2007 Project Abstract
For the Period Ending June 30, 2010

PROJECT TITLE: Threat of Emerging Contaminants to Upper Mississippi Walleye
PROJECT MANAGER: Dr. Heiko L. Schoenfuss
AFFILIATION: St. Cloud State University
MAILING ADDRESS: 720 Fourth Avenue South, WSB-273
CITY/STATE/ZIP: St. Cloud, MN 56301
PHONE: (320) 308-3130
E-MAIL: hschoenfuss@stcloudstate.edu
WEBSITE: web.stcloudstate.edu/aquatictox
FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION:

M.L. 2009, Chapter 143, Section 2, Subd. 16. Carryforward
The availability of the appropriations for the following projects is extended to June 30, 2010:
5) Laws 2007, chapter 30, section 2, subdivision 5, paragraph (m), threat of emerging contaminants to Upper Mississippi walleye.

APPROPRIATION AMOUNT: $97,000

Overall Project Outcome and Results

In this combined field and laboratory study we assessed whether populations of native walleye in the Upper Mississippi River experienced altered genetic diversity correlated with the exposure to estrogenic endocrine active compounds. We collected fin-clips for genetic analysis from almost 600 walleye (13 sites) and sub-sampled over 360 of these fish (6 sites) for blood and reproductive organs. We further enhanced our sample size by adding genetic data from over 900 walleye analyzed for previous studies. Finally, we caged male fathead minnows at three of the sample sites to confirm the presence of estrogenic endocrine active compounds. Our findings indicate that male walleye in four river segments produce measurable concentrations of plasma vitellogenin (an egg-yolk protein and, when expressed in male fish, a biomarker of acute estrogenic exposure), a finding consistent with the presence of estrogenic endocrine active compounds and consistent with published historical data for at least three of these study sites (Grand Rapids, Pool 2, Lake Pepin). Patterns of vitellogenin induction were consistent for native walleye and caged fathead minnows. No widespread occurrence of histopathological changes such as intersex was found. To assess the genetic diversity of the walleye populations at the study sites, we DNA fingerprinted individual fish using molecular genetic markers. Genetic differences were observed between populations, however, these differences were consistent with geographic distance between populations (greater geographic distance=greater genetic difference) with the largest observed difference in genetic diversity found between fish upstream and downstream of St. Anthony Falls (and/or Lock and Dam 1 of the Mississippi River), a historical barrier to fish movement. In summary, while the persistent occurrence of endocrine disruption in wild fish populations is troubling, this insult has not resulted in the degradation of reproductive organs in individual walleye or alteration in genetic diversity of walleye populations.

Project Results Use and Dissemination

Project results have been provided to the LCCMR on a semi-annual basis and in this final report. A related report on some of the genetic findings is also being prepared for the MN Department of Natural Resources.

We plan to present the results of this study to the scientific community in form of a peer-reviewed manuscript in the near future.

Furthermore, we will present our results to the regional scientific community and stakeholders at upcoming fisheries (i.e., Annual Meeting of the American Fisheries Society, Minnesota Chapter) and toxicological (i.e., Annual Meeting of the Society for Environmental Toxicology & Chemistry, Midwest Chapter) meetings.

We have also provided limited project information on the website of the Aquatic Toxicology Laboratory at St. Cloud State University (web.stcloudstate.edu/aquatictox) and will provide a more extensive review of the study after approval of the final report by the LCCMR.

Trust Fund 2007 Work Program Final Report

Date of Report: August 16, 2010
Final Report
Date of Work program Approval:
Project Completion Date: June 30, 2010

The completion data of the project has been extended by one year to June 30, 2010 per M.L. 2009, Chapter 143, Section 2, Subd. 16. Carryforward.

I. PROJECT TITLE: Threat of Emerging Contaminants to Upper Mississippi Walleye

Project Manager: Dr. Heiko L. Schoenfuss
Affiliation: St. Cloud State University
Mailing Address: 720 Fourth Avenue South, WSB-273
City / State / Zip : St. Cloud / MN / 56301
Telephone Number: (320) 308-3130
E-mail Address: hschoenfuss@stcloudstate.edu
FAX Number: (320) 308-4166
Web Page address: web.stcloudstate.edu/aquatictox

Location: Upper Mississippi Watershed

Total Trust Fund Project Budget:	**Trust Fund Appropriation:**	**$** 97,000
	Minus Amount Spent:	**$** 97,000
	Equal Balance:	**$0**

Legal Citation:

M.L. 2009, Chapter 143, Section 2, Subd. 16. Carryforward
The availability of the appropriations for the following projects is extended to June 30, 2010:
5) Laws 2007, chapter 30, section 2, subdivision 5, paragraph (m), threat of emerging contaminants to Upper Mississippi walleye.

