0.000	0.001	0.002	0.005	0.001	0.010	0.008
DCC2-2	0.003	0.001	0.001	0.003	0.014	0.002	0.020	0.019
DCC2-3	0.004	0.000	0.001	0.003	0.010	0.002	0.019	0.015
GL1	0.006	0.000	0.001	0.007	0.024	0.002	0.048	0.024
GL2	0.002	0.000	0.000	0.001	0.003	0.000	0.008	0.003
UJC1-1	0.026	0.006	0.009	0.025	0.143	0.030	0.105	0.139
UJC1-2	0.025	0.013	0.009	0.024	0.123	0.023	0.105	0.139
UJC1-3	0.016	0.006	0.008	0.018	0.159	0.025	0.157	0.176
A007	0.011	0.005	0.005	0.014	0.105	0.020	0.107	0.112
A008	0.014	0.008	0.005	0.016	0.103	0.017	0.107	0.130
DJC2-1	0.025	0.015	0.007	0.028	0.152	0.023	0.135	0.173
DJC2-2	0.021	0.016	0.007	0.022	0.132	0.023	0.133	0.164
DJC2-3	0.021	0.017	0.006	0.023	0.137	0.024	0.131	0.173
UCE2-1	0.012	0.003	0.003	0.006	0.041	0.005	0.025	0.030
UCE2-2	0.007	0.003	0.002	0.005	0.037	0.005	0.023	0.030
UCE2-3	0.001	0.000	0.001	0.002	0.015	0.003	0.008	0.011
UCE2-4	0.006	0.003	0.003	0.007	0.047	0.006	0.028	0.034
AS29	0.010	0.004	0.003	0.009	0.048	0.045	0.082	0.072
MCE2-1	0.025	0.005	0.005	0.013	0.070	0.016	0.050	0.063
MCE2-2	0.012	0.006	0.005	0.015	0.062	0.058	0.048	0.059
MCE2-3	0.019	0.004	0.005	0.014	0.077	0.018	0.068	0.075
S28	0.008	0.004	0.002	0.007	0.035	0.032	0.038	0.033
DCE3-1	0.006	0.005	0.002	0.007	0.044	0.005	0.061	0.067
DCE3-2	0.009	0.004	0.002	0.009	0.046	0.006	0.073	0.072
DCE3-3	0.003	0.005	0.002	0.009	0.049	0.007	0.073	0.080
A001	0.001	0.000	0.000	0.000	0.001	0.000	0.001	0.001
A005	0.085	0.049	0.029	0.039	0.605	0.120	1.110	1.120
A006	0.001	0.000	0.000	0.001	0.005	0.001	0.005	0.004
A53	0.004	0.003	0.001	0.006	0.031	0.004	0.045	0.047
A003	0.000	ND	ND	0.000	0.000	0.000	0.001	0.000
S57	0.024	0.006	0.010	0.014	0.097	0.013	0.124	0.109

