United States Department of the Interior

FISH AND WILDLIFE SERVICE
East Lansing Field Office (ES)
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IN REPLY REFER TO:

Mr. David Bareham
Manager Environment
Enbridge Energy, Limited Partnership
333 South Kalamazoo Avenue
Marshall, MI 49068

Dear Mr. Bareham:

Attached please find a final report from the U.S. Geological Survey entitled "*Health Assessment and Histopathologic Analyses of Fish Collected from the Kalamazoo River, Michigan, Following Discharges of Diluted Bitumen Crude Oil from the Enbridge Line 6B*". This report and the work it describes were completed as part of the Natural Resource Damage Assessment (NRDA) related to the July 2010 Enbridge Pipeline 6B oil discharges and associated removal efforts. U.S. Geological Survey completed this work on behalf of the Trustees for this NRDA: the United States Department of the Interior, acting through the Fish and Wildlife Service and the Bureau of Indian Affairs; the United States Department of Commerce, acting through the National Oceanic and Atmospheric Administration; the State of Michigan, acting through the Department of Environmental Quality, the Department of Natural Resources, and the Department of Attorney General; the Nottawaseppi Huron Band of the Potawatomi; and the Match-E-Be-Nash-She-Wish Band of Pottawatomi.

In brief, the study showed that fish collected from the three oiled sites had significant adverse changes relative to fish collected from upstream, including reduced condition factors, greater numbers and severity of anomalies and lesions, increased mucous producing cells and cytochrome P4501A activity in the gills, and increased macrophage aggregates in the spleen.

If you have any questions, please feel free to contact me at 517-351-8324.

Sincerely,

Lisa L. Williams, Ph.D.
Lead Administrative Trustee, Enbridge Line 6B NRDA
U.S. Fish and Wildlife Service

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Health Assessment and Histopathologic Analyses of Fish Collected from the Kalamazoo River, Michigan, Following Discharges of Diluted Bitumen Crude Oil from the Enbridge Line 6B

By Diana M. Papoulias, Vanessa Veléz, Diane K. Nicks, and Donald E. Tillitt

Administrative Report 2014

U.S. Department of the Interior
U.S. Geological Survey

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Acknowledgments

Funding for this study was provided by the US Fish and Wildlife Service (FWS), East Lansing Field Office, East Lansing, Michigan. We were assisted in the field and with logistics by personnel from the FWS (Edward Karecki and Stephanie Millsap), Michigan Department of Natural Resources (Olen Gannon, Matt Smith, Mike Wilson, Ed Pearce, Scott Hanshue, Jay Wesley, and Kregg Smith), and Enbridge contractor (John Hess). Lisa Williams (FWS) and Jessica Winters (National Oceanic and Atmospheric Administration) provided valuable comments on an early draft.

We were assisted in the laboratory by personnel from the Columbia Environmental Research Center (CERC), Biochemistry and Physiology Branch (Mandy Annis, Rachel Claunch, Claire McMenamy, and Ashley White). Fish biologists (CERC) Christopher Schmitt and Aida Farag provided valuable comments on an early draft.

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Conversion Factors

Multiply	By	To obtain
	Length	
millimeter (mm)	0.03937	inch (in.)
inch (in.)	25.4	millimeter (mm)
mile (mi)	1.609	kilometer (km)
	Volume	
cubic millimeter (mm^3)	3.3814×10^{-5}	ounce
deciiliter (dL)	0.02642	gallon (gal)
	Mass	
gram (gr)	0.03527	ounce, avoirdupois (oz)

Temperature in degrees Celsius (°C) may be converted to degrees Fahrenheit (°F) as follows:
°F=(1.8×°C)+32

Abbreviations

AFS American Fisheries Society (AIFRB)

AIFRB American Institute of Fishery Research Biologists

ANOVA analysis of variance

ASIH American Society of Ichthyologist and Herpetologists

BEST Biomonitoring of Environmental Status and Trends

CERC Columbia Environmental Research Center

CF condition factor

CYP1A cytochrome P450 1A

EROD ethoxyresorufin-O-deethylase

FWS U.S. Fish and Wildlife Service

g gravitational

G:L ratio granulocytes to lymphocytes

GRH golden redhorse sucker

H&E hematoxylin and eosin

HAI health assessment index

Hb hemoglobin

HB Historic Bridge

HSI hepatosomatic index

LR Legacy Ranch

MA macrophage aggregates

MI Marshall Impoundment

NBF neutral buffered formalin

NOAA National Oceanic and Atmospheric Administration

p probability

PAH polyaromatic hydrocarbon

PHAH planar halogenated aromatic hydrocarbons

PROC GLM general linear model procedure

SB Shady Bend

SMB smallmouth bass

USGS U.S. Geological Survey

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By Diana M. Papoulias, Vanessa Veléz, Diane K. Nicks, and Donald E. Tillitt

Abstract

On July 25, 2010, a 30-inch diameter pipeline ruptured near Marshall, Michigan, and began discharging diluted bitumen crude oil that flowed for approximately 38 miles down the Kalamazoo River. The U.S. Fish and Wildlife Service requested assistance from the U.S. Geological Survey Columbia Environmental Research Center in assessing the adverse health effects of this accident on fish at various locations in the Kalamazoo River downstream from the oil discharge. The objective was to compare fish collected from three oiled sites against fish collected from a reference site for a suite of biological indicators (bioindicators). The bioindicators of adverse health effects were selected to target multiple levels of biological organization and included an evaluation of condition, tissue histopathology, immunotoxicity, induction of CYP1A, and a necropsy-based assessment of health. The results for these bioindicators were overall similar for both species and among the three oiled sites. Most of the bioindicator results for fish from the oiled sites contrasted with and were different from the bioindicator results obtained for fish collected from a reference site above and unaffected by the oil discharge. Moreover, results from this evaluation are consistent with both laboratory studies of polyaromatic

hydrocarbon (PAH) toxicity to fish and field assessments of catastrophic oil spills or chronic PAH contamination from multiple anthropogenic sources.

Introduction

On July 25, 2010, at Enbridge Line 6B, a 30-inch diameter pipeline owned by Enbridge Energy ruptured near Marshall, Michigan, and began discharging diluted bitumen crude oil into a wetland adjacent to Talmadge Creek. The oil flowed through Talmadge Creek into the Kalamazoo River, a Lake Michigan tributary. The Kalamazoo River was in flood stage at the time of the discharge and the oil flowed into its flood plain and down the river for approximately 38 miles to Morrow Lake. The U.S. Fish and Wildlife Service (FWS) requested that the U.S. Geological Survey (USGS) Columbia Environmental Research Center (CERC) conduct a necropsy-based evaluation to aid their assessment of the adverse health effects on fish at various locations in the Kalamazoo River downstream from the oil discharge site.

The objective of this evaluation was to compare fish from these oiled sites against fish from a site that was not oiled for a suite of biological indicators (bioindicators). There is wide acceptance for the use of bioindicators to evaluate biological harm to aquatic organisms resulting from catastrophic oil spills (Martinez-Gomez and others, 2010; Law and others, 2011). The sublethal indicators of adverse effects on fish were selected based on their known association with degraded environments or polycyclic aromatic hydrocarbon (PAH) exposure. Golden redhorse (*Moxostoma erythrurum*), a benthic feeder, and smallmouth bass (*Micropterus dolomieu*), a piscivore, were the fish species chosen for collection. These fish species were collected because they represent different trophic levels and are commonly found in the affected areas of the Kalamazoo River as well as upstream and downstream from the affected area. Additionally, sucker and bass species have been commonly studied at sites used for biological monitoring, including PAH-impacted sites (Schmitt and others, 1993).

The bioindicators selected for this assessment target multiple levels of biological organization: the individual, tissue, cell, and biochemical levels. Bioindicators at the level of the individual fish included in this assessment are the biomarkers: condition, the relation of the liver weight to the body weight (hepatosomatic index [HSI]), and the calculation of a fish health index. Fish condition is commonly

assessed using weight and length measurements (Blackwell and others, 2000). Variation from an expected weight for a given length may be due to nutrition, environmental quality, or stage of reproductive maturity. Abnormally small HSI values may reflect depleted energy reserves and may indicate poor fish health. Abnormally large HSI values may be associated with increased production of xenobiotic metabolizing enzymes, an increased parasite load, or other pathological changes (Schmitt and others, 1999). Health indices integrate observations of gross abnormalities during the necropsy into a single numerical value that can be scaled and used to assess general fish health (Schmitt and others, 1999).

Polycyclic aromatic hydrocarbons have been reported to suppress the immune system and therefore spleen, head kidney, and blood were examined (Hart and others, 1998; Reynaud and Deschaux, 2006; Reynaud and others, 2008; Uribe and others, 2011). In addition to spleen and head kidney histopathology, macrophage aggregates were quantified. Macrophage aggregates are collections of phagocytic macrophages containing cellular debris collected in the body and are found in fish spleen and head kidney (Fournie and others, 2001). Macrophage aggregates typically contain pigments such as hemosiderin, lipofuscin, ceroid, and melanin according to the type of degraded material engulfed. Macrophage number and size are used as biomarkers because these measurements have been determined to be associated with a number of environmental stressors including exposure to polycyclic aromatic hydrocarbons (Wolke, 1992; Agius and Roberts, 2003). Hematological biomarkers diagnostic of immune system dysfunction included leukocyte counts. Leukocyte profiles provide a prognostic evaluation of the level of stress an organism is experiencing (Davis and others, 2008). Hemoglobin was also measured; low hemoglobin is an indicator of anemia and has been associated with exposure to PAHs (Martinez and others, 2008).

Gills serve important respiratory and excretory functions that require a highly vascular and large surface area. The gill tissues are in direct contact with the surrounding water and therefore a primary site of contaminant contact and uptake. Mucus cells on gill lamellae proliferate when stimulated by irritants

and a number of gill histopathologies are induced by exposure to polycyclic aromatic hydrocarbons (Martinez and others, 2008). Production of cytochrome P450 1A (CYP1A) is a well-known response to exposure to PAHs and has been determined to be produced in gills of PAH-exposed fish (Stegeman and Lech, 1991; Moore and others, 2003). In gills, the CYP1A biomarker can be detected with immunohistochemistry.

This report details the results of work by the CERC for the following:

- collection and preservation of gill, spleen, head kidney, liver, bile, and plasma from 110 fish;
- preparation of whole blood smears and conduct of differential analysis of leukocytes;
- calculation of a health assessment index;
- identification of histopathologic lesions in gill, spleen, and head kidney; and
- preservation and storage of samples for possible future analysis.

Methods

A biologist from the CERC Biochemistry and Physiology Branch was onsite August 19 and 20, 2010, to conduct necropsies and collect tissues. Michigan Department of National Resources, FWS, and Enbridge personnel collected fish using boat electrofishing, and used net pens to hold the captured fish in the river until necropsy.

Ten to 15 individuals of two fish species, a mid-water piscivore species, smallmouth bass (SMB), and a benthic sucker species, golden redhorse (GRH), were targeted for collection at four sites on the Kalamazoo River. The size of GRH targeted were 10 inches (in.) to 15 in. and the size of SMB targeted were greater than or equal to 14 in. Sites were selected to ensure a fish collection from each of the three different river divisions as segmented by Incident Command, which roughly corresponded to the degree of observed river oiling. An upstream reference fish collection was also selected. The Historic Bridge (Division C), Legacy Ranch (Division D), and Shady Bend (Division E) sites were downstream (9 miles,

22 miles, and 27 miles, respectively) from the oil discharge. The Marshall Impoundment location was upstream of the oil discharge location and served as a reference site (fig. 1).

Necropsy

Field necropsy procedures generally followed those used by USGS in the Biomonitoring of Environmental Status and Trends (BEST) program (Schmitt and others, 1999). A blood sample was obtained from the posterior caudal vein using a previously prepared heparinized needle (20 gauge) and syringe kept cold on wet ice. A part of the collected whole blood was used for blood smear slides and hemoglobin measurements. An additional part of the whole blood used for hormone and protein analyses was decanted from the syringe barrel into a heparinized vacutainer and held on wet ice. After blood collection, the fish was euthanized with a sharp blow to the head, weighed (grams [gr]), and the total length was measured (in millimeters [mm]). Visual observations were made of external features and grossly visible tissue anomalies were recorded; some abnormal tissues were dissected and preserved in 10-percent neutral buffered formalin (NBF) for histopathologic analysis. A gill arch, generally the second arch of the left gill, of each fish was removed, placed in a histo-cassette, and preserved in 10-percent NBF. The liver of each SMB was carefully removed with the gall bladder intact. The gall bladder was dissected from the liver, bile collected in a clean cryovial, and the entire liver was weighed. The gall bladders of GRH were dissected directly and bile collected. Because GRH have a diffuse liver that is difficult to accurately weigh, liver weight could not be obtained. Duplicate subsamples of the liver tissue from each fish were collected and flash frozen in a dry ice/ethanol slurry for ethoxyresorufin-O-deethylase (EROD) analysis. Liver, spleen, gonad, head kidney, and hind kidney were examined for abnormalities. A single piece of the spleen and head kidney were removed, placed in separate histo-cassettes and preserved in 10-percent neutral buffered formalin. Upon completion of the internal examination, the fish carcass and pieces of organs were wrapped in aluminum foil for proper and secure disposal. Work surfaces and instruments were cleaned with 70-percent ethanol followed with an acetone rinsing. Chilled blood

samples were centrifuged at 3,000 g (gravitational force) for 15 minutes and plasma was aspirated into duplicate cryovials and flash frozen in a dry ice/ethanol slurry. Samples were stored in a secured location under appropriate preservation conditions until shipment. All samples were shipped to CERC at the end of the field sampling period and stored in a secure location under appropriate preservation conditions until analysis.

All field data were recorded on datasheets. Originals were digitally scanned at CERC, copies were sent to FWS East Lansing Field Office, and originals were archived at CERC (appendix 1). Samples collected in the field were transferred to CERC with chain-of-custody documentation (appendix 2).

Condition Factor and Hepatosomatic Index

Fulton's condition factor was calculated as the body weight (grams) x 10^5 ÷ (body length (mm))^3 (Thompson (1917). Hepatosomatic Index (HSI) was calculated as the liver weight (grams) ÷ body weight (grams) x 100. Hepatosomatic Index was calculated only for SMB.

Health Assessment Index

Numerical values were assigned to internal and external observations of lesions recorded in the field, and a necropsy-based fish health assessment index (HAI) score was calculated for each fish by summing the values for all organs (Schmitt and others, 1999). The HAI score ranges from 1 (healthy) to 220 (unhealthy). The suite of gross abnormalities selected for this assessment was chosen to be consistent with abnormalities commonly assessed in fish health monitoring programs (Fournie and others, 1996). Examples of abnormalities assessed included grossly visible disorders of the eye (exophthalmia, hemorrhage, opacity, emboli, missing), opercles (shortening, deformities, parasites), body and fin surfaces (ulcers, parasites, discolored areas or raised growths), and disorders of the gills and skeleton.

Hematology

Triplicate drops of whole blood were used to make three fresh-preparation blood smear slides. Slides were air-dried then immersed in 100-percent methanol for 10 minutes for preservation. One blood

smear slide was stained and viewed for each fish to evaluate the leukocyte (white blood cell) population. Cells were stained using the three-step Quick-Dip 3 differential stain (Mercedes Medical, Sarasota, Florida). Leukocytes (lymphocytes, monocytes, and granulocytes) and thrombocytes were counted using a Nikon 90i® and NIS-Elements® digital imaging software (v 4.10; Nikon Instruments Inc., Melville, New York) at 600x or 1,000x magnification. Cell counting continued until an approximate total of 200 leukocyte and thrombocyte cells had been counted and categorized.

Hemoglobin measurements were made following manufacturer's instructions by placing a droplet of blood onto a HemoCue® cassette and inserting the cassette into a HemoCue 201+ analyzer (Brea, California).