ML 2007, [Chap. 30], Sec.[2], Subd. 5m.

Appropriation Language:
$97,000 is from the trust fund to St. Cloud State University to assess whether the genetic diversity of walleye in the Upper Mississippi is negatively impacted by emerging contaminants at pollution hotspots where feminized male fish have been identified.

II. and III. FINAL PROJECT SUMMARY

In this combined field and laboratory study we assessed whether populations of native walleye in the Upper Mississippi River experienced altered genetic diversity correlated with the exposure to estrogenic endocrine active compounds. We collected fin-clips for genetic analysis from almost 600 walleye (13 sites) and sub-sampled over 360 of these fish (6 sites) for blood and reproductive organs. We further enhanced our sample size by adding genetic data from over 900 walleye analyzed for previous studies. Finally, we caged male fathead minnows at three of the sample sites to confirm the presence of estrogenic endocrine active compounds. Our findings indicate that male walleye in four river segments produce measurable concentrations of plasma vitellogenin (an egg-yolk protein and, when expressed in male fish, a biomarker of acute estrogenic exposure), a finding consistent with the presence of estrogenic endocrine active compounds and consistent with published historical data for at least three of these study sites (Grand Rapids, Pool 2, Lake Pepin). Patterns of vitellogenin induction were consistent for native walleye and caged fathead minnows. No widespread occurrence of histopathological changes such as intersex was found. To assess the genetic diversity of the walleye populations at the study sites, we DNA fingerprinted individual fish using molecular genetic markers. Genetic differences were observed between populations, however, these differences were consistent with geographic distance between populations (greater geographic distance=greater genetic difference) with the largest observed difference in genetic diversity found between fish upstream and downstream of St. Anthony Falls (and/or Lock and Dam 1 of the Mississippi River), a historical barrier to fish movement. In summary, while the persistent occurrence of endocrine disruption in wild fish populations is troubling, this insult has not resulted in the degradation of reproductive organs in individual walleye or alteration in genetic diversity of walleye populations.

IV. OUTLINE OF PROJECT RESULTS*:*

Rationale. In 2006 St. Cloud State University in collaboration with the US Geological Survey conducted a study of fish health in the Upper Mississippi River from Lake Itasca to the Iowa border. Our study sampled 43 sites and included fish samples from four species, including walleye and smallmouth bass, as well as water and sediment samples from each location. This survey of fish health in the context of emerging contaminants, especially endocrine disruptors and pharmaceuticals, represents the largest such effort in North America to date.

Our results indicate that there are several “hotspots” where fish health in the Mississippi River is impaired in a fashion that is consistent with the effects of emerging contaminants (see map). These effects include the feminization of male fish, which has been linked to intersex (hemaphrodism) and reduced reproductive ability in male fish. The long-term health of Minnesota fish populations may be at risk due to the impacts of these emerging contaminants on fish health. This is especially true since recent genetic research has demonstrated that long-term fish

exposure to treated wastewater effluents can alter the genetic structure of fish populations and potentially result in genetic bottlenecks that can cripple a population. Fish populations that may appear healthy could in fact be approaching a critical deficit in genetic diversity to overcome subsequent environmental challenges. This is a crisis that is not obvious to the naked eye until it is too late.

We propose a combined field and laboratory approach that would for the first time, link the occurrence of emerging contaminants in the Mississippi River to feminization in male fish and explore the possibility of reduced genetic diversity in populations of walleye. The study is designed to make use of existing data and the broad expertise of our collaborative group, including an aquatic toxicologist (HLS), a fish geneticist (LMM), and aquatic ecologist (MLJ). Results of this study would allow resource managers and agencies to focus resources on particularly vulnerable populations of walleye and could be extrapolated to fish populations in all Minnesota waters.

Result 1: Field Collection of Feminized Fish

Description: We will confirm the feminizining effects of Mississippi River waters on walleye and fathead minnows at four contamination "hotspots" identified in our extensive existing data set. We will also identify two reference sites where fish and water samples showed no indication of the presence of emerging contaminants. All six sites will be re-sampled for walleye (up to 60 fish) and fathead minnows to generate a larger fish sample than was possible in our previous study (we will be able to add archived samples from the previous study). In addition we will cage male fathead minnows at each sites for seven days, an US EPA recommended length of time. The caging of fathead minnows will serve two purposes, first, it will confirm the presence of emerging contaminants at the identified hotspot sites before the commencement of the costly genetic analysis, and second, it will allow us to link our field studies to an existing large body of laboratory research on the effects of emerging contaminants on fish. All walleye collected will be assessed for their reproductive health (Result 2) and genetic diversity (Result 3).