Site	B(a)A	C&T	B(b)F	B(k)F	B(a)P	IP	D(a,h)A	B(g,h,i)P	sumPAH
A004	0.000	0.002	0.000	0.000	ND	ND	ND	ND	0.02
A010	0.028	0.049	0.018	0.008	0.021	0.013	0.008	0.024	0.41
S14	0.007	0.018	0.005	0.003	0.004	0.004	0.000	0.009	0.16
S15	0.100	0.112	0.084	0.035	0.097	0.079	0.014	0.086	1.31
S24	0.030	0.054	0.025	0.009	0.028	0.023	0.005	0.035	0.47
S25	0.031	0.045	0.025	0.012	0.027	0.027	0.004	0.031	0.44
S27	0.066	0.080	0.046	0.024	0.059	0.048	0.009	0.045	0.69
UBC1-1	0.055	0.085	0.032	0.012	0.046	0.028	0.010	0.040	0.71
UBC1-2	0.053	0.078	0.033	0.013	0.042	0.027	0.009	0.039	0.66
UBC1-3	0.072	0.103	0.046	0.016	0.054	0.035	0.012	0.049	0.90
DBC2-1	0.058	0.083	0.051	0.018	0.054	0.049	0.017	0.060	0.78
DBC2-2	0.059	0.081	0.057	0.019	0.061	0.050	0.012	0.064	0.78
DBC2-3	0.067	0.093	0.053	0.019	0.065	0.058	0.021	0.072	0.88
UCC1-1	0.026	0.043	0.019	0.011	0.022	0.020	0.004	0.021	0.38
UCC1-2	0.014	0.032	0.012	0.004	0.009	0.008	0.002	0.012	0.24
UCC1-3	0.010	0.029	0.009	0.004	0.007	0.006	0.001	0.009	0.19
A009	0.065	0.109	0.095	0.029	0.081	0.101	0.026	0.099	0.99
DCC2-1	0.002	0.007	0.003	0.002	0.001	0.002	0.000	0.003	0.05
DCC2-2	0.005	0.015	0.008	0.005	0.004	0.009	0.001	0.009	0.12
DCC2-3	0.005	0.014	0.007	0.005	0.003	0.006	0.001	0.007	0.10
GL1	0.008	0.020	0.006	0.011	0.001	0.014	0.001	0.009	0.18
GL2	0.001	0.003	0.001	0.002	0.000	ND	ND	ND	0.02
UJC1-1	0.083	0.109	0.052	0.017	0.064	0.050	0.021	0.069	0.95
UJC1-2	0.079	0.101	0.044	0.016	0.058	0.036	0.021	0.056	0.87
UJC1-3	0.112	0.132	0.081	0.031	0.108	0.081	0.025	0.086	1.22
A007	0.068	0.098	0.042	0.020	0.059	0.041	0.008	0.045	0.76
A008	0.075	0.097	0.060	0.018	0.071	0.051	0.015	0.057	0.85
DJC2-1	0.100	0.130	0.087	0.024	0.094	0.079	0.025	0.093	1.19
DJC2-2	0.102	0.132	0.102	0.033	0.107	0.119	0.035	0.114	1.26
DJC2-3	0.101	0.135	0.097	0.032	0.106	0.114	0.030	0.116	1.26
UCE2-1	0.010	0.023	0.009	0.005	0.003	0.004	0.001	0.011	0.19
UCE2-2	0.009	0.021	0.007	0.004	0.000	0.004	0.001	0.008	0.17
UCE2-3	0.002	0.006	0.000	0.001	0.000	ND	ND	ND	0.05
UCE2-4	0.011	0.027	0.009	0.005	0.002	0.005	0.002	0.013	0.21
AS29	0.036	0.056	0.053	0.016	0.032	0.047	0.008	0.052	0.57
MCE2-1	0.037	0.054	0.025	0.009	0.024	0.017	0.011	0.030	0.45
MCE2-2	0.028	0.045	0.022	0.005	0.022	0.015	0.006	0.027	0.43
MCE2-3	0.045	0.055	0.033	0.013	0.044	0.034	0.009	0.040	0.55
S28	0.016	0.033	0.023	0.009	0.016	0.026	0.005	0.036	0.32
DCE3-1	0.031	0.050	0.040	0.013	0.024	0.033	0.009	0.034	0.43
DCE3-2	0.038	0.060	0.052	0.015	0.037	0.054	0.011	0.047	0.54
DCE3-3	0.039	0.057	0.048	0.013	0.029	0.041	0.010	0.042	0.51
A001	ND	0.001	ND	0.000	ND	ND	ND	ND	0.01
A005	0.746	0.773	0.799	0.360	0.650	0.888	0.190	0.599	8.16
A006	0.001	0.003	0.000	0.001	ND	ND	ND	ND	0.02
A53	0.020	0.035	0.021	0.009	0.017	0.016	0.005	0.020	0.29
A003	ND	0.000	ND	ND	ND	ND	ND	ND	0.00
S57	0.052	0.080	0.056	0.026	0.039	0.043	0.008	0.052	0.75