Histopathology

Gill, spleen, and head kidney were preserved in 10-percent neutral buffered formalin. Before tissue processing, tissues were rinsed twice in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, once in 50-percent ethanol, and then transferred to 70-percent ethanol. Gills were decalcified with Cal-Ex II™ (Thermo Fisher Scientific, Waltham, Massachusetts) for 45 minutes. Tissues were processed and infiltrated with paraffin using a Shandon Excelsior® Tissue Processor through a typical xylene and ethanol series (Thermo Fisher Scientific). Infiltrated tissues were embedded in paraffin using a Microm® EC350-2 (Thermo Fisher Scientific) embedding center. Sections were cut at 7 microns on a Leica RM 2235 microtome (Leica Microsystems, Wetzlar, Germany). All microscopy was accomplished using a Nikon 90i with digital imaging capability. NIS Elements software was used to process and analyze digital images. The examiner made evaluations and counts without knowing from which site the fish were collected, except for the viewing of organs of a few individuals of each species from the reference site, which were used to orient and educate the examiner unfamiliar with the histomorphology of these species.

Two slides were made of each spleen of each fish. One slide was stained with hematoxylin and eosin (H&E), a general nuclear stain, and the other slide was stained with Pearl's stain (Luna, 1968) for macrophage aggregates. Macrophage aggregates for each fish were quantified by area and number in five fields of view at 20x and reported as a fraction of the total area of tissue viewed and as the average area of an individual macrophage aggregate (MA). Pigments associated with MAs and whether MAs were loose or clustered into centers were noted. Spleens were evaluated for presence or absence of lipid deposits, necrosis, fibrosis, and parasites; identification of parasites was not attempted. The prominence of lymphoid tissue was scored from 1 to 3 with 1 being low, 2 being moderate, and 3 being high prevalence; scores of individual fish were averaged to obtain a site score for each species.

Head kidney tissue sections were stained with H&E (Luna, 1968). Head kidney was evaluated for (1) presence or absence of parasites; (2) MAs; (3) enlarged blood vessels; (4) increased leukocytes; (5) interrenal cell hyperplasia, pyknosis, and vacuolation; and (6) chromaffin cell proliferation and vacuolation. Five individuals of each species from each site were evaluated.

Gills were evaluated for lesions, mucus cells, and for immunohistochemical staining of cytochrome P450 1A. Sections for evaluation of lesions were stained with H&E (Luna, 1968). Lesions were scored for occurrence and severity as follows: 0 equals none, 1 equals low, 2 equals moderate, 3 equals high. Scores were summed for each fish species and lesion type and percent of fish with a lesion was reported. Mucus cells were specifically stained with periodic acid Schiff and alcian blue (pH 2.5) and counted on five secondary lamellae (Alvarado and others, 2006). Numbers of mucocytes were normalized to total length along lamellae on which cells were counted. Gill sections were semiquantified for CYP1A staining. Paraffin sections of gill tissue for CYP1A were deparaffinized and rehydrated before heat-induced antigen epitope retrieval following the citrate buffer method (http://www.ihcworld.com/_protocols/epitope_retrieval/citrate_buffer.htm). A mouse anti-cod CYP1A antibody (Biosense Laboratories, Bergen, Norway), previously demonstrated to be broadly cross-reactive

among fish species, was applied following the vendor's suggestions for use in immunohistochemistry (Ueng and others, 1992). Gill showing positive CYP1A staining was semiquantified by counting the areas of positive staining on five primary lamellae for five fish from each species from each site. In cases where more than five lamellae were attached to the gill arch and present on the slide, all were counted and a correction factor applied.

Additional Samples

Additional samples, collected but not analyzed, are being stored at CERC. Liver samples collected for EROD, blood samples collected for hormone or protein analysis, and bile samples for biomarkers of PAH exposure were stored at -80 degrees Celsius (^{o}C) at CERC. An additional 19 bile samples collected by FWS were included for storage with the samples CERC collected.

Statistics

All measurements were tested for significant differences among sites by one-way (site as fixed effect) Analysis-of-Variance (ANOVA) using a general linear model procedure (PROC GLM) in SAS v.9.3 (SAS Institute, Inc., Cary, North Carolina). Significance level used to judge statistical significance was p=0.05. When no sex-related differences (Student's t-test) were detected for a measurement, data from both sexes were combined. Percent thrombocytes and leukocytes were log-transformed for statistical analyses. Condition factor, HSI, and ratio of leukocytes were arcsine transformed. If measurements did not meet assumptions of normality and homogeneity of variance after transformation, a Kruskal-Wallis rank test was used. The percentages of fish with spleen parasites or with a specific splenic or gill lesion were analyzed for differences between the reference site and the sites downstream from the oil discharge by computing an odds ratio with a chi-square statistic. No statistics were provided on gill scores. Significance level used to judge statistical significance was p=0.05. Only summary descriptive statistics are provided for CYP1A immunohistochemistry in gill tissue because staining conditions were not optimal. This endpoint was added late in the study and after tissues had been field-collected. For

optimal staining, preservation of gills should have followed a different procedure than was followed in this study. Results indicate presence or absence of CYP1A induction and relative intensity among sites.

All research was conducted in accordance with the procedures described by the American Society of Ichthyologist and Herpetologists (ASIH), American Fisheries Society (AFS), and American Institute of Fishery Research Biologists (AIFRB), "Guidelines for Use of Fishes in Field Research" (American Fisheries Society and others, 2004); and with all CERC guidelines for the humane treatment of test organisms during culture and experimentation.

Results

A total of 110 fish were collected for necropsy: 64 adult females, 45 adult males, and one juvenile. The targeted number of fish for each species (15) was collected at every site except Shady Bend where only 10 fish of each species were collected because of field sampling time constraints (table 1). Greater numbers of females than males of both species were collected at all sites except Legacy Ranch, although ratios of males to females were close to one except at Marshall Impoundment for both species and at Shady Bend for golden redhorse (table 1). No site-specific significant differences were observed in lengths of males and of females of both species or weights of smallmouth bass (table 1); however, site-specific differences in weights of GRH females were observed. Female GRH were heaviest at Historic Bridge and Marshall Impoundment and weighed the least at Shady Bend (table 1).

Condition Factor

Condition factors (CF) did not differ between the sexes in either species at any site. Overall, fish from the oiled sites, Legacy Ranch and Shady Bend, had significantly smaller CFs than those from the reference site, Marshall Impoundment (table 2). Condition factors for SMB, but not GRH, from Historic Bridge were also less than at Marshall Impoundment (table 2).

Hepatosomatic Index

There were no significant differences in HSI between male and female SMB; therefore, the data were combined. Hepatosomatic index was greatest for SMB from Historic Bridge and least for those at Marshall Impoundment the reference site (table 3).

Health Assessment Index

Fish from the oiled sites generally had more anomalies and lesions, and thus greater HAI scores, than those from the reference area. Marshall Impoundment was the only site at which there were significant sex-related differences, in both species, of the health assessment index (table 4). This statistical difference between the sexes may be because fewer males than females, of each species, were captured and available for evaluation at Marshall Impoundment in contrast to the other sites (except GRH at Shady Bend) which had equal numbers of males and females. To be consistent among sites, HAI scores were calculated for each site and species with the sexes combined. Marshall Impoundment fish had the lowest HAI scores compared to the other sites (table 4). Scores tended to be numerically higher in GRH than smallmouth bass. The few anomalies observed on fish from Marshall Impoundment included those associated with the eyes, body surface, and fins. During visual assessment, the fish from sites downstream from the oil discharge had relatively more lesions on these same areas of the body and, in addition, had lesions on their gills.

Hematology

Hemoglobin

Mean hemoglobin values ranged from 2.7 to 6.2 gr/dL (grams/deciliter) in both species and sexes, collectively. Golden redhorse from Historic Bridge had the smallest hemoglobin values. Hemoglobin values for SMB did not differ significantly among sites. Hemoglobin concentrations in male and female GRH from Historic Bridge were lower than at other sites; however, a statistically significant difference was found only with females from Marshall Impoundment and with females from Legacy Ranch (table 5).

Leukocytes and thrombocytes

Significant differences in leukocytes, but not thrombocytes, were found between the fish collected at sites below the oil discharge and those collected from the reference site. Percent lymphocytes of GRH from Historic Bridge and Legacy Ranch, the two sites closest to the oil discharge, were statistically, but likely not biologically, smaller than for fish from Marshall Impoundment, whereas lymphocytes of GRH from Shady Bend were greater (table 6). Granulocytes and monocytes of GRH were not significantly different between Marshall Impoundment and the affected sites (table 6). In contrast to GRH, more lymphocytes and fewer granulocytes were counted in blood of SMB from Historic Bridge and Legacy Ranch compared to SMB from Marshall Impoundment (table 6). Monocytes were slightly and significantly elevated in SMB from Shady Bend relative to those from Marshall Impoundment (table 6). The ratio of granulocytes to lymphocytes was different between the two species but similar between the sexes within a species at most sites (table 6). Ratios for GRH were significantly larger at Legacy Ranch and smaller at Shady Bend compared to Marshall Impoundment (table 6). Ratios for SMB were significantly smaller at all sites for both sexes except for females at Shady Bend.

Head Kidney

Lesions in head kidney were minor and not remarkable across species and sites. Histopathology of head kidneys of fish from the reference site was similar to that observed in head kidneys of fish from the oiled sites.

Spleen

Numbers or sizes of MAs in spleen tissue of fish from sites downstream from the discharged oil were larger than those for MAs of fish from the reference site, Marshall Impoundment. Macrophage aggregates in spleen tissues of SMB were significantly larger in size in fish from Historic Bridge and Shady Bend than those fish from Marshall Impoundment and Legacy Ranch (table 7); however, average

MA size in spleen tissues did not differ significantly among sites for GRH (table 8). Total MA area as a fraction of the area of spleen examined was greater in GRH, but not SMB, from Historic Bridge compared to fish from Marshall Impoundment (tables 7 and 8). Additionally, both species from Shady Bend had a greater total MA area than those from Marshall Impoundment (tables 7 and 8). Numerically, fish from oiled sites had more MAs than did fish from the Marshall Impoundment unaffected by the oil discharge; however, this relation was only significant for GRH with Historic Bridge and Legacy Ranch sites (tables 7 and 8).

Macrophage aggregates in all fish contained varying amounts (not quantified) of hemosiderin and lipofuscin/ceroid pigments. The most commonly observed pigment was hemosiderin. Macrophage aggregates from Historic Bridge fish contained melanin, but melanin was not observed in fish from any other site. Qualitatively, the density of pigments in MAs for both species followed a high to low pattern as follows: Historic Bridge > (greater than) Legacy Ranch > Shady Bend >Marshall Impoundment. Most of the MAs in all fish were present as encapsulated centers, although greater than 30 percent of GRH from Historic Bridge and Legacy Ranch also had many loose macrophage aggregates. Loose MAs are more typical of the type of MA found in soft-rayed fishes (that is, GRH) than in spiny-rayed fish (that is, SMB). Loose MAs may also indicate a more recent appearance of an MA before formation of an encapsulated center.

Splenic parasite numbers were variable between species and among sites downstream from the oil discharge relative to the reference site. Parasites were commonly found in SMB spleens but were uncommon in golden redhorse. Smallmouth bass from Shady Bend had more parasites than bass from Marshall Impoundment but fewer parasites were found in SMB from Historic Bridge compared to Marshall Impoundment (figs. 2 and 3). No parasites were observed in GRH from Marshall Impoundment and occurred in 10 percent or less of GRH from the other sites (fig. 2).

Scores for prominence of lymphoid tissue were lower for fish from oiled sites compared to those from Marshall Impoundment for both species but significantly different only for the golden redhorse (figs. 4 and 5).

Splenic lesions were more numerous in fish from sites downstream from the oil discharge than in fish from the reference site. No fibrosis or necrosis and a low incidence of lipid deposits were observed in Marshall Impoundment fish in contrast to fish from oiled sites. Lipid deposits were not observed in GRH from Marshall Impoundment but this splenic lesion was observed in a few SMB at this site. The incidence of lipid deposits, necrosis, and fibrosis varied between fish species from oiled sites and among oiled sites but overall were significantly greater in both species at all oiled sites relative to the reference site. Relative to other lesions, lipid deposits in SMB and fibrosis in GRH were the more prominent lesions (figs. 6, 7, 8, 9).

Gill

Sites downstream from where oil was discharged had more fish with gill lesions than did the reference site where fish were not exposed to oil. Overall, seven types of gill lesions were observed: aneurisms, blood congestion, epithelial cell hyperplasia, epithelial lifting, curling of secondary lamellae, fusion of secondary lamellae, and parasites. At no site were all fish completely lesion-free, and one or more fish of each species at a site was observed to have at least one of these lesions (table 9). No gill lesions were found in 7 of 30 fish from Marshall Impoundment, 1 of 30 fish from Historic Bridge, and 4 of 30 fish from Legacy Ranch. Only 1 of the 20 fish from Shady Bend had no gill lesions (table 9). Epithelial lifting and hyperplasia, aneurisms, congestion, and parasites were significantly more prevalent in gills of GRH from the oiled sites than in those from the reference site, and these effects were more severe in fish from the oiled sites than for fish from the reference site (table 9). Gill aneurisms, blood congestion, curling, and fusion were significantly more prevalent in SMB, at some but not all oiled sites, than those at the reference site (table 9).

Mucous producing cells and cells producing the CYP1A protein were more numerous on gills of fish at the sites downstream from the oil discharge than at the reference site. Significantly fewer mucocytes were measured at the reference site, Marshall Impoundment, than at the three oiled sites for both species (figs. 10 and 11). CYP1A produced by gill cells, as detected by immunohistochemistry staining, was observed in fewer GRH individuals, fewer cells were stained in fish of both species, and staining was overall much lighter in fish from Marshall Impoundment as compared to fish from the oiled sites (table 10). Two individuals of each species from Historic Bridge had extensive areas of CYP1A-positive staining that was unlike any other fish examined.

Discussion

Three weeks after the Enbridge Line 6B discharged oil into Talmadge Creek and the Kalamazoo River, two species of fish were collected from three locations downstream from the spill and from an upstream reference site. The objective of these collections was to compare fish from oiled sites to fish from a site that was not oiled for a suite of bioindicators. These indicators of adverse effects on fish were selected based on their known association with degraded environments or reported association with PAH exposure.

Interpretation of some bioindicators (for example, condition) can be affected by large differences in size, maturity, and age. There were no species-specific length differences among sites although GRH females from Legacy Ranch and Shady Bend tended to weigh less than GRH from Historic Bridge or Marshall Impoundment. Fish were collected after the reproductive season, and as a result during field necropsy, gonads of all individual fish were determined to be at an intermediate stage between ripe and spent. Age was not determined for these fish, but an estimate of age of SMB based on local data of the relation between length and age was made (Jay Wesley, Michigan Department of Natural Resources, oral commun., December 2012). Estimated age of SMB was variable at all sites, but on average females were

younger (3–5 years old) than males (5–6 years old) (table 11). No age-length data were available to make a comparable estimate of age for golden redhorse. The HAI scores from Marshall Impoundment indicate that the reference site provided sufficiently healthy fish and therefore bioindicator results at this site could be reliably used as benchmarks of normal when evaluating results from sites downstream from the oil discharge.

Timing and location of fish collections in relation to when and where the oil was discharged may have affected the severity of the bioindicator response. Polycyclic aromatic hydrocarbons are also common in aquatic environments found near human activity. Therefore, a background level of response is to be expected. Fish for this health assessment were collected 3 weeks after the pipeline break and between 9 and 27 miles below the point of oil discharge. Total extractable hydrocarbon (TEH) concentrations measured in Kalamazoo River surface water were elevated downstream from the oil discharge point relative to concentrations upstream from the discharge (Stephanie Millsap, US Fish and Wildlife Service, oral commun., December 2012). Concentrations of TEH in surface water decreased during the 3 weeks before fish collections but remained elevated compared to upstream concentrations at the time of fish collections. Consistently, surface water at Historic Bridge, 9 miles below the discharge, contained slightly greater concentrations of TEHs than Legacy Ranch and Shady Bend, which were another 13–18 miles downstream (Stephanie Millsap, FWS, oral commun., December, 2012). Heavier fractions of the diluted bitumen from this spill sank to the bottom of the river and became associated with sediments. At the time that the fish were collected, the submerged oil was likely acting as a source of polycyclic aromatic hydrocarbons (PAHs) to benthic invertebrates, the overlying water, and aquatic organisms in this stretch of river. Because of the discharge of oil, the Kalamazoo River was closed to the public, but the oil spill response resulted in heavy boat traffic from john boats with small outboard motors and from airboats, every day until well after fish were sampled for this study.