Summary Budget Information for Result 1:	**Trust Fund Budget:**	**$** 19,000
	Amount Spent:	**$ 19,000**
	Balance:	**$ 0**

Deliverable	**Completion Date**	**Budget**	**Status**
1. *Collecting Walleye I*	*Sept. 30, 2007*	*$5,000*	*$0*
2. *Cage Fatheads*	*Dec. 31, 2007*	*$9,000*	*$0*
3. *Collect Walleye II*	*Sept. 30, 2008*	*$5,000*	*$0*

Completion Date: December 31, 2009

Final Report Summary:
During three field season (2007-2009) we collected 377 adult walleye from six field sites in the Mississippi River between Lake Winnibigoshish and Lake Pepin, including several previously identified "hotspot" sites (Grand Rapids, Clearwater,

Pool 2, Lake Pepin) and two control sites (Downstream of Lake Winnibigoshish, Sartell) (Table 1, below). Fish were collected by various means with a sizeable portion being donated by anglers on the Mississippi River on walleye openers in 2007 and 2008. Additional collections were made by the St. Cloud State University Aquatic Toxicology Laboratory and the MN Department of Natural Resources. Although our collections targeted male fish, we were able to assess parameters related to endocrine disruption in 66 female fish (18% of the total catch). We also proceeded with the deployment of caged fathead minnows on the Mississippi River (Table 2), however drought conditions in 2007, the flooding in 2008 and concerns about *viral hemorrhagic septicemia* limited the success of this effort to three sites.

Table 1. Summary of walleye collected in this study for assessment of endocrine disruption and genetic diversity.

Sampling location	river mile[1]	**2007**		**2008**		**2009**		**Sum**	
		Morphology & physiology	genetics[2]	Morphology & physiology	genetics[2]	Morphology & physiology	genetics[2]	Morphology & physiology	genetics[2]
Lake Bemidji	1282		30[3]						**30**
Upstream of Grand Rapids	1,202	79	81	25	20	30	24	**134**	**125**
Grand Rapids	1,183	40	39	67	59	37	33	**144**	**131**
Aitkin	1,060		25						**25**
Brainerd–above dam	1008		28						**28**
Brainerd-below dam	1005		34						**34**
Little Falls	965		19						**19**
Sartell	929	27	30					**27**	**30**
Clearwater	923	9	3					**9**	**3**
Monticello	898				18				**18**
Pool 2	836	22	47					**22**	**47**
Red Wing	792						47		**47**
Lake Pepin	771	15	15	26	32			**41**	**47**

[1] River mile as measured above Cairo, IL

[2] Genetic sample size differs from morphology/physiology sample due to extra fin clips added to the study from other sources and occasional DNA amplification failure.

[3] Sampled in 2006 by MN DNR

Figure 1. Map of the Upper Mississippi River in Minnesota with sites from which walleye were collected.

Our attempts to collect walleye for his study provided some important insights for future studies of this important species in Minnesota waters: (i) collecting large numbers of walleye nearly concurrently at multiple sites in large river system is extremely challenging as weather and water conditions (i.e., ice coverage) will vary across the State, as fish move into deeper water shortly after spawning in early

spring, and as these fish seldom occur in large densities. (ii) Out of necessity and in correspondence with the MN Department of Natural Resources, we resorted to collecting fish tissues from walleye captured by anglers on walleye opener. This technique was very successful, engaged the local fishing community and provided a temporally very concise sample. We recommend the involvement of local fishing organizations for future studies with similar parameters.

Summary of field methods applied: Three to 5 milliliters (mL) of blood was drawn from the caudal vasculature and transferred into a hematocrit tube that was stored on wet ice. The fish was then sacrificed, and measurements were recorded for the fish's weight, total and standard lengths. Body Condition Indices (BCIs) were calculated as [(total weight/standard length^3)*1000000]. The BCI is considered a measure to examine whether fish are of similar nutritional condition across field sites.

Several testis samples were collected for histological analysis, and placed into histological cassettes. In male fish, both testes were removed and a representative sample was collected and placed into a histological cassette. If gravid ovaries were present in the abdominal cavity, the sex was noted on the data sheets as female, but no attempt was made to weigh or collect these tissues for later histological analysis. The rationale for the exclusion of female reproductive tissue was that a gravid female ovary was too fragile to be removed intact and that histological analysis would not yield any further information. All histological cassettes were then placed into a site-specific container with 4 percent formalin. During the collection, an effort was made to return collected fish samples (blood and testis) to the laboratory within 15 hours but not more than 36 hours, from collection time. All specimens were maintained on ice until they could be processed according to analysis needs in the laboratory.