Appendix C: Engagement Summary

Updated: *Nov 2019*

Re-designation Step	Activity	Comments	Date	Completed (Yes/No)
Initial draft.	Initial draft report prepared.	Initial draft report prepared by Environment and Climate Change Canada and circulated to Co-lead and RAP Coordordinator for comment.	December 12, 2018	Yes
	MECP Review	Yes - provided comments/edits. See above.	January 4, 2019	
	ECCC Review	ECCC expert reviewed and provided comments.	January 8, 2019	
	Expert Review	No. Used expert reports to form foundation of the report.	NA	
CRIC	Initial draft provided to CRIC for review.	Sent by RAP Coordinator to CRIC members for June CRIC meeting 2019.	June 8, 2019	Yes
	Presentation to CRIC	June 2019 CRIC meeting		
	Comments from the CRIC	No written comments but WIFN suggested that BUI evaluation is based on internal (not external) lesions should be very clear.		
	CRIC Endorsement/Support for Re-designation	June 2019 CRIC meeting		
WIFN engagement - as per Engagement Strategy	Community engagement meeting regarding additional sampling of brown bullheads in the bays and channels of WIFN bays.	Presentation to the WIFN community on proposal to collect additional fish in collaboration with the WIFN Heritage Centre.	July 6, 2012	Yes
	Approval provided and sampling of Brown Bullhead started using local anglers.	In collaboration with the WIFN Heritage Centre, local fishermen caught brown bullheads. Sampled in 2013 and 2014.	Spring 2013 and 2014	Yes
	Newsletter articles in community newspaper (Nin.da.waab.jib News)	Re: Brown Bullhead study and AOC	May 2013, June, July and Sept 2014, October 2017, January 2018	Yes
	Social Media	Short video clip of field work.	July 1, 2014	Yes
	Science Symposium 2016 and 2019	Community events were co-organized and sponsored by the CRIC and WIFN. Presentation provided on results of brown bullhead project and basis for a not impaired status.	April 2016 and April 2019	Yes
	Focus Group	Presented at 2 community information sessions the results of studies conducted to assess BUI (pm and lunch session).	March 28 and 29 2019	Yes
	Heritage Committee Meeting Presentation	Present results of studies conducted to assess this BUI.	TBD	
	Heritage Committee Concurrence/Motion	TBD	TBD	

	Chief and Council Concurrence/Motion			
AFN Consultation	Draft Status Report	Provided to AFN at CRIC meeting in June 2019.	June 1, 2019	Yes
	Community engagement	Environment Committee utilized River Day, Earth Day and Enviro-Fest, social media and Annual reports to advise community of AOC related projects and BUI status. No additional community engagement requested by AFN to the CRIC. Community engagement handled by Environment Committee.	2016-2019	Yes
	Science Symposium 2016 and 2019	Community event co-organized and sponsored by CRIC/AFN. Presentation made on results of brown bullhead project and studies are basis for a not impaired status recommendation.	April 2016 and April 2019	Yes
	Environment Committee Presentation	Presenting findings of studies conducted by ECCC to assess the status of this BUI.	November 5, 2019	Yes
	Environment Committee Concurrence/Motion	TBD		
	Chief and Council Concurrence/Motion	TBD		
BPAC Consultation	Draft Assessment Report Provided to BPAC	Provided hard copies at September meeting and electronic copies in October 2019 for Nov meeting.	November 5, 2019	Yes
	Presentation to the BPAC	Presented overview of the BUI and summarized studies undertaken to assess this BUI in the SCR AOC. Motion deferred to Nov 2019 meeting to allow report updating and distribution.	September 1, 2019	Yes
	Motion	NI status supported at November meeting.	November 2019	Yes
Four Agency Managers Work Group	Draft Status Report provided to Four Agency Managers Work Group	Provided draft report and presentation.	July 1, 2019	Yes
	Presentation to FAMC WG	Presented summary of research findings to assess the status of this BUI.	July 23, 2019	Yes
	Comments from FAMC	No comments from State/Comments from USEPA, US F & W.	August 2019.	Yes
	Completed Revisions	Revisions complete	September 30, 2019	Yes
General Public	Posted/Open for public review and comment	Posted on website.		
	Feedback (if any)	Nil.		
	Completed Revisions	NA		
COA Annex 1 Leads for Review	Assessment Report provided to COA Annex 1 Leads	TBC		

Official Status Designation	Letter from Party (ECCC) to CRIC, Agency leads, FAMC and IJC advising of status.	TBD.
U.S Side		
Impaired	BPAC endorsement for BUI removal	13-Jul-16 BUI Not Impaired