General health metrics (CF, HSI, HAI score) consistently indicated poorer health of fish from sites downstream from the oil discharge relative to fish from the reference site. Moreover, these results were comparable to other studies that used these same biomarkers to assess fish health after exposure to PAHs. Lower condition factors were reported for flatfishes from a location near an oil refinery terminal and for sea bass exposed in the laboratory to crude oil (Kahn, 2003; Kerambrun and others, 2012). The flatfish in Kahn's (2003) evaluation also had heavier livers and therefore increased HSI values and an increased incidence of epidermal lesions especially on skin, gills, and fins compared to flatfish from a reference site. Increased numbers of fish with fin and gill lesions and the severity of those lesions were the variables that most strongly affected the high HAI scores in Kalamazoo River fish downstream from the oil discharge site. Nevertheless, the degree to which habitat differences between the impoundment, where the reference fish were collected, and the more freely flowing river reaches where the oiled fish were collected cannot be evaluated solely from the data obtained in this study. Condition, weight, and length data from SMB and GRH fish surveys on the Kalamazoo River before the Enbridge oil discharge incident may provide useful comparative information for example.

Organ-related differences were similar across species and across sites, and are consistent with literature reports of PAH effects. Macrophage aggregates were either larger or more numerous in fish from oiled sites than fish from the reference site. This same response of MAs has been reported for fish collected from chronically oiled locations (Haensly and others, 1982; Marty and others, 1999; Kahn, 2003). Macrophage aggregates are a good indicator of the general stress of a fish, which may or may not be due to contaminant exposure. In this study, the fish were exposed to contaminants and may also have been experiencing stress from the disturbance of the response activities. Marty and others (1999) attributed increased MAs to the old age of Pacific herring (*Clupea pallasii*) caught in the vicinity of the Exxon Valdez oil spill. Throughout the life of a fish, MAs collect and isolate debris that cannot be metabolized and eliminated; therefore, older fish would tend to accumulate higher concentrations of debris

and MAs (Fournie and others, 2001). In the present assessment, age estimated from length (only for SMB) was similar across sites and linear regression analyses did not support a relation between MAs and age (data not shown).

Spleen lesions, gill lesions, and mucocytes on gill lamellae were all more prevalent in fish from sites downstream from the oil discharge compared to reference fish. Gill lesions, aneurisms, and blood congestion were consistently elevated in fish from sites downstream from the oil discharge and these same types of lesions were observed in fish exposed to water-soluble diesel oil (Simonato and others, 2008). Kahn (2003) found flatfishes from marine areas contaminated with oil had severe gill epithelial cell hypertrophy and fusion, lesions which were observed infrequently or less severely in Kalamazoo River fish perhaps because of the shorter period of exposure. In contrast, gill mucocytes were elevated in both the present study and Kahn (2003) and also Haensly and others (1982) perhaps because these cells are a first line of defense against irritants.

The biomarker CYP1A was clearly identified in gills of both species of fish from all Kalamazoo River sites; however, the response was strongest at sites downstream from the oil discharge site. Cytochrome P450 1A expression is the classic biomarker for chemicals that work through the aryl hydrocarbon receptor biochemical pathway (Whyte and others, 2000). These chemicals include PAHs as well as planar halogenated aromatic hydrocarbons (PHAH). The CYP1A protein is produced primarily in fish hepatocytes and to a lesser extent in kidney and gill (Tuvikene, 1995). Immunohistochemistry of fish gills using antibodies to CYP1A has been successfully used to identify field exposure of fishes to polycyclic aromatic hydrocarbons (Moore and others, 2003). Most recently, it was used to demonstrate a graded exposure response over time to oil spilled during the Deepwater Horizon accident (Dubansky and others, 2013). The low intensity of CYP1A expression at the reference site is not unexpected because of the prevalence and stochastic widespread distribution of PAHs in aquatic habitats.

The blood diagnostic indicators in the present assessment were somewhat inconsistent between species and among sites. This is perhaps explained by taxonomic differences in fish physiology and species-specific habitat and trophic characteristics. Hemoglobin concentrations within normal ranges are diagnostic of general health and the values measured in this study are consistent with values reported in the literature for seemingly healthy fish (Powers and others, 1939; Schmitt and others, 1993). Anemia is indicated by abnormally low hemoglobin concentrations but the etiology of this condition varies (Heath, 1995). A few reports associate lowered hemoglobin concentrations in fish with exposure to crude oil or water-soluble diesel oil (Alkindi and others, 1996; Kahn, 2003; Simonato and others, 2008; Hedayati and Jahanbakhshi, 2012). In contrast, fish collected in a petroleum-contaminated area by Kahn (1998) did not have lowered hemoglobin concentrations, but did have increased levels of hemosiderin compared to reference fish. Exposure of fish to oil is postulated to cause lysis of blood cells and thus the release of hemosiderin, a pigment commonly found in macrophage aggregates (Alkindi and others, 1996). The qualitative MA pigment observations in the present study are consistent with this effect. Although hemoglobin concentrations from the site closest to the oil discharge were statistically less than those from the reference site only for GRH females, together the hemoglobin concentrations and hemosiderin biomarkers support an exposure to oil in the present evaluation.

Parasite infestation was also somewhat inconsistent among species and sites. The effect of environmental stressors on parasitism in fish can be variable and complex in part because the stressor may increase the fishes' vulnerability to parasitic infection and have adverse effects directly on the parasite at some stage in its lifecycle. Moreover, ectoparasites are thought to increase and endoparasites to decrease in fish exposed to chemical contaminants (Kahn, 2003 and references therein). In the present fish health evaluation, macroscopically visible ectoparasites were infrequent on both species; however, fin erosion, which was frequently noted in fish from sites downstream from the oil discharge, may have been due to microscopic ectoparasites that would not have been seen during necropsy. Also, gill parasites were not

noted during necropsy but were observed during histopathology evaluations and were increased in GRH at Historic Bridge, the site closest to the oil discharge. Compared to fish from the reference site, splenic endoparasites in SMB were lowest at the two sites closest to the oil discharge and greatest at the site farthest from the oil whereas, in GRH, splenic parasites were sparse in fish from oiled sites and absent in reference fish. These results can be interpreted as a general response to stress given the limited macroscopic evaluation of parasite infestation of the collected fish from the Kalamazoo River, the relatively brief time the fish would have been exposed to the discharged oil, and potential habitat differences between the free-flowing stretch of the river and the impounded reservoir where the reference fish were caught. Another factor to be considered is that the presence of significant boat traffic as part of the oil spill response may have also stressed fish in the areas downstream from the discharge.

Polycyclic aromatic hydrocarbons are generally expected to suppress the fish immune system. The characteristic leukocyte profile reported for fish exposed to PAHs includes a decrease in lymphocytes (Kahn, 2003; Hedayati and Jahanbakhshi, 2012; McNeil and others, 2012); however, there is an incomplete understanding of the mechanisms of immunotoxicity and species-specific effects (Reynaud and Deschaux, 2006). Immunosuppression can hinder fish from fighting bacterial and viral infections, and this can lead to lesions on the external surfaces such as the frayed fins and gill hyperplasia observed in fish from some of the Kalamazoo River oiled sites (McNeil and others, 2012). The spleen is a primary immune system organ in fish and the location of lymphocyte production. On average, spleen lymphoid tissue appeared reduced in fish from Kalamazoo River sites downstream from the oil discharge. A similar observation was made for anterior kidney of fish exposed to select PAH compounds (Hart and others, 1998; Holladay and others, 1998); however, Reynaud and Deschaux (2006) presented contradictory results wherein PAHs stimulated and suppressed lymphocyte proliferation depending on chemical, dose, and species. Counts and ratios of circulating leukocytes in the blood are also common measurements for assessing general environmental stress effects on fish (Davis and others, 2008). A popular diagnostic of

general stress is the ratio of circulating granulocytes to lymphocytes wherein granulocytes increase and lymphocytes decrease. In contrast to PAH-induced immunosuppression, general stress-related lymphopenia is usually accompanied by an increase in granulocytes (Davis and others, 2008). The immune system response patterns observed in fish from the Kalamazoo River study are mixed. Circulating lymphocytes were only slightly depressed in GRH at the Historic Bridge site and granulocytes and the granulocyte-to-lymphocyte (G:L) ratio were not different when compared to GRH from the reference site. Golden redhorse from Legacy Ranch did show the general stress pattern in G:L ratio relative to GRH from Marshall Impoundment. In comparison to the reference site, Shady Bend (the site farthest from the discharge; fig. 1) GRH had a slight increase in lymphocytes, no difference in granulocytes, and a resulting decrease in the G:L ratio. Golden redhorse monocytes were few and not significantly different among sites, suggesting that the fish were not likely diseased. In contrast to the blood results, splenic lymphoid tissue was significantly reduced in all GRH from Kalamazoo River oiled sites. Together, GRH immune system suppression attributed to PAH exposure is suggested by the qualitatively observed lower lymphocyte production in the spleen but at the time of sampling was not reflected by the distribution of leukocyte types in the blood.

Results for SMB are even more difficult to interpret. In contrast to GRH, circulating lymphocytes in SMB from the two sites closest to the oil discharge tended to be greater compared to lymphocyte levels in SMB from the reference site. Granulocytes were fewer in SMB at the two sites closest to the discharge and G:L ratios less, excepting that for the female from Shady Bend, compared to the SMB from the reference site. Monocytes, which phagocytize foreign particles, were elevated only in SMB at Shady Bend and this is consistent with the elevated splenic parasites observed for SMB at this site. Assuming the leukocyte profile in fish from Marshall Impoundment is representative of normal, it seems that PAH effects an increase in SMB lymphocytes. The fact that splenic lymphoid tissue was not statistically reduced in SMB at oiled sites as it was for GRH may be related to these leukocyte results. Although

differences in blood leukocyte patterns exist between the oiled fish and the reference fish, a lack of information on the normal and stressed profiles in SMB and GRH hinders a clear interpretation of these immunological endpoints.

A number of health parameters, from the biochemical level to the individual fish level were evaluated in fish collected in the Kalamazoo River downstream from the Enbridge Line 6B discharge point. The results for these bioindicators were overall similar for both species and among the three oiled sites. Most of the bioindicator results for fish from the oiled sites contrasted with and were significantly different from the bioindicator results obtained for fish collected from a reference site above and unaffected by the oil discharge. Moreover, results from this evaluation are consistent with both laboratory studies of PAH toxicity to fish and field assessments of catastrophic oil spills or chronic PAH contamination from multiple anthropogenic sources. The present fish health evaluation has demonstrated that fish at sites as far as 27 miles downstream from the Enbridge Line 6B pipeline discharge point were less healthy than fish from above the discharge point, showed signs of generalized stress, and showed effects in specific endpoints that were consistent with the adverse effects expected from exposure to crude oil.

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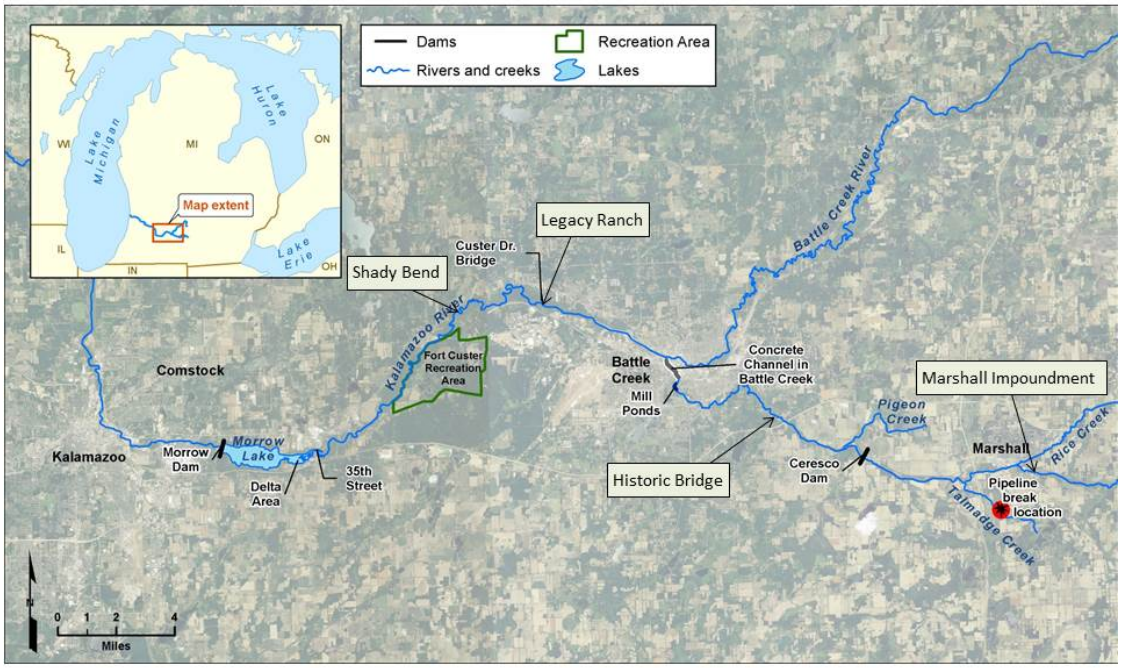

Figure 1. Approximate location of sample sites along the Kalamazoo River in Michigan. The Enbridge Line 6B pipeline break occurred near Talmadge Creek, which enters the Kalamazoo River downstream from Marshall, Michigan. The upstream reference area is in the Marshall Impoundment (MI), and the sampling sites within the Kalamazoo River downstream from the oil discharges are Historic Bridge (HB) Legacy Ranch (LR), and Shady Bend (SB).

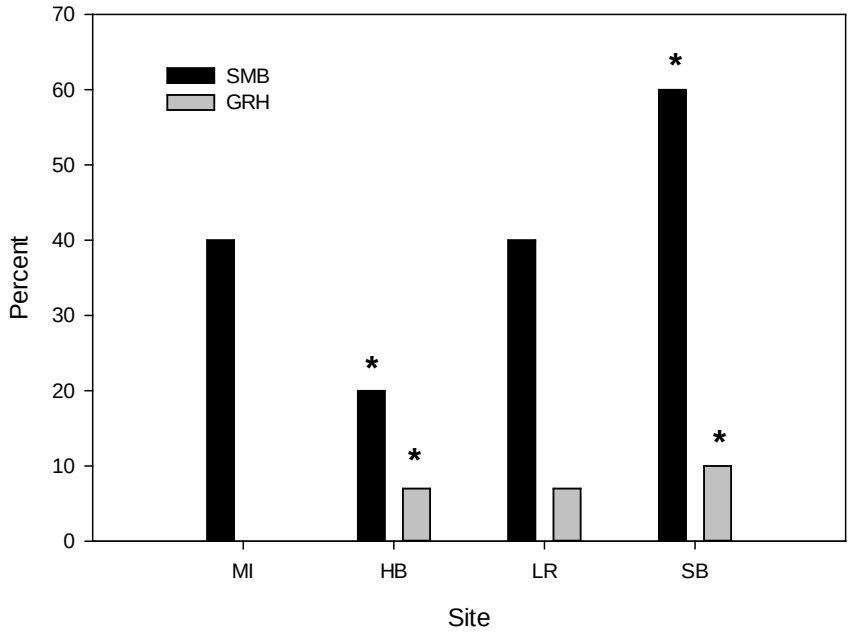

Figure 2. Percent of smallmouth bass (SMB) and golden redhorse (GRH) with splenic parasites from Historic Bridge (HB), Legacy Ranch (LR), Shady Bend (SB) and a reference site, Marshall Impoundment (MI). An asterisk above a bar indicates a significant difference ($p \leq 0.05$) between fish of a given species at the oiled site and at the reference site. [$\leq$ is less than or equal to]