Result 2: Laboratory Assessment of Fish Health

Description: We will (1) confirm the presence of emerging contaminants at the field site and (2) link the findings in the wild caught walleye to more defined laboratory endpoints in the fathead minnow. The SCSU Aquatic Toxicology Laboratory is well equipped to analyze the reproductive health of all captured and caged fish. We have extensive expertise in documenting the likely effects of fish exposure to emerging contaminants in a timely and cost efficient manner. All fish captured and caged will undergo a histopathological analysis of the reproductive organs to test for the occurrence of hemaphrodism, a blood plasma analysis for the female egg yolk protein in male fish (a bioindicator of acute exposure to emerging contaminants), and will be measured for morphometric endpoints. The inclusion of caged fathead minnows will provide a linkage between relevant field and laboratory data. This unique study design would greatly increase the interpretive power by establishing a cause and effect relationship between emerging contaminants and fish samples instead of merely allowing for correlations to be made.

Summary Budget Information for Result 2: **Trust Fund Budget:** **$** 29,000
Amount Spent: **$ 29,000**
Balance: **$ 0**

Deliverable	Completion Date	Budget	Status
1. *Walleye VTG I*	*Dec. 31, 2007*	*$4,000*	*$0*
2. *Walleye Histology I*	*March 31, 2008*	*$6,000*	*$0*
3. *Fathead Minnow VTG/Histology*	*Dec. 31, 2008*	*$9,000*	*$0*
4. *Walleye VTG II*	*March 31, 2009*	*$4,000*	*$0*
5. *Walleye Histology II*	*June 30, 2009*	*$6,000*	*$0*

Completion Date: June 30, 2010

Final Report Summary:
We collected mature walleye from six sites on the Upper Mississippi River and assessed the potential for physiological or morphological responses consistent with the exposure to endocrine active compounds (Figure 2). In addition, we assessed similar parameters in male fathead minnows that were caged at three of the six field sites (Table 2).

Figure 2. (A), (B) Physiological (vitellogenin induction [µg/mL]), and (C), (D) morphological (Body Condition Index) characteristics of walleye collected in the Mississippi River 2007-09.

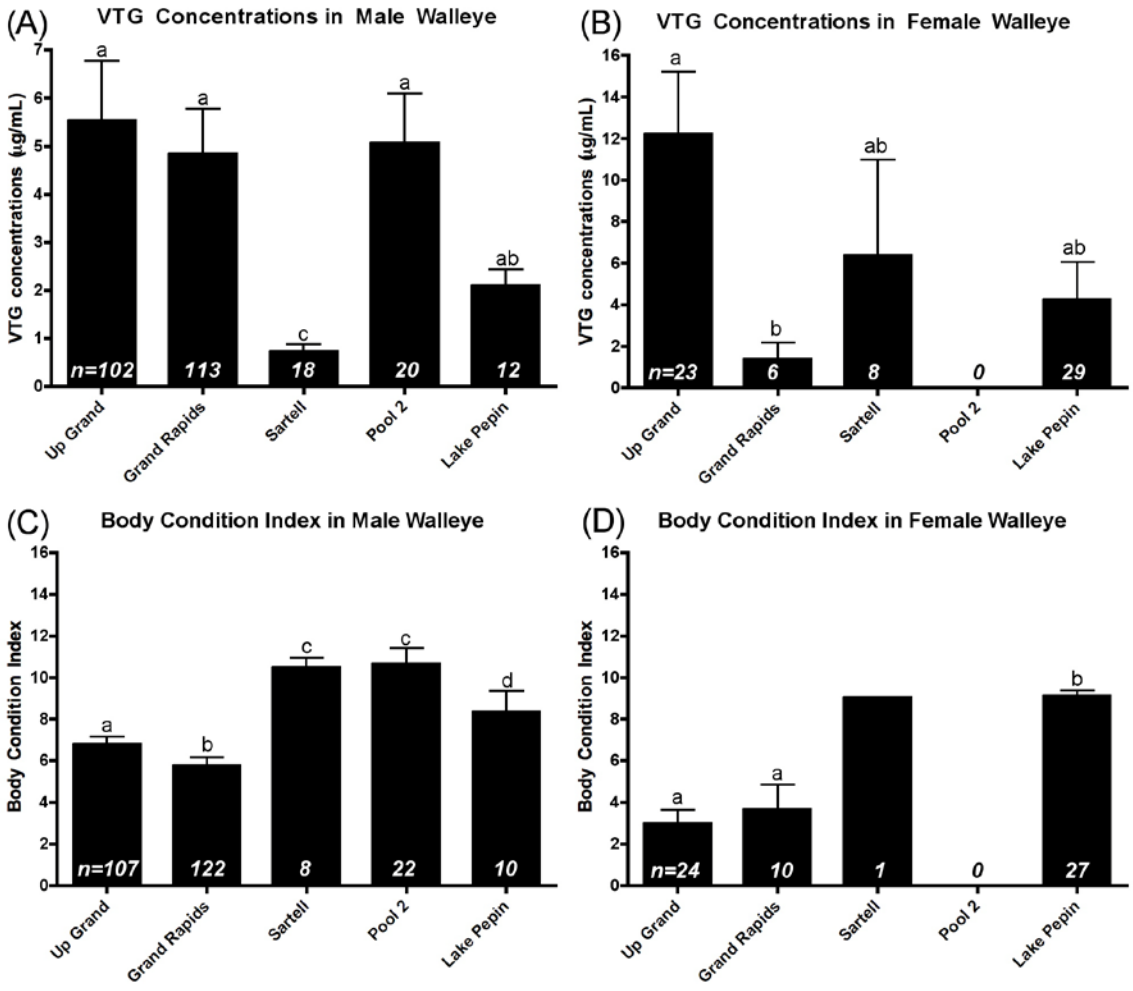


Table 2. Physiological and morphological assessment of male fathead minnows caged at three sites in the Upper Mississippi River.

Caging Location	n	Plasma vitellogenin mean ± standard error	Body Condition Factor mean ± standard error
Upstream Grand Rapids	0	cages lost in 2008 flooding	
Grand Rapids	0	cages lost in 2008 flooding	
Sartell	7	2.2 ± 0.54	11 ± 0.43
Clearwater	20	24 ± 8.8	11± 0.25
Pool 2	19	152 ± 120	11 ± 0.23
Lake Pepin	0	no fish caged due to concerns over *viral hemorrhagic septicemia*	

Our analysis of the collected walleye and caged fathead minnows revealed that fish in several sections of the Upper Mississippi River in Minnesota are exposed to estrogenic endocrine active compounds that are persistent enough to result in physiological responses (vitellogenin induction), but not severe enough to cause morphological (body condition index, histopathology) changes to the exposed fish. We observed the induction of plasma vitellogenin in four of the six river segments from which we collected fish: Upstream of Grand Rapids, Grand Rapids, Pool 2 in St. Paul, MN, and in Lake Pepin. No induction was seen in Clearwater, downstream of St. Cloud, where plasma vitellogenin concentrations were near the detection limit for wild caught walleye as well as caged male fathead minnows. Vitellogenin induction in male fish was consistent for walleye and caged fathead minnows in Pool 2. Interestingly, three of the four sites that were found to contain wild male walleye with measurable concentrations of vitellogenin have been reported to be estrogenic in past studies (Grand Rapids, Pool 2 and Lake Pepin) (Hinck et al. 2009, Aquatic Tox; Barber et al. Aquatic Tox 2007, 82:36-46; Lee et al. 2000, USGS Report 00-4202; Folmar et al. 2001 Arch Environ Contam Toxicol 40:392-398). At none of these sites was induction of vitellogenin correlated with histopathological changes to reproductive organs. In fact, only sporadic histopathological changes were observed across all walleye collected in this study.

The fourth site to exhibit estrogenic activity as assessed by vitellogenin induction in male walleye was just upstream of Grand Rapids (below the outfall of Lake Winnibigoshish). No clear source of estrogenicity is apparent upstream of this collection site. However, other studies have suggested non-point sources such as septic systems, agricultural runoff, and phytoestrogens released from decomposing leave litter may contribute to estrogenicity at specific sites.

Differences in vitellogenin induction and in body condition indices in female walleye across the study sites are likely related to differences in reproductive state of these fish. As most fish were collected on the same day during each of the three field seasons (Walleye Opener), fish in the southern most sites (Lake Pepin, Pool 2) were further past spawning then fish at the northern most sites (Upstream of-, and in Grand Rapids).