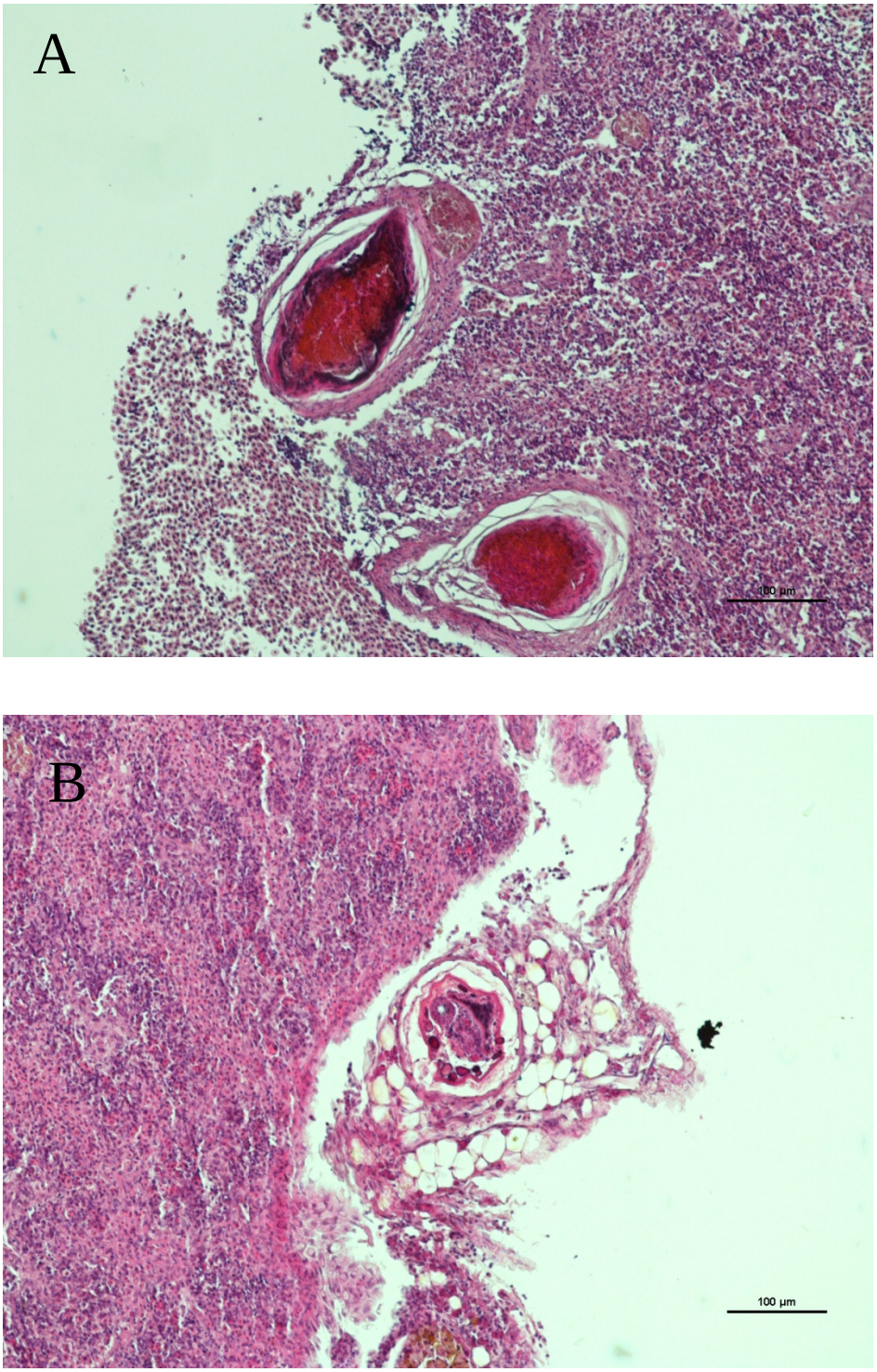

Figure 3. Example of parasites found in histological sections of smallmouth bass spleen from *A*, Marshall Impoundment and *B*, Shady Bend. Scale bar represents 100 microns.

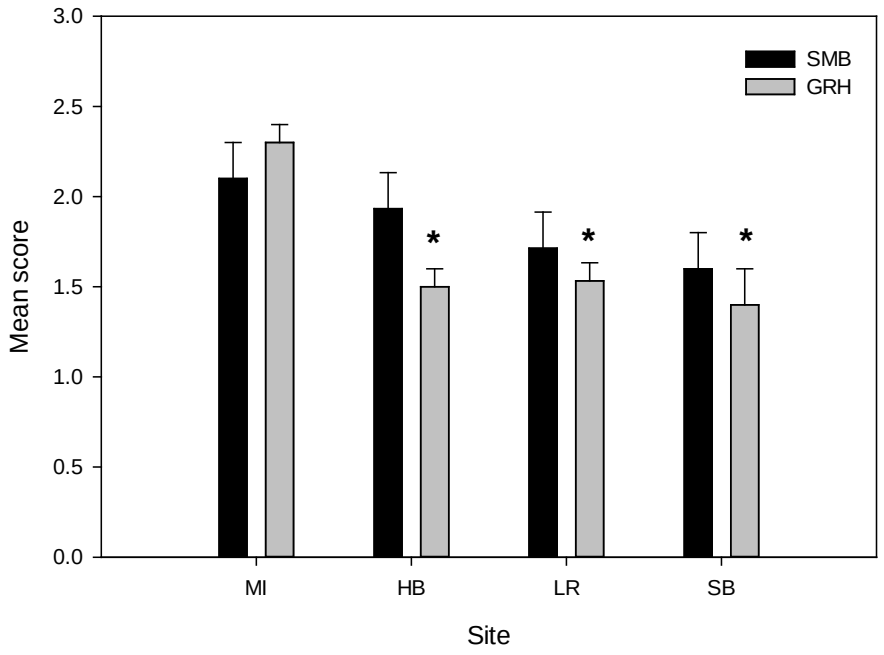

Figure 4. Mean score indicating the prevalence of splenic lymphoid tissue for smallmouth bass (SMB) and golden redhorse (GRH) from Historic Bridge (HB), Legacy Ranch (LR), Shady Bend (SB), and a reference site, Marshall Impoundment (MI). Error bar represents one standard error (SE) of the mean. An asterisk above a bar indicates a significant difference ($p \leq 0.05$) between the respective species and the reference site.[$\leq$ is less than or equal to]

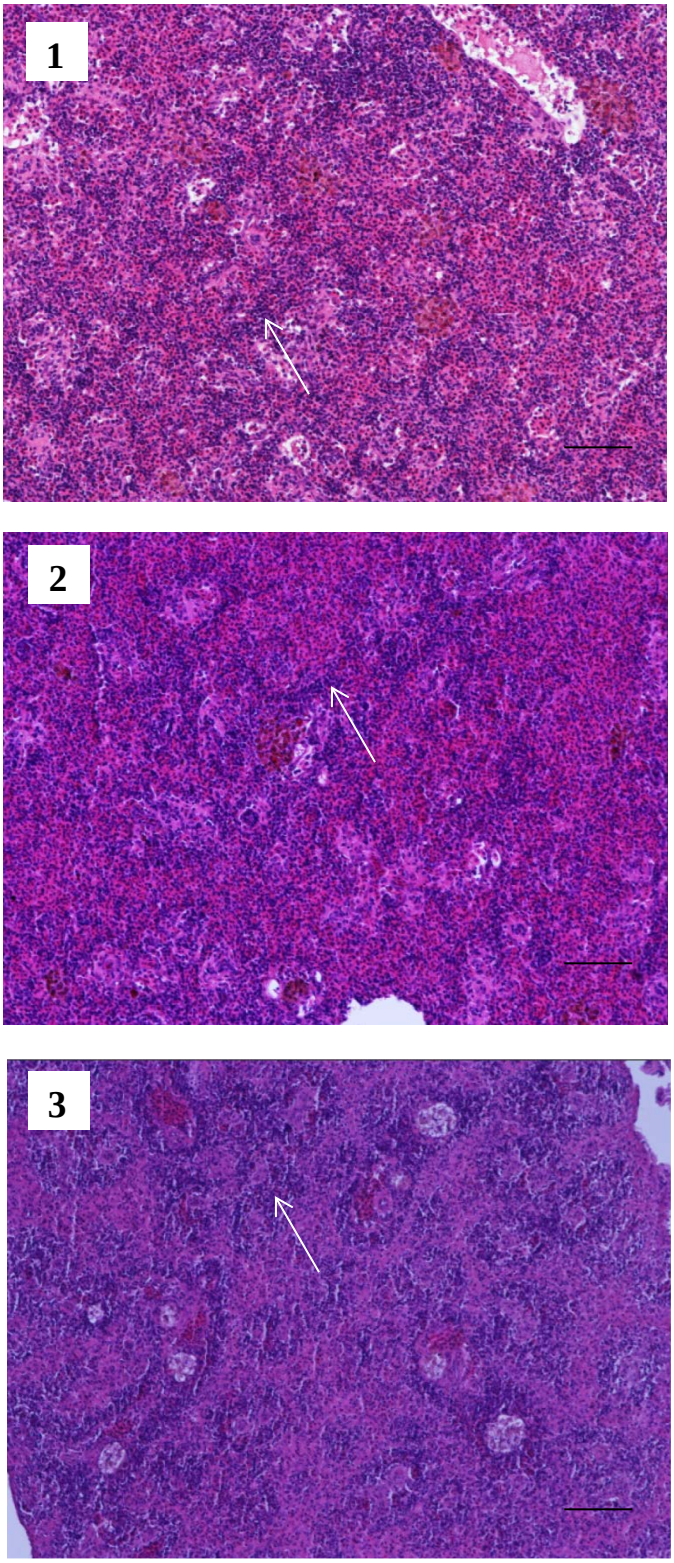

Figure 5. Histological sections of golden redhorse sucker spleen. Spleens were classified 1–3 according to the amount of lymphoid tissue (arrows) present. Scale bar represents 100 microns.

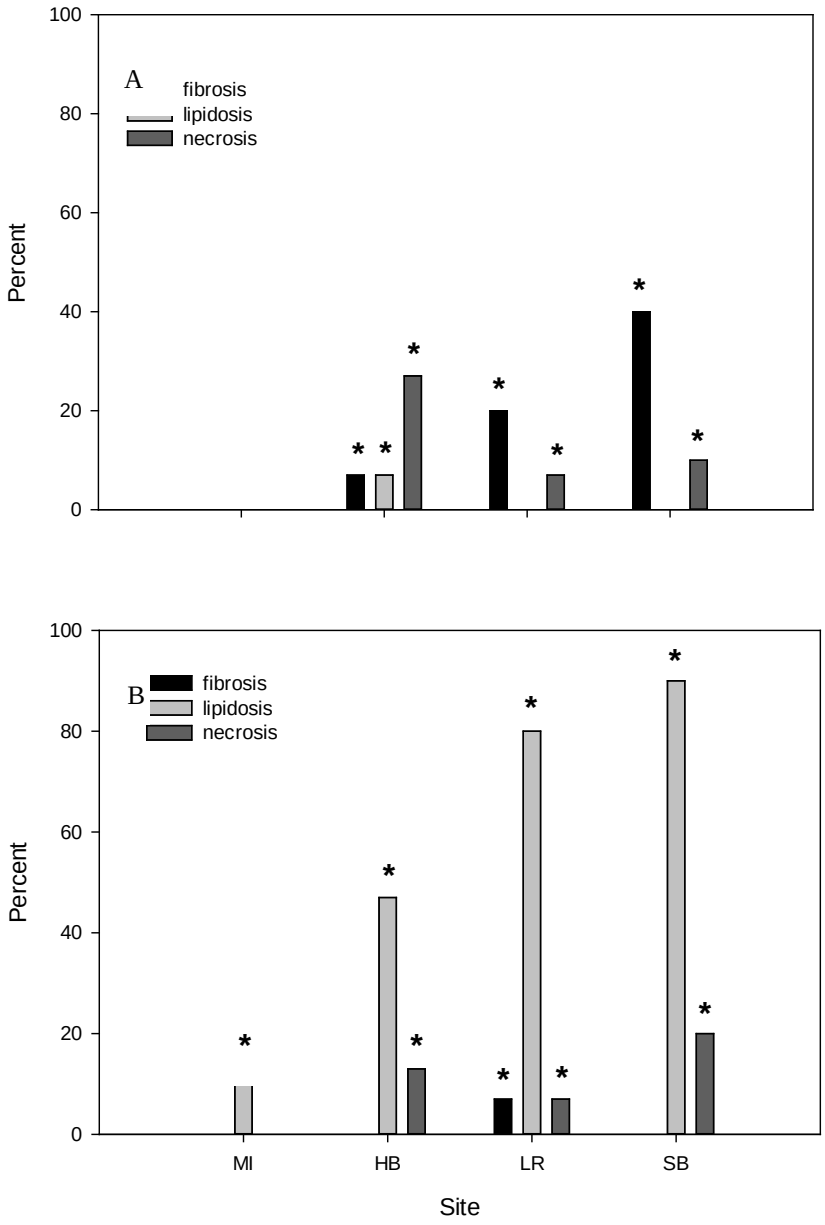

Figure 6. Percent of *A*, golden redhorse sucker and *B*, smallmouth bass with fibrosis, lipid deposits, or necrosis in spleens from three sites Historic Bridge (HB), Legacy Ranch (LR), and Shady Bend (SB) affected by the Enbridge Line 6B oil discharge and a reference site, Marshall Impoundment (MI). An asterisk above a bar indicates a significant difference ($p \leq 0.05$) between the oiled site and the reference site. [$\leq$ is less than or equal to]

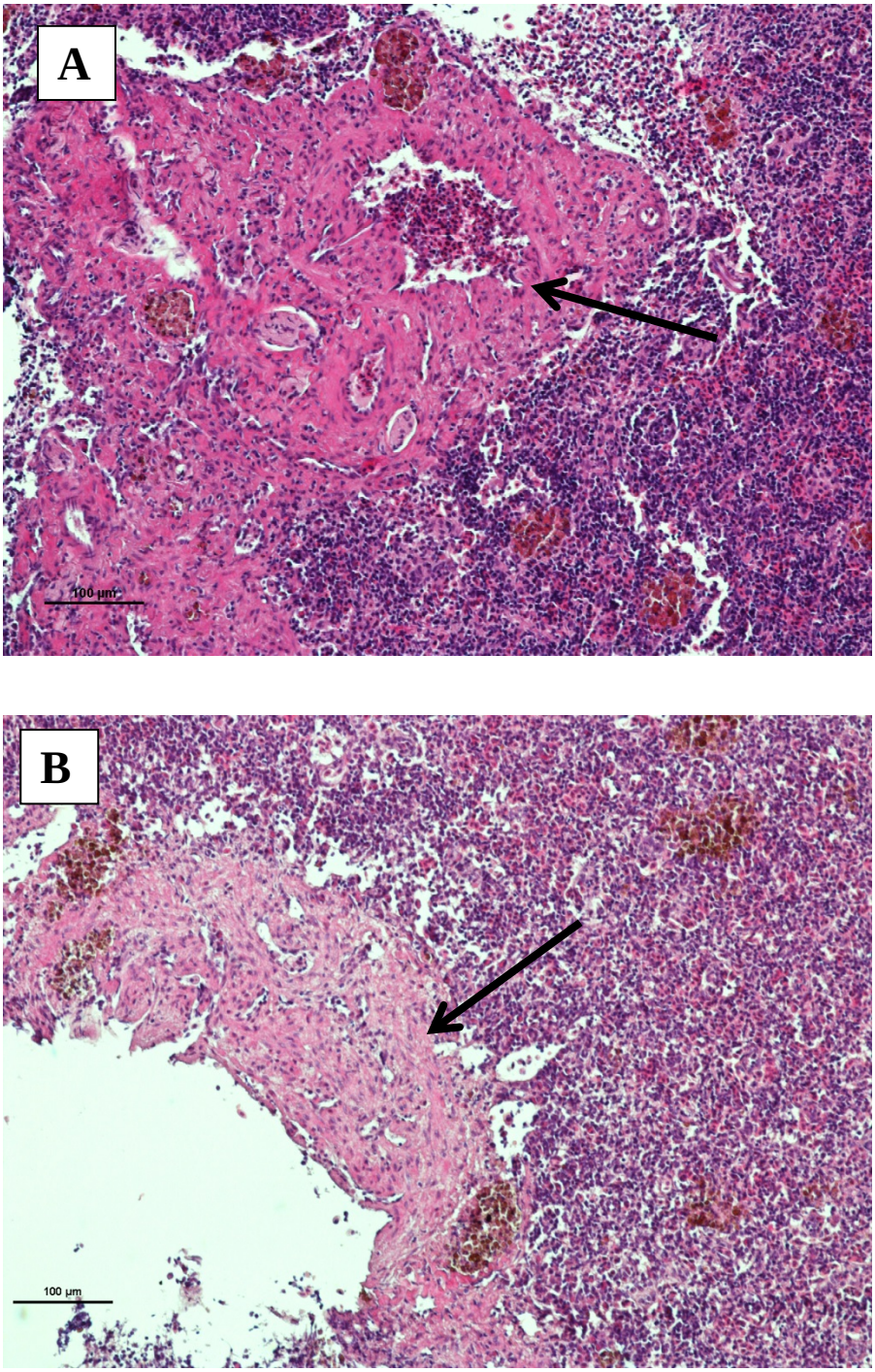

Figure 7. Example of fibrosis (arrow) in histological section of spleen of golden redhorse sucker from *A*, Shady Bend, and *B*, Historic Bridge. Scale bar represents 100 microns.