Summary of laboratory methods applied: Vitellogenin concentrations were determined in fish plasma using enzyme-linked immunosorbent assay (ELISA) techniques. Whole blood samples were centrifuged in hematocrit tubes (Phoenix Research Products, Hayward, California) for 5 minutes at 5,800 x g, and two aliquots of more than 1 mL plasma were retained for analyses. Triplicate aliquots from each sample were stored in two separate -80 ºC freezers before analyses. An ELISA antibody for striped bass was used to analyze vitellogenin concentrations in walleye plasma (Biosense Laboratories, Bergen, Norway). Standard curves were calculated based on five to seven dilution points (after removing the highest and lowest dilution points). Each ELISA plate included two blanks and a series of purified vitellogenin standards at 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.95, 0.97, 0.18, and 0.024 micrograms per milliliter (µg/mL). The r-square values for the standard curves were greater than 0.99.

Testis of all male (or perceived male) fish were removed and processed for histological analysis. Histological cassettes were processed in a Jung TP1050 automated tissue processor (Leica, Wetzlar, Germany) according to an established histological protocol of dehydration and embedding in paraffin wax (Gabe, Histological Techniques, 1976). Once embedded, histological sections (three sections per histological cassette) were produced and stained with hematoxylin and eosin stains (two sections). The slides were examined for reproductive condition (immature, gravid, spawned out) and occurrence of intersex or other histopathological findings (i.e., parasitic cysts). Fish were designated as intersex if microscopic evaluation determined the simultaneous occurrence of ovarian and testicular tissue.

Result 3: Emerging Contaminant Population-Level Genetic Effects

Description: Using DNA-based genetic markers, we will assess patterns of genetic diversity within and between walleye populations collected at the contaminated "hotspot" sites and the uncontaminated reference sites. Evaluation of multiple sites along the length of the river will determine if consistent patterns emerge in relation to pollution levels. These assessments will determine if the documented biological effects on individual fish are also translating to genetic and ecological effects at the population level, possibly threatening the genetic integrity and persistence of fish populations. The AquaGen Laboratory at the University of Minnesota is a well-established molecular genetic laboratory focusing specifically on fish population genetics and has a long history of analyzing population level genetic diversity in game fish.

Summary Budget Information for Result 3:	**Trust Fund Budget:**	**$** 49,000
	Amount Spent:	**$ 49,000**
	Balance:	**$ 0**

Deliverable	**Completion Date**	**Budget**	**Status**
1. Walleye DNA Year 1	Dec. 31, 2007	$15,000	$0
2. Data analysis Year 1	June 30, 2008	$5,000	$0
3. Walleye DNA Year 2	Dec. 31, 2008	$22,000	$0
4. Data analysis Year 2	June 30, 2009	$7,000	$0

Completion Date: June 30, 2010

Final Report Summary:

Summary of laboratory methods and data analysis applied:

We genotyped walleye from 12 sites in the Mississippi River (Table 1, above) and added additional reference sites from prior studies (Table 3, below) to determine if patterns in genetic diversity were related to levels of endocrine disruptor effects. The most plausible genetic effect of exposure to endocrine active compounds is a reduction in genetic diversity due to a bottleneck, which can be caused by reductions

in population size and altered mating success among individuals. We found genetic differences among walleye along the length of the river but no patterns correlated with effects consistent with exposure to endocrine active compounds. Most of the population differentiation was attributed to a split between samples collected above and below the Twin Cities. This split may be due to historical barriers to fish movement, and thus genetic exchange, at St. Anthony Falls in Minneapolis. Populations above the Twin Cities, both hot spot and reference site samples, had lower genetic diversity than those below, with a trend toward increasing diversity going downstream. Nearby reference and hot spot samples had no significant differences in genetic diversity. While endocrine active compounds may alter the reproductive physiology of individual walleye as determined in Result 2, they are not causing population bottlenecks severe enough to detectably alter genetic diversity throughout the Mississippi River.

Table 3. Location, sample size (N), and major drainage for samples of walleye with data from previous research by L. Miller. These samples had data for only 8 of 10 microsatellite DNA markers used in the current project.

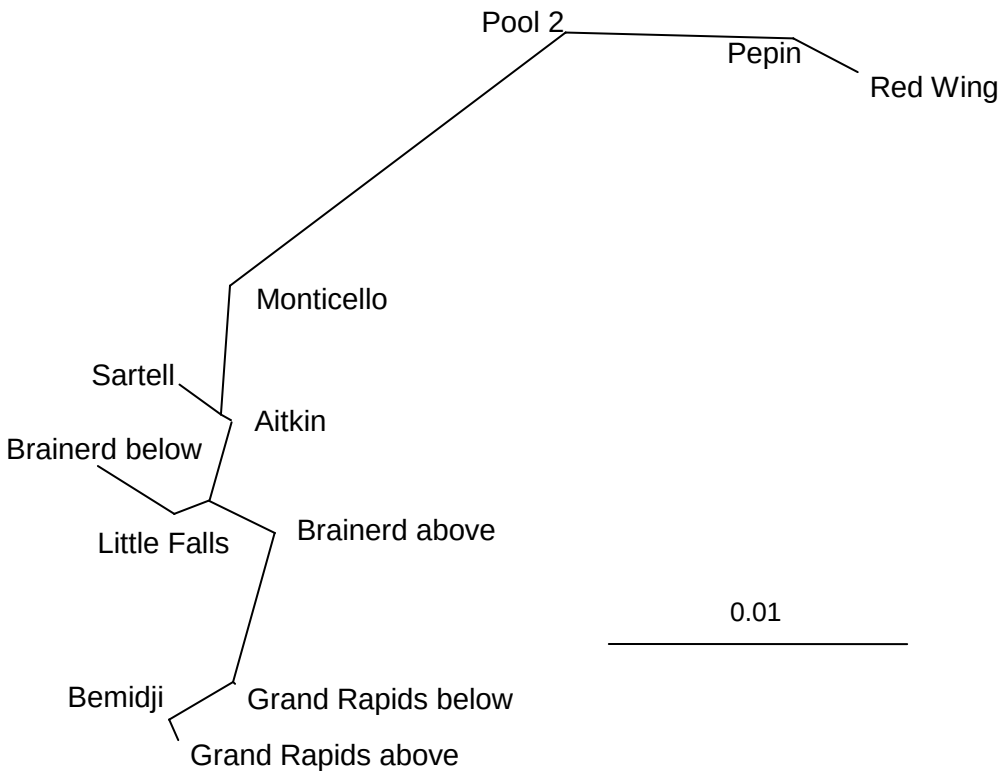
Sample location	N	Drainage
Cutfoot/Lake Winnibigoshish	182	Mississippi
Leech Lake	82	Mississippi
Little Boy River	71	Mississippi
Pine River	91	Mississippi
St. Louis River	171	Great Lakes
Pike River	178	Rainy/Hudson
Red Lake	169	Hudson

Result details

We genotyped each walleye using 10 genetic markers of types known as microsatellite DNA loci. Microsatellite markers typically reveal high genetic variation among individuals and populations, and have been used to study relationships between toxins and genetic diversity (e.g., Whitehead et al. 2003; Bourret et al. 2008). Each locus, which represents a small region of DNA on the pairs of chromosomes of the fish, was amplified by the polymerase chain reaction (PCR) to make millions of copies that could be visualized. The PCR fragments were scored based on their lengths. Fragments with different lengths, called alleles, indicate differences in DNA sequences. The allele scores across all 10 genetic markers make up the DNA fingerprint of the individual. The genetic diversity of individuals and populations can be measured by the number of different alleles (allelic richness) or the number of loci with different alleles on each of the pair of chromosomes (heterozygosity). Population genetic structure (i.e., genetic diversity among populations) can be measured in various ways generally based on allele frequency differences among populations.

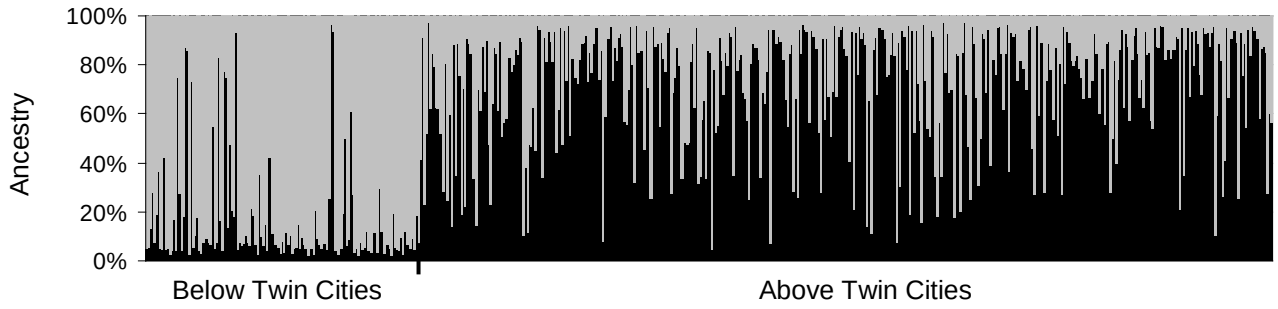
Several approaches to analyzing genetic population structure indicated that there are genetic differences among walleye along the length of the river but that most of the differentiation is attributed to a split between samples collected above and below the Twin Cities. The measure F_{st}, which is a sensitive measure of differences in allele frequencies among populations, was small and sometimes statistically non-significant in comparisons among populations above (F_{st} range 0-0.021) or below (F_{st} range 0-0.008) the Twin Cities. In contrast, F_{st} was larger and significant for all comparisons between above and below Twin Cities populations (Fst range 0.012-0.046), except for the Monticello and Pool 2 samples. It is not surprising that some populations above the Twin Cities had slight genetic differences as they came from over 320 river miles with numerous dams between some of them. A tree diagram depicting genetic relationships among populations revealed a similar picture: large branching between populations above and below the Twin Cities and slightly separated clusters of upper Mississippi (Grand Rapids and Bemidji) samples and middle Mississippi (Aitkin to Monticello) samples (Figure 3).