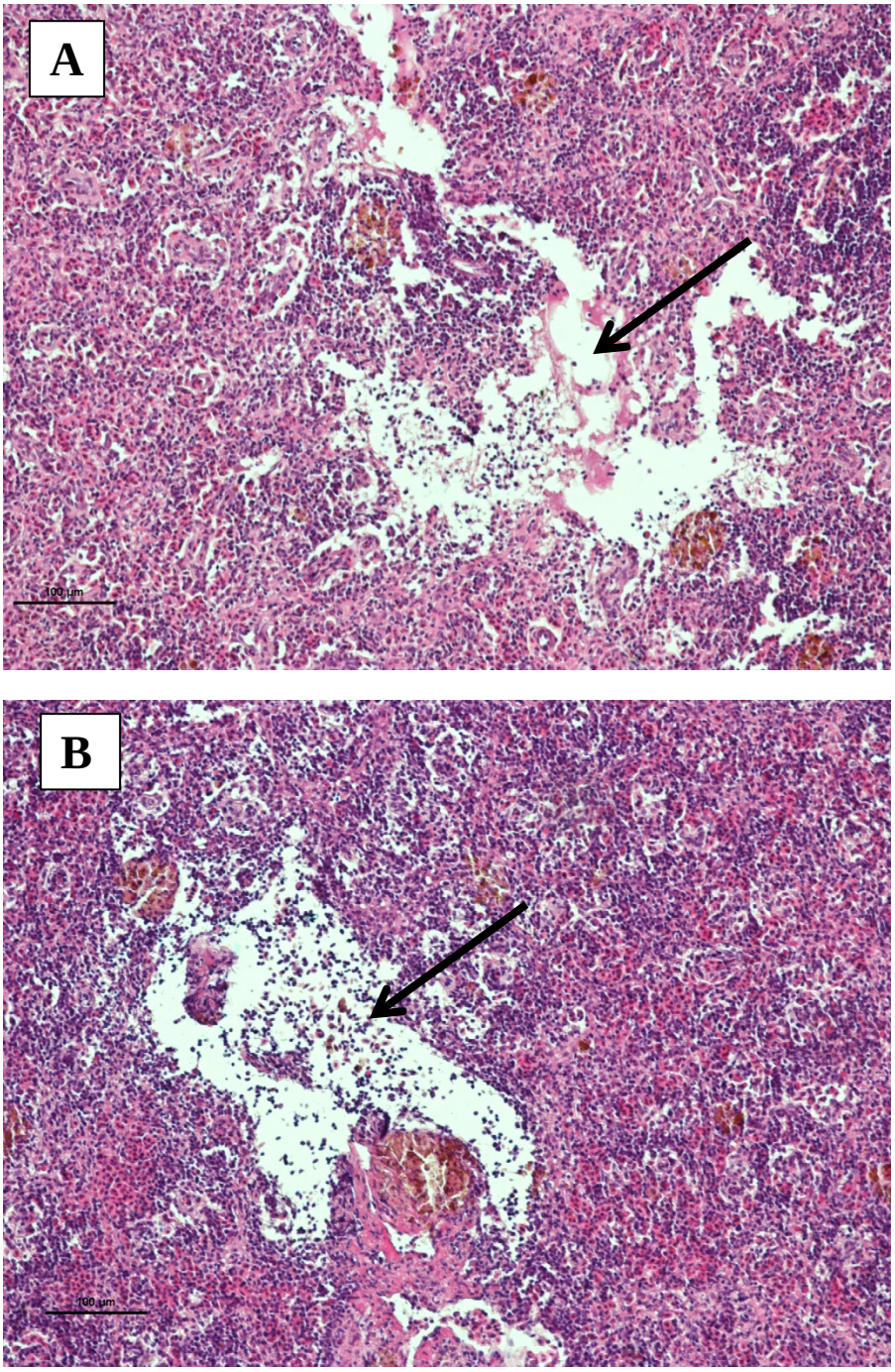

Figure 8. Example of necrosis (arrow) in histological section of spleen of golden redhorse sucker from *A*, Legacy Ranch and *B*, Historic Bridge. Scale bar represents 100 microns.

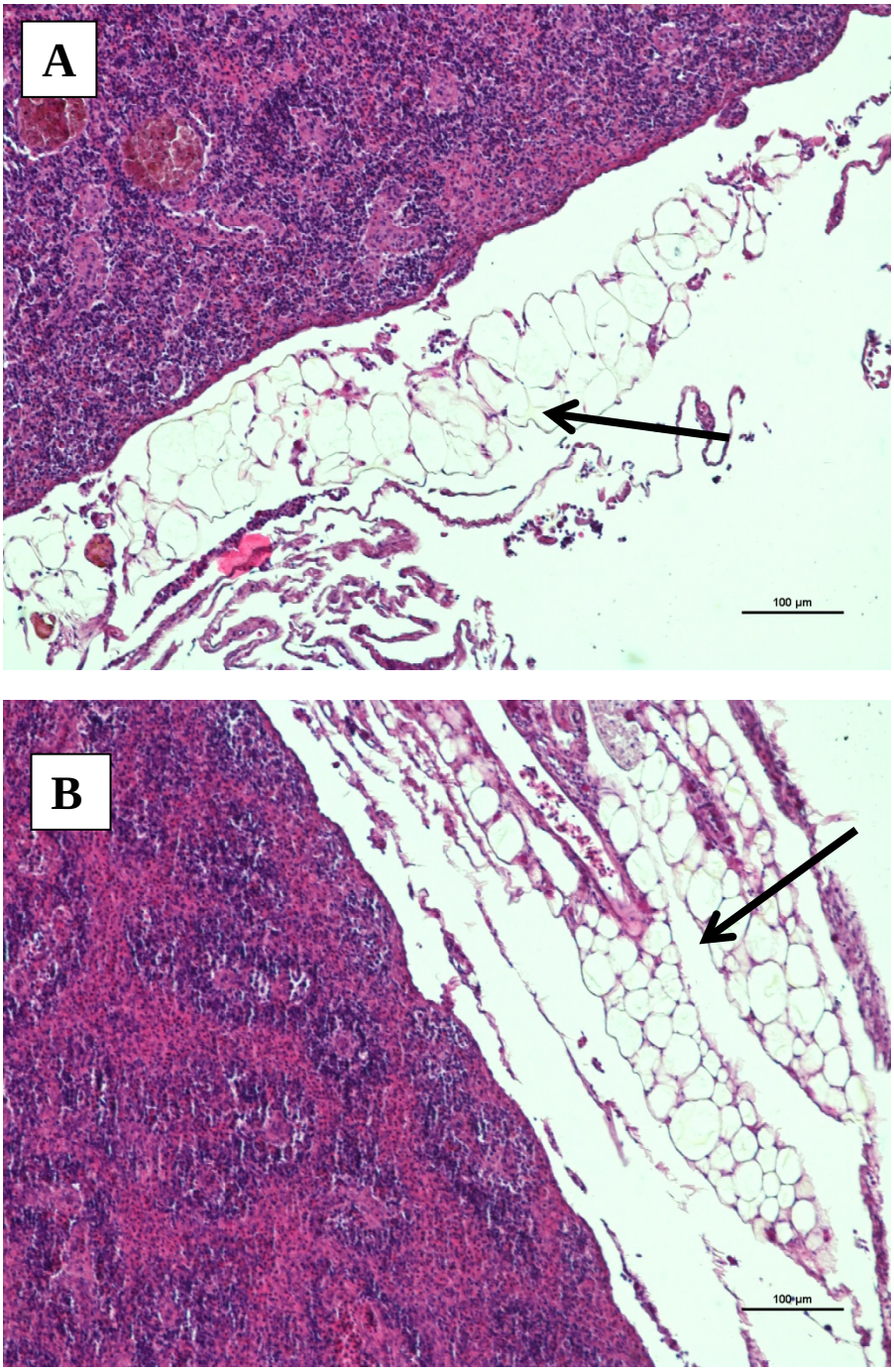

Figure 9. Example of lipid (arrow) encapsulating spleen in histological section from golden redhorse sucker from *A*, Marshall Impoundment and *B*, Shady Bend. Scale bar represents 100 microns.

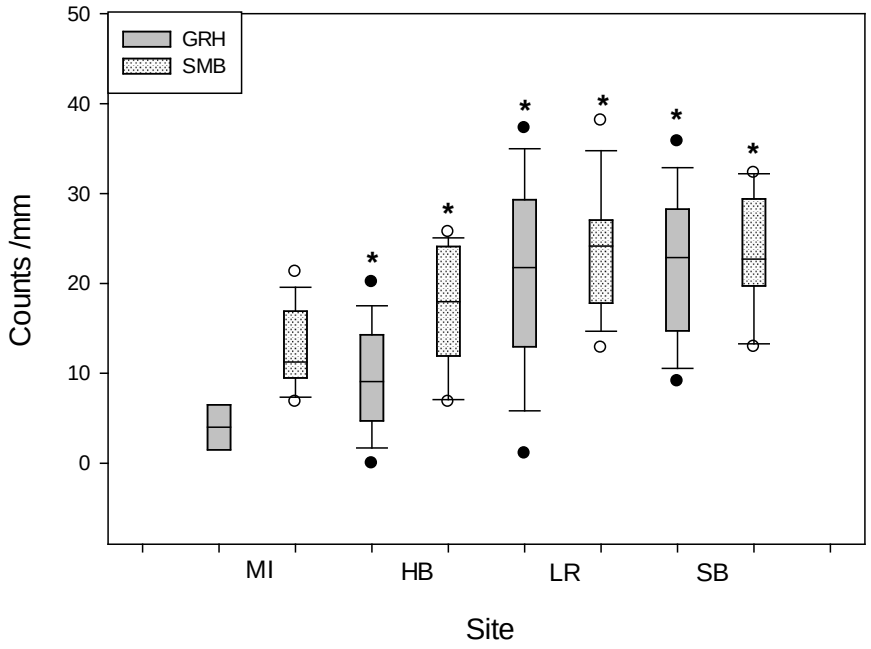

Figure 10. Mucus cell counts per millimeter gill filament in golden redhorse sucker (GRH) and smallmouth bass (SMB) from Historic Bridge (HB), Legacy Ranch (LR), Shady Bend (SB), and a reference site, Marshall Impoundment (MI). The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers (error bars) above and below the box indicate the 90th and 10th percentiles, and symbols outside the box indicate outlying points. An asterisk above a bar indicates a significant difference ($p \leq 0.05$) between the respective species and the reference site. [$\leq$ is less than or equal to]

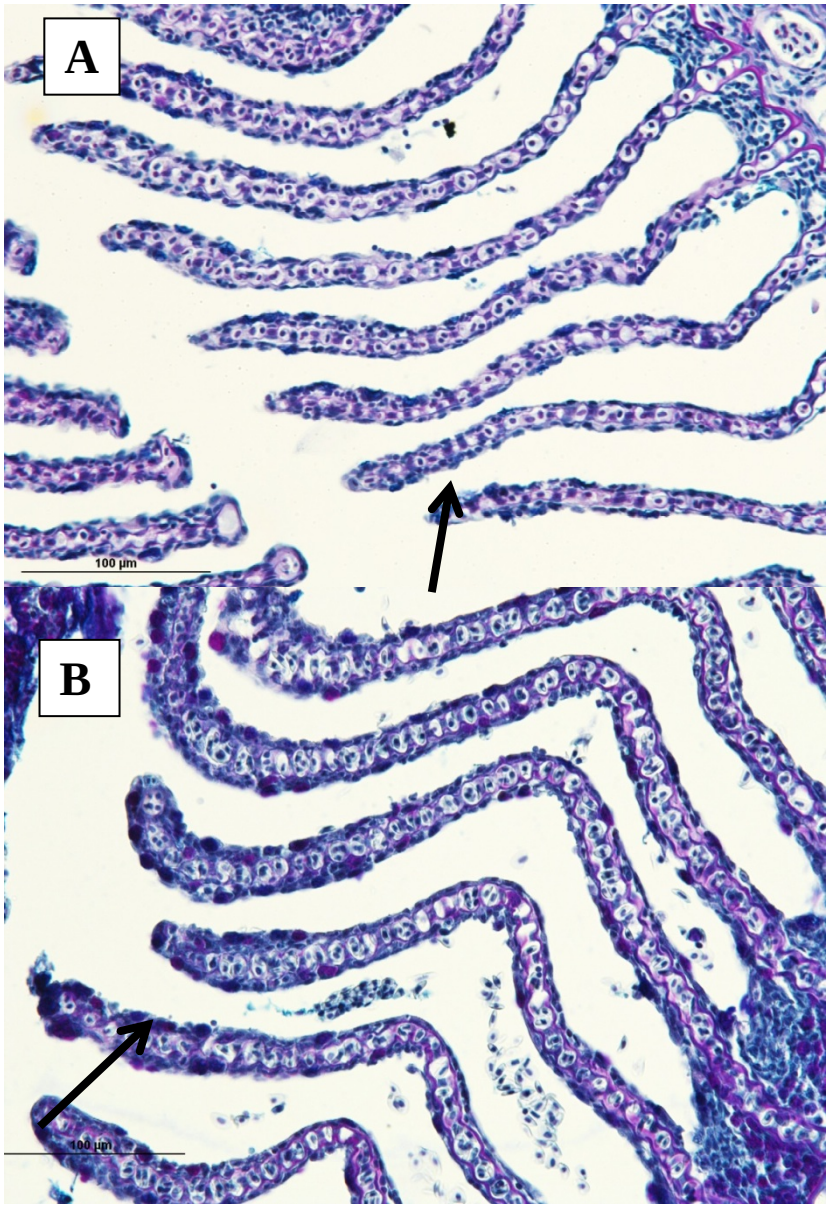

Figure 11. Example of mucus cells (arrows) on secondary lamellae in histological sections of gills from golden redhorse sucker collected from *A*, Marshall Impoundment and *B*, Historic Bridge. Scale bar represents 100 microns.

Table 1. Mean size of smallmouth bass (SMB) and golden redhorse sucker (GRH) from Marshall Impoundment (reference site) and three sites affected by the Enbridge Line 6B oil discharge. [millimeter = mm; number = N; grams = gr; standard deviation = SD]

Site	Species	Sex	N	Length (mm) mean (SD)	Weight (gr) mean (SD)	Significant differences[1]
Marshall Impoundment	SMB	M	5	339 (50)	602 (264)	
	SMB	F	10	315 (62)	475 (260)	
	GRH	M	3	391 (28)	634 (102)	
	GRH	F	12	427 (23)	827 (177)	AC
Historic Bridge	SMB	M	7	364 (71)	706 (364)	
	SMB	F[2]	7	283 (53)	261 (122)	
	GRH	M	7	371 (67)	570 (263)	
	GRH	F	8	440 (50)	933 (262)	A
Legacy Ranch	SMB	M	8	318 (63)	401 (185)	
	SMB	F	7	267 (43)	263 (118)	
	GRH	M	8	370 (26)	486 (94)	
	GRH	F	7	412 (27)	686 (166)	BC
Shady Bend	SMB	M	5	345 (61)	690 (297)	
	SMB	F	5	398 (78)	514 (398)	
	GRH	M	2	377 (28)	410 (85)	
	GRH	F	8	324 (29)	635 (134)	B

[1] Males and females were significantly different ($p \leq 0.05$) in length and weight and were therefore analyzed separately. The only site-related effects were for weight of GRH females. The same letter indicates no significant site differences ($p > 0.05$) among weights of GRH females.
[2] An eighth female was captured but not included because it was juvenile.

Table 2. Mean condition factor (CF) of smallmouth bass (SMB) and golden redhorse sucker (GRH) from Marshall Impoundment (reference site) and three sites affected by the Enbridge Line 6B oil discharge. [number = N; standard deviation = SD]

Site	Species	Sex	N	CF mean (SD)	Significant differences[1]
Marshall Impoundment	SMB	M	5	1.46 (0.17)	A
	SMB	F	10	1.40 (0.07)	
	GRH	M	3	1.06 (0.06)	A
	GRH	F	12	1.05 (0.07)	
Historic Bridge	SMB	M	7	1.36 (0.09)	B
	SMB	F	7	1.15 (0.33)	
	GRH	M	7	1.05 (0.04)	A
	GRH	F	8	1.06 (0.07)	
Legacy Ranch	SMB	M	8	1.22 (0.25)	B
	SMB	F	7	1.32 (0.12)	
	GRH	M	8	0.95 (0.05)	B
	GRH	F	7	0.97 (0.12)	
Shady Bend	SMB	M	5	1.21 (0.09)	B
	SMB	F	5	1.27 (0.12)	
	GRH	M	2	0.99	B
	GRH	F	8	0.99 (0.03)	

[1] Sexes were not significantly different (p>0.05) and were combined within a species for significance testing among sites. The same letter within a species indicates no significant differences (p>0.05).

Table 3. Mean hepatosomatic index (HSI) for smallmouth bass (SMB) from Marshall Impoundment (reference site) and three sites affected by the Enbridge Line 6B oil discharge. [number = N; standard deviation = SD]

Site	Species	Sex	N	HSI mean (SD)	Significant differences[1]
Marshall Impoundment	SMB	M	5	0.54 (0.06)	A
	SMB	F	10	0.53 (0.09)	
Historic Bridge	SMB	M	7	0.75 (0.09)	B
	SMB	F	7	1.19 (1.02)	
Legacy Ranch	SMB	M	8	0.75 (0.54)	AC
	SMB	F	7	0.57 (0.07)	
Shady Bend	SMB	M	5	0.63 (0.07)	C
	SMB	F	5	0.57 (0.11)	

[1] Sexes were not significantly different and were combined for significance testing among sites.
The same letter indicates no significant difference (p>0.05).

Table 4. Mean scores for the Health Assessment Index (HAI) for smallmouth bass (SMB) and golden redhorse sucker (GRH) from Marshall Impoundment (reference site) and three sites affected by the Enbridge Line 6B oil discharge. [(number = N; standard deviation = SD].

Site	Species	Sex	N	HAI mean (SD)	Significant differences[1]
Marshall Impoundment	SMB	M	5	0 (0)	A
	SMB	F	10	1 (3)	
	GRH	M	3	23 (23)	A
	GRH	F	12	6 (9)	
Historic Bridge	SMB	M	7	26 (11)	B
	SMB	F	7	30 (0)	
	GRH	M	7	59 (17)	B
	GRH	F	8	60 (11)	
Legacy Ranch	SMB	M	8	36 (11)	C
	SMB	F	7	33 (5)	
	GRH	M	8	43 (10)	C
	GRH	F	7	44 (21)	
Shady Bend	SMB	M	5	48 (22)	C
	SMB	F	5	36 (5)	
	GRH	M	2	25 (7)	C
	GRH	F	8	45 (18)	

[1]Sexes (except fish from Marshall Impoundment) were not significantly different (p>0.05) and were combined within a species for significance testing among sites. The same letter within a species indicates no significant difference (p>0.05).

Table 5. Mean hemoglobin (Hb) for smallmouth bass (SMB) and golden redhorse sucker (GRH) from Marshall Impoundment (reference site) and three sites affected by the Enbridge Line 6B oil discharge. [grams/deciliter = gr/dL; number = N; standard deviation = SD]

Site	Species	Sex	N	Hb[1] (gr/dL) mean (SD)	Significant differences[2]
Marshall Impoundment	SMB	M	5	5.8 (0.5)	
	SMB	F	10	5.4 (0.8)	
	GRH	M	3	3.7 (0.8)	
	GRH	F	11	5.0 (0.6)	A
Historic Bridge	SMB	M	7	6.2 (1.4)	
	SMB	F	7	5.6 (1.4)	
	GRH	M	6	2.7 (0.9)	
	GRH	F	8	3.0 (1.7)	B
Legacy Ranch	SMB	M	8	6.0 (1.3)	
	SMB	F	7	4.7 (1.8)	
	GRH	M	7	4.4 (1.3)	
	GRH	F	7	5.2 (1.1)	A
Shady Bend	SMB	M	5	6.2 (1.2)	
	SMB	F	4	5.2 (0.8)	
	GRH	M	2	4.4	
	GRH	F	7	4.3 (0.9)	AB

[1]Hemoglobin values were corrected following Clark and others (2008).
[2]Sexes were not significantly different ($p>0.05$) for both species and all stations except GRH from Marshall Impoundment and SMB from Legacy Ranch. Sexes were analyzed separately within a species for significance testing among sites. Significant differences ($p\leq0.05$), found only among GRH females, are indicated by different letters.

Table 6. Thrombocytes, leukocytes, and the ratio of granulocytes (G) to lymphocytes (L) in blood smears of smallmouth bass (SMB) and golden redhorse sucker (GRH) from a Marshall Impoundment (reference site) and three sites associated with the Enbridge Line 6B oil discharge. Monocytes, granulocytes, and lymphocytes are reported as a percent of total leukocytes. Thrombocytes are reported as a percent of total thrombocytes plus leukocytes. [number = N; stand deviation = SD].

				Leukocytes[1]			
Site	Species Sex	N	Thrombocytes[1] mean (SD)	Monocytes mean (SD)	Lymphocytes mean (SD)	Granulocytes mean (SD)	G:L Ratio[2]
Marshall Impoundment	SMB F	10	3 (4)	3 (4)	72 (12)	26 (11)	0.32
	SMB M	5					0.53
	GRH F	12	15 (8)	1 (1)	93 (8)	6 (7)	0.07
	GRH M	3					
Historic Bridge	SMB F	7	2 (2)	1 (1)	94 (4)*	4 (4)*	0.05 *
	SMB M	7					0.05 *
	GRH F	8	16 (13)	1 (1)	91 (5)*	7 (5)	0.08
	GRH M	7					
Legacy Ranch	SMB F	7	1 (2)	1 (1)	96 (3)*	3 (3)*	0.03 *
	SMB M	8					0.04 *
	GRH F	7	24 (16)	1 (1)	86 (10)*	13 (9)	0.16 *
	GRH M	8					
Shady Bend	SMB F	5	3 (2)	5 (7)*	81 (17)	14 (12)	0.36
	SMB M	5					0.07 *
	GRH F	8	17 (11)	2 (2)	97 (3)*	2 (2)	0.02 *
	GRH M	2					

[1]Sexes were not significantly different ($p>0.05$) and were combined within a species for significance testing among sites. Within a column, means with an asterisk are significantly different ($p<0.05$) from respective species from Marshall Impoundment.
[2]Sexes were only significantly different ($p\leq0.05$) for SMB. Smallmouth bass sexes were analyzed separately for significance testing among sites whereas, GRH were combined for the analysis. Means with an asterisk are significantly different ($p<0.05$) from respective species and sex from Marshall Impoundment.

Table 7. Macrophage aggregates (MA) in spleens of smallmouth bass from a reference site (Marshall Impoundment) and three sites affected by the Enbridge Line 6B oil discharge. Within a column, means with the same letter are not significantly different (p>0.05). [square microns = µm2; number = N; standard deviation = SD, > is greater than]

Site	N	Total MA Area / μm^2 mean ± SD (range)	Size of MA (μm^2) mean ± SD (range)	Number MAs / μm^2 mean ± SD (range)
Marshall Impoundment	15	0.008±0.008 A (0–0.03)	1106±895 A (0–3044)	$4.9x10^{-6}$±$4.5x10^{-6}$ A ($0–1.3x10^{-5}$)
Historic Bridge	14	0.02±0.01 AB (0–0.05)	2523±1529 B (834–6695)	$5.6x10^{-6}$±$2.5x10^{-6}$ A ($1.90x10^{-6}–1.0x10^{-5}$)
Legacy Ranch	15	0.01±0.008 AB (0–0.02)	1505±845 A (0–3946)	$7.2x10^{-6}$±$5.3x10^{-6}$ A ($0–2.3x10^{-5}$)
Shady Bend	10	0.02±0.01 B (0.01–0.05)	3190±1484 B (1222–6059)	$7.7x10^{-6}$±$2.6x10^{-6}$ A ($3.3x10^{-6}–1.1x10^{-5}$)

Table 8. Macrophage aggregates (MA) in spleens of golden redhorse sucker from a reference site (Marshall Impoundment) and three sites affected by the Enbridge Line 6B oil discharge. Within a column, means with the same letter are not significantly different (p>0.05). [square microns = µm2; number = N; standard deviation = SD, > is greater than]

Site	N	Total MA Area / μm^2 mean ± SD (range)	Size of MA (μm^2) mean ± SD (range)	Number MAs / μm^2 mean ± SD (range)
Marshall Impoundment	15	0.02±0.01 A (0–0.04)	1232±567 A (0–2202)	$1.1x10^{-5}$±$6.9x10^{-6}$ B ($0–2.5x10^{-5}$)
Historic Bridge	15	0.05±0.06 BC (0–0.28)	2788±3674 A (365–16120)	$1.7x10^{-5}$±$6.2x10^{-6}$ A ($4.0x10^{-6}$–$2.5x10^{-5}$)
Legacy Ranch	15	0.02±0.01 AC (0.01–0.06)	1330±379 A (764–2127)	$1.9x10^{-5}$±$1.0x10^{-5}$ A ($8.8x10^{-6}$– $4.2x10^{-5}$)
Shady Bend	10	0.03±0.02 BC (0–0.07)	1714±1197 A (0–4809)	$1.7x10^{-5}$ $8.9x10^{-6}$ AB ($0–3.6x10^{-5}$)

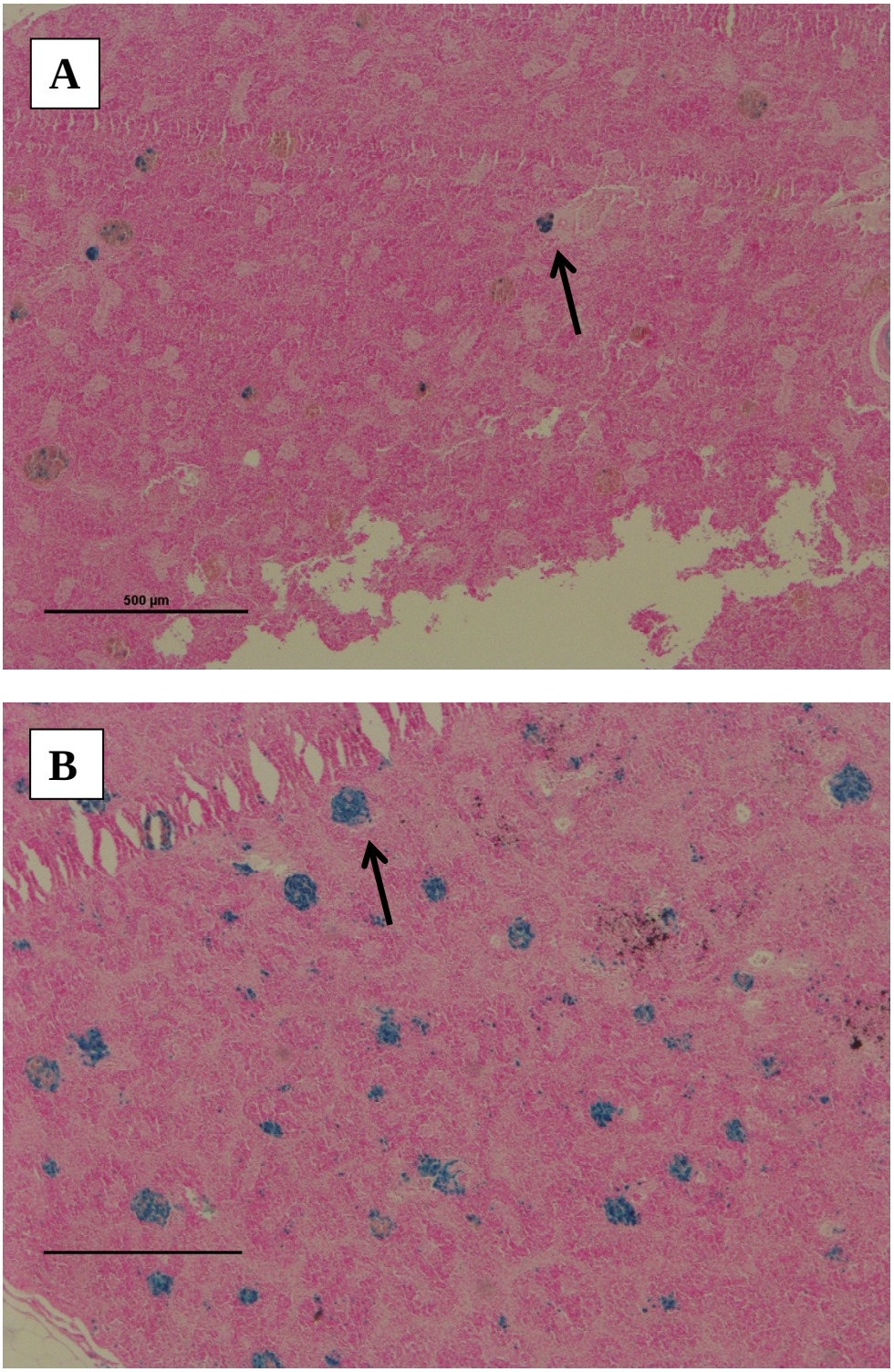

Figure 12. Example of macrophage aggregates (arrow) in histological section of spleen from smallmouth bass from *A*, Marshall Impoundment and *B*, Shady Bend. Scale bar represents 500 microns.

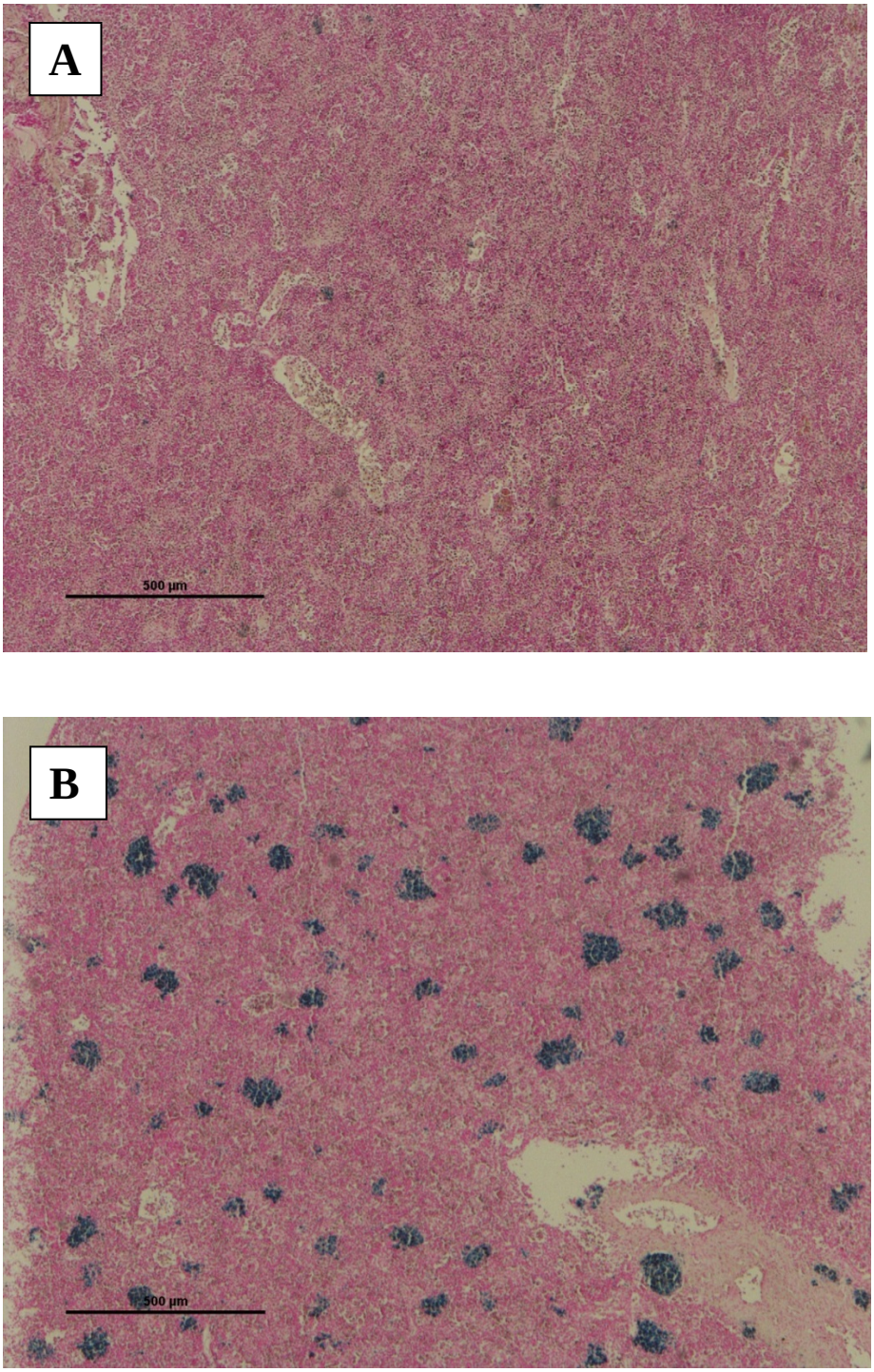

Figure 13. Example of macrophage aggregates (arrow) in histological section of spleen from golden redhorse from *A*, Marshall Impoundment and *B*, Historic Bridge. Scale bar represents 500 microns.

Table 9. Gill lesions in fish from Marshall Impoundment (MI), Historic Bridge (HB), Legacy Ranch (LR), and Shady Bend (SB) as percent of fish with a lesion (Percent) and severity score (Score; see text for explanation; maximum score is 45 and a higher number indicates lesion is more severe). Percent fish from oiled sites within brackets [ ] are significantly different ($p \leq 0.05$) from the reference site, Marshall Impoundment. [$\leq$ is less than or equal to]

	Golden redhorse sucker		Smallmouth bass	
Lesion	Percent (**MI**-HB-LR-SB)	Score (**MI**-HB-LR-SB)	Percent (**MI**-HB-LR-SB)	Score (**MI**-HB-LR-SB)
Epithelial Lifting	**13**-[27-47-50]	**2**-11-12-12	**20**-20-[33]-10	**3**-3-5-2
Epithelial hyperplasia	**47**-[67]-53-0	**8**-19-12-0	**60**-53-60-40	**11**-11-12-9
Aneurism	**33**-[60-60-70]	**10**-19-16-17	**27**-[60]-27-20	**6**-19-4-5
Curling	**40**-33-40-40	**9**-5-7-8	**7**-13-0-[20]	**1**-2-0-1
Congestion	**33**-40-[53-50]	**6**-12-12-11	**13**-[27-27-50]	**3**-5-4-9
Parasites	**7**-[20-0-0]	**1**-4-0-0	**0**-0-0-0	**0**-0-0-0
Fusion secondary lamellae	**13**-7-7-[0]	**2**-1-2-0	**7**-[20]-7-[0]	**1**-4-1-0

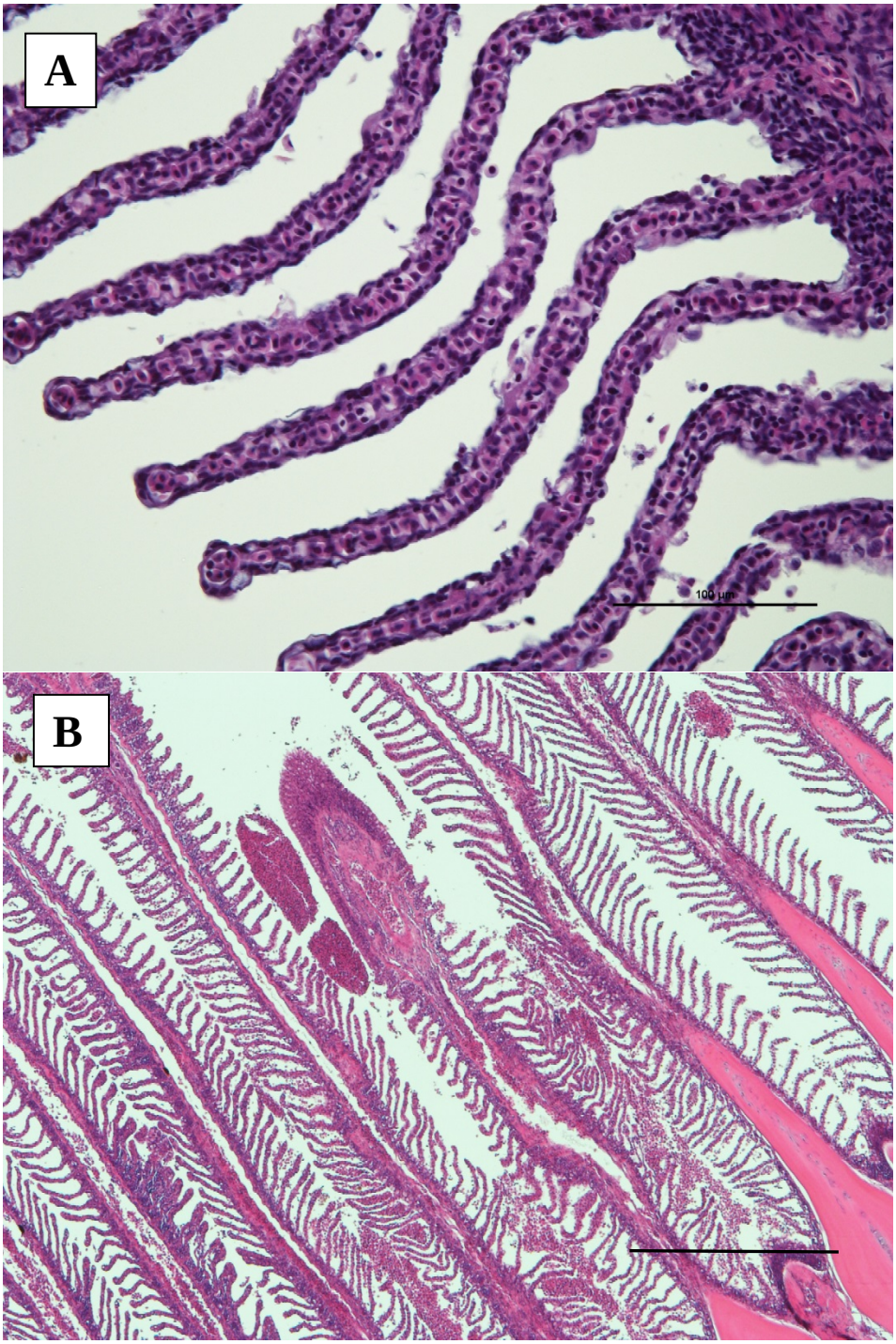

Figure 14. Example of histological section of *A*, normal gill secondary lamellae and a section *B*, showing many primary lamellae one of which is shortened. Scale bar in top panel represents 100 microns and scale bar in bottom panel represents 500 microns.

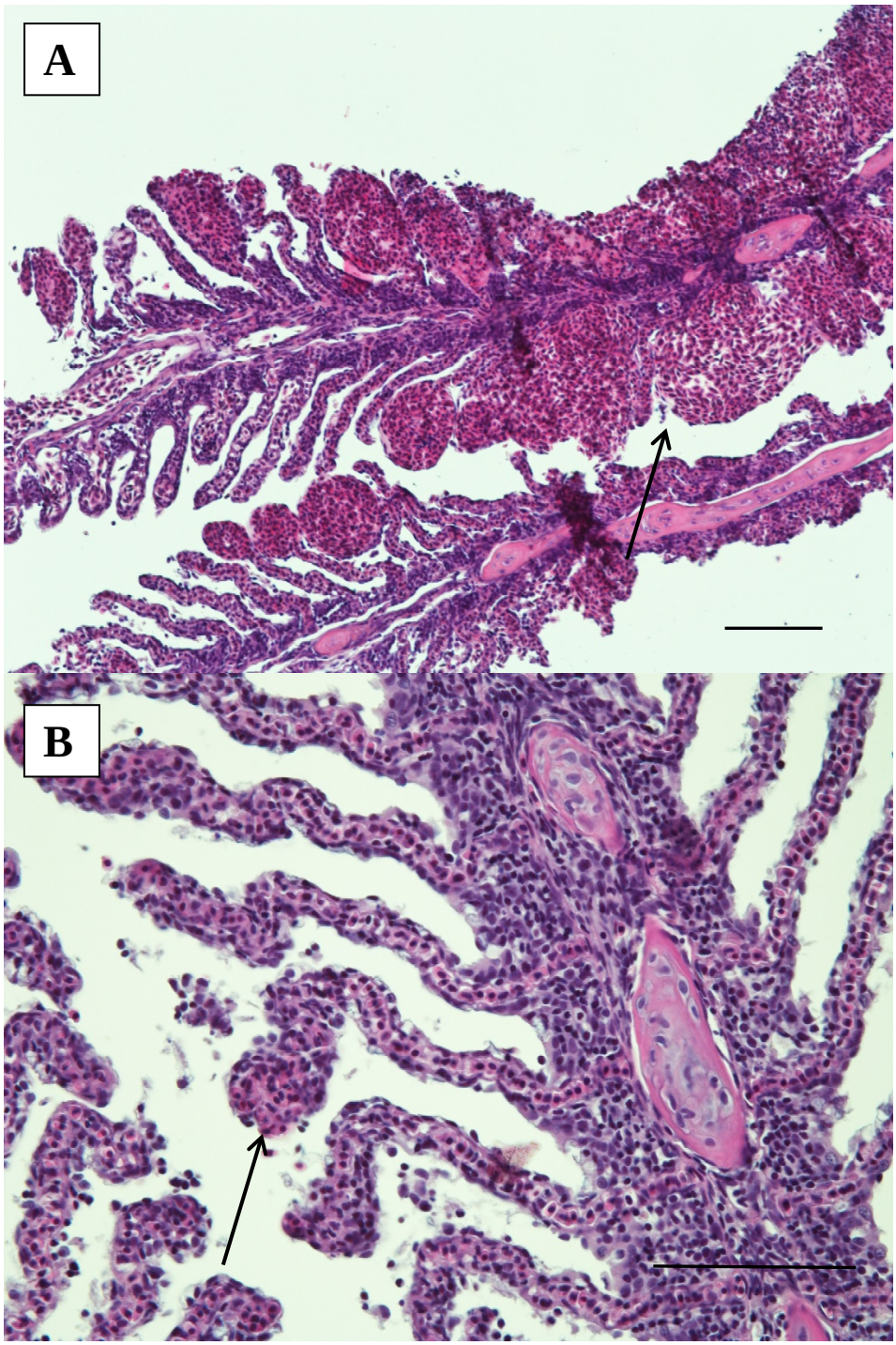

Figure 15. Example of aneurism (arrow) in secondary lamellae of an *A*, histological gill section and *B*, blood congestion (arrow) in secondary lamellae. Scale bars represent 100 microns.

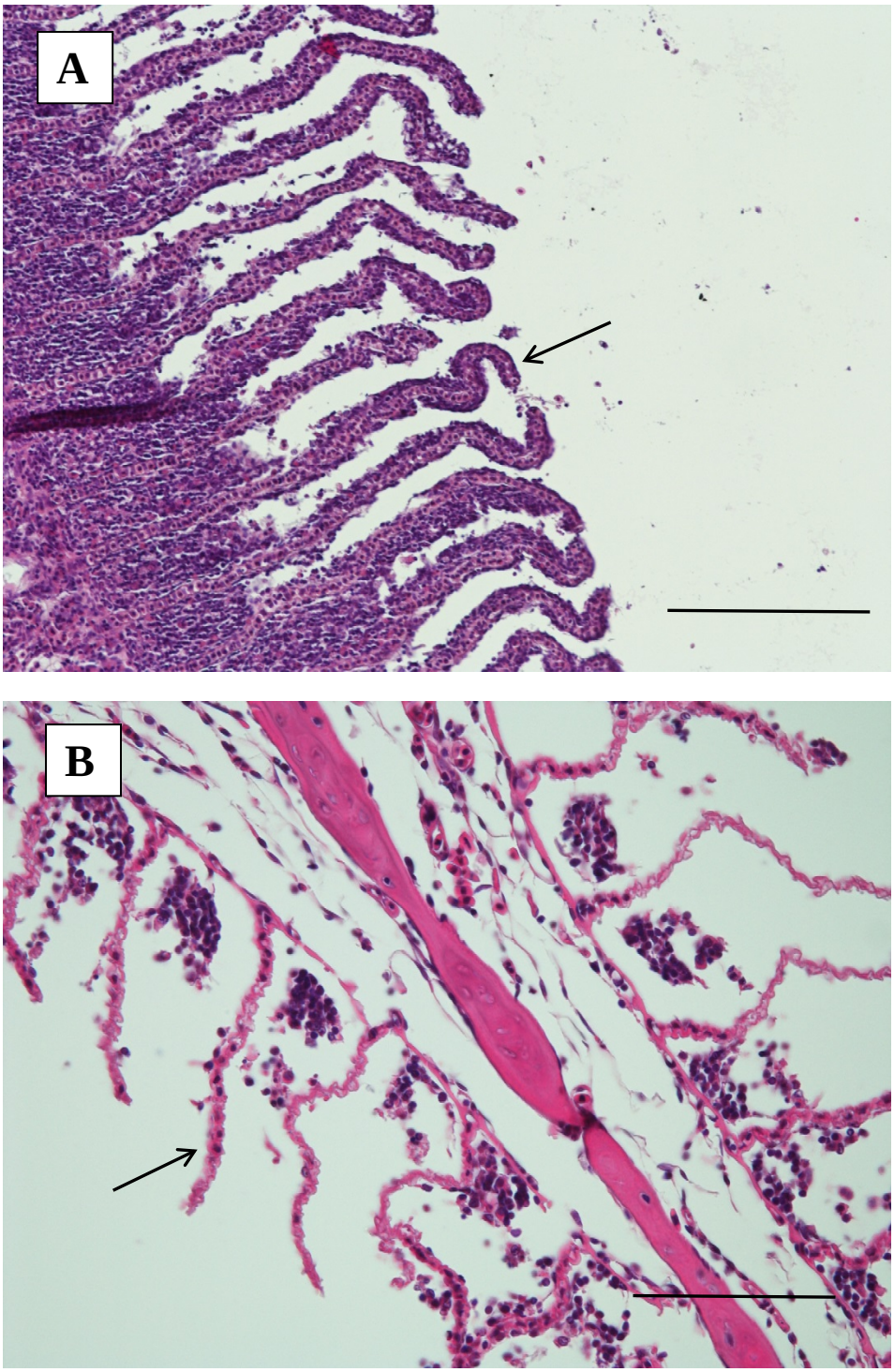

Figure 16. Example of histological section of gill showing *A*, curling at ends of secondary lamellae (arrow) and *B*, degenerated secondary lamellae stripped of epithelial cells (arrow). Scale bar represents 100 microns.

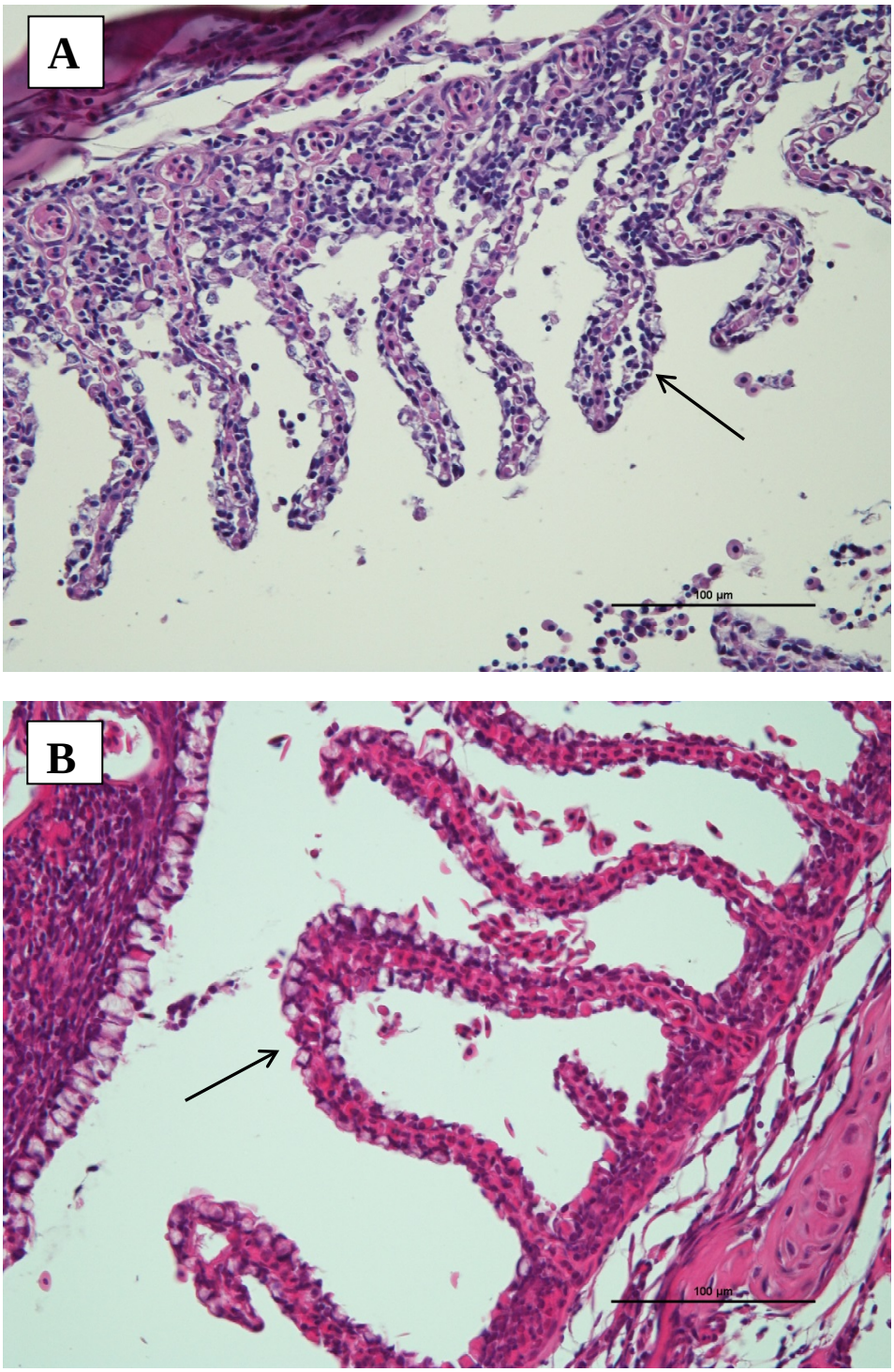

Figure 17. Example of epithelium on *A*, secondary lamellae lifting away from gill (arrow) and *B*, secondary lamellae fusing together (arrow). Scale bar represents 100 microns.

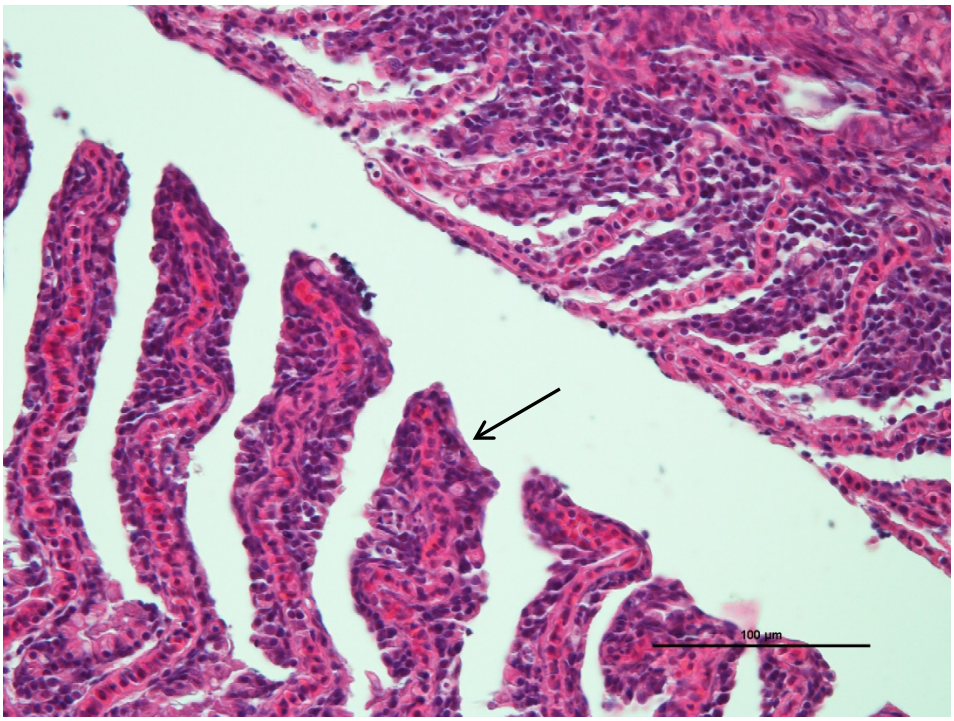

Figure 18. Example of epithelial hyperplasia in secondary lamellae (arrow). Scale bar represents 100 microns.

Table 10. Immunohistochemical staining for CYP1A in gills of smallmouth bass (SMB) and golden redhorse sucker (GRH) from Marshall Impoundment (MI; reference site), Historic Bridge (HB), Legacy Ranch (LR), and Shady Bend (SB). Areas of positive staining were counted on approximately five primary lamellae of five fish[1] for each species and site. Results are reported as mean number of stained areas (Mean), number of fish that did not show any staining (No stain), and the minimum and maximum (Range) of stained cells observed in the sample of fish from a given site.

	Species						
	SMB			GRH			
Site	Mean	No stain	Range	Mean	No stain	Range	Comment
MI	3	1	2–6	2	4	3–6	Stain light
HB	5	0	2–9	5	1	1–23	Large areas stained
LR	5	1	6–8	11	0	2–16	
SB	5	0	2–8	5	0	3–15	

[1]Seven GRH were evaluated for site MI.

Table 11. Estimated age (years) of smallmouth bass from four sites on the Kalamazoo River. (number = N; coefficient of variation = CV).

Site	Sex	N	Mean	Range	CV
Marshall Impoundment	F	10	4	3–7	3
	M	5	5	4–7	4
Historic Bridge	F	7	4	3–7	3
	M	7	6	4–9	3
Legacy Ranch	F	7	3	2–4	6
	M	8	5	3–7	3
Shady Bend	F	5	5	3–10	2
	M	5	6	48	4

Appendix 1. Field datasheets

See attached electronic files or CD

Appendix 2. Chain-of-custody forms

USGS
science for a changing world

U.S.Department of the Interior
U.S.GEOLOGICAL SURVEY
Columbia Environmental Research Center
4200 New Haven Road
Columbia, Missouri 65201

Chain-of-Custody Record **Attachment 1**

Study No.

Study Name: Enbridge Oil Spill near Marshall, MI Fish Health Assessment

Control No.

Samplers: (Signatures) Diane K. Nicks, John Hess & Employees of MI DNRE

page 1 of 3

Sample Identification	Date	Time	Type *	Remarks and Observations
1-30 tissues	8-18-10		F	Marshall Impoundment Samples, See inventory
				Attached.
31-60 tissues	8-19-10		F	Historic Bridge Park samples, see inventory
				Attached.
61-90 tissues	8-19-10 8-20-10		F	Legacy Ranch Samples, see inventory
				attached.
91-110 tissues	8-20-10		F	Shady Bend Samples, see inventory
				attached.

Relinquished by: (Signature)	Date/Time	Received by: (Signature)	Relinquished by: (Signature)	Date/Time	Received by: (Signature)
Diane K. Nicks	8-20-10 9:30 pm	8-24-10 9:39 AM Diane K. Nicks			
Relinquished by: (Signature)	Date/Time	Received by: (Signature)	Relinquished by: (Signature)	Date/Time	Received by: (Signature)
Relinquished by: (Signature)	Date/Time	Received for Laboratory by:(Signature)	Date/Time	Remarks	

* W=water, S=sediment, P=plant, F=fish, B=benthos, O=other, define in remarks

Appendix 2. (continued)

Page 2 of 3

Enbridge Oil Spill near Marshall, MI Fish Health Assessment Sample Inventory (x indicates sample present)

			Histological Samples (10% NBF)			Frozen (-80°C)					blood smear slides			
Site	Fish #	Species	gill (left)	spleen	head kidney	liver A	liver B	bile	plasma A	plasma B	A	B	C	Comments
Marshall Impoundment	1	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	Not noted on data sheet
Marshall Impoundment	2	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Marshall Impoundment	3	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Marshall Impoundment	4	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Marshall Impoundment	5	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Marshall Impoundment	6	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Marshall Impoundment	7	golden redhorse sucker	x	x	x	x	x	x	x		x	x	x	
Marshall Impoundment	8	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Marshall Impoundment	9	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Marshall Impoundment	10	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Marshall Impoundment	11	golden redhorse sucker	x	x	x	x	x		x	x	x	x	x	
Marshall Impoundment	12	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Marshall Impoundment	13	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	Liver A in Bile A vial & Bile in Liver A vial
Marshall Impoundment	14	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Marshall Impoundment	15	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Marshall Impoundment	16	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	
Marshall Impoundment	17	smallmouth bass	x	x	x	x	x	x	x		x	x	x	
Marshall Impoundment	18	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	
Marshall Impoundment	19	smallmouth bass	x	x	x	x	x	x	x		x	x	x	
Marshall Impoundment	20	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	
Marshall Impoundment	21	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	
Marshall Impoundment	22	smallmouth bass	x	x	x	x	x	x			x	x	x	sample may be labeled as head kidney
Marshall Impoundment	23	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	
Marshall Impoundment	24	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	
Marshall Impoundment	25	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	
Marshall Impoundment	26	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	
Marshall Impoundment	27	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	
Marshall Impoundment	28	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	
Marshall Impoundment	29	smallmouth bass	x	x	x	x	x	x	x		x	x	x	small sample
Marshall Impoundment	30	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	
Historic Bridge Park	31	smallmouth bass	x	x	x	x	x	x	x		x	x	x	
Historic Bridge Park	32	smallmouth bass	x	x	x	x	x	x	x		x	x	x	small sample, Liver A in Bile A vial & Bile in Liver A vial
Historic Bridge Park	33	smallmouth bass	x	x	x	x	x	x	x		x	x	x	small sample
Historic Bridge Park	34	smallmouth bass	x	x	x	x	x		x		x	x	x	small sample w/ RBC's ?, Liver A in Bile vial
Historic Bridge Park	35	smallmouth bass	x	x	x	x	x	x	x		x	x	x	small sample w/ RBC's ?
Historic Bridge Park	36	smallmouth bass	x	x	x	x	x				x	x	x	
Historic Bridge Park	37	smallmouth bass	x	x	x	x	x	x	x		x	x	x	
Historic Bridge Park	38	smallmouth bass	x	x	x	x	x	x	x		x	x	x	
Historic Bridge Park	39	smallmouth bass	x	x	x	x	x	x			x	x	x	
Historic Bridge Park	40	smallmouth bass	x	x	x	x	x	x	x		x	x	x	small sample
Historic Bridge Park	41	smallmouth bass	x	x	x	x	x	x	x		x	x	x	small sample w/ RBC's ?
Historic Bridge Park	42	smallmouth bass	x	x	x	x	x	x	x		x	x	x	small sample
Historic Bridge Park	43	smallmouth bass	x	x	x	x	x	x	x		x	x	x	Liver A in Bile A vial & Bile in Liver A vial
Historic Bridge Park	44	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	
Historic Bridge Park	45	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	
Historic Bridge Park	46	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Historic Bridge Park	47	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Historic Bridge Park	48	golden redhorse sucker	x	x	x	x	x		x		x	x	x	
Historic Bridge Park	49	golden redhorse sucker	x	x	x	x	x	x	x		x	x	x	
Historic Bridge Park	50	golden redhorse sucker	x	x	x	x	x	x			x	x	x	Not noted on data sheet
Historic Bridge Park	51	golden redhorse sucker	x	x	x	x	x	x	x		x	x	x	not good sample?
Historic Bridge Park	52	golden redhorse sucker	x	x	x	x	x	x	x		x	x	x	Not noted on data sheet
Historic Bridge Park	53	golden redhorse sucker	x	x	x	x	x		x		x	x	x	
Historic Bridge Park	54	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Historic Bridge Park	55	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	Not noted on data sheet
Historic Bridge Park	56	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Historic Bridge Park	57	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Historic Bridge Park	58	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Historic Bridge Park	59	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Historic Bridge Park	60	golden redhorse sucker	x	x	x	x	x		x	x	x	x	x	

Appendix 2. (continued)

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Enbridge Oil Spill near Marshall, MI Fish Health Assessment Sample Inventory (x indicates sample present)

			Histological Samples (10% NBF)			Frozen (-80°C)					blood smear slides			
Site	Fish #	Species	gill (left)	spleen	head kidney	liver A	liver B	bile	plasma A	plasma B	A	B	C	Comments
Legacy Ranch	61	smallmouth bass	x	x	x	x	x	x	x		x	x	x	
Legacy Ranch	62	smallmouth bass	x	x	x	x	x	x	x		x	x	x	
Legacy Ranch	63	smallmouth bass	x	x	x	x	x	x	x		x	x	x	small sample
Legacy Ranch	64	smallmouth bass	x	x	x	x	x	x	x		x	x	x	
Legacy Ranch	65	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	Not noted on data sheet
Legacy Ranch	66	smallmouth bass	x	x	x	x	x	x	x		x	x	x	jellied
Legacy Ranch	67	smallmouth bass	x	x	x	x	x	x	x		x	x	x	sample had very little plasma all RBC's ?
Legacy Ranch	68	smallmouth bass	x	x	x	x	x	x	x		x	x	x	sample had very little plasma all RBC's ?
Legacy Ranch	69	smallmouth bass	x	x	x	x	x	x	x		x	x	x	sample had very little plasma all RBC's ? Bile sample collected from work surface
Legacy Ranch	70	smallmouth bass	x	x	x	x	x	x	x		x	x	x	small sample
Legacy Ranch	71	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	
Legacy Ranch	72	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	
Legacy Ranch	73	smallmouth bass	x	x	x	x	x	x	x		x	x	x	
Legacy Ranch	74	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	
Legacy Ranch	75	smallmouth bass	x	x	x	x	x	x	x		x	x	x	
Legacy Ranch	76	golden redhorse sucker	x	x	x	x	x	x	x		x	x	x	
Legacy Ranch	77	golden redhorse sucker	x	x	x	x	x		x	x	x	x	x	
Legacy Ranch	78	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Legacy Ranch	79	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	there is an extra #79 plasma vial??
Legacy Ranch	80	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Legacy Ranch	81	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Legacy Ranch	82	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Legacy Ranch	83	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Legacy Ranch	84	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Legacy Ranch	85	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Legacy Ranch	86	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Legacy Ranch	87	golden redhorse sucker	x	x	x	x	x		x	x	x	x	x	
Legacy Ranch	88	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Legacy Ranch	89	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Legacy Ranch	90	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	
Shady Bend	91	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	
Shady Bend	92	smallmouth bass	x	x	x	x	x	x	x		x	x	x	
Shady Bend	93	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	Not noted on data sheet
Shady Bend	94	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	Not noted on data sheet
Shady Bend	95	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	Not noted on data sheet
Shady Bend	96	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	Not noted on data sheet
Shady Bend	97	smallmouth bass	x	x	x	x	x	x	x		x	x	x	Not noted on data sheet
Shady Bend	98	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	Not noted on data sheet
Shady Bend	99	smallmouth bass	x	x	x	x	x	x	x	x	x	x	x	Not noted on data sheet
Shady Bend	100	smallmouth bass	x	x	x	x	x	x	x		x	x	x	Not noted on data sheet
Shady Bend	101	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	Not noted on data sheet
Shady Bend	102	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	Not noted on data sheet
Shady Bend	103	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	Not noted on data sheet
Shady Bend	104	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	Not noted on data sheet
Shady Bend	105	golden redhorse sucker	x	x	x	x	x	x	x		x	x	x	Not noted on data sheet
Shady Bend	106	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	Not noted on data sheet
Shady Bend	107	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	Not noted on data sheet
Shady Bend	108	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	Not noted on data sheet
Shady Bend	109	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	Not noted on data sheet
Shady Bend	110	golden redhorse sucker	x	x	x	x	x	x	x	x	x	x	x	Not noted on data sheet