Figure 3. Tree diagram of genetic relationships among Mississippi River walleye populations based on the genetic distance F_{st}. Longer branch lengths indicate greater genetic differences.



The approach to identifying genetic clusters with the program STRUCTURE identified only two main groupings. The two clusters were not perfectly distinguishable (as indicated by the presence of both colors in each sample in Figure 4), but populations below the Twin Cities averaged 74-90% assignment to one cluster while those above averaged 58-79% to the second cluster, indicating that the strongest genetic structuring of Mississippi River walleye occurs between populations above and below the Twin Cities. The lack of distinguishing power for samples above the Twin Cities was likely a result of only slight differences among samples and an isolation-by-distance pattern of genetic structure.

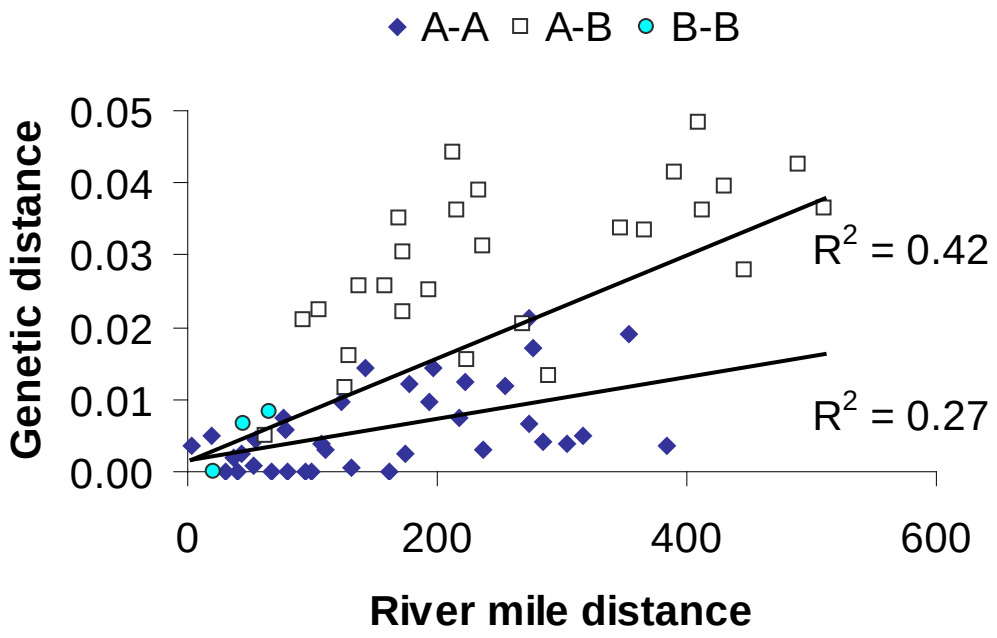
Figure 4. Assignment of ancestry based on STRUCTURE analysis for 12 samples of Mississippi River walleye. Each vertical bar represents a single fish with the proportion of ancestry assigned to each of two genetically distinct clusters indicated by color.



The F_{st} and STRUCTURE approaches to assessing genetic structure among populations do not work well when populations have subtle differences along a geographic gradient, as can occur along a river. This phenomenon is known as isolation-by-distance (IBD). When IBD occurs, a sample does not represent an isolated genetic unit. Instead, some movement and gene exchange tends to occur at shorter distances but less so with increasing distance. Distant locations along the river may develop genetic differentiation but in fact may have genetic exchange over many generations as descendents move up or down the river.

Statistical tests provided strong evidence for patterns of isolation-by-distance in Mississippi River walleye (Figure 5). For all sample pairs, river distance between them was significantly correlated with genetic distance ($p = 0.0012$, R^2 0.42), but this was driven in part by the higher genetic differentiation between samples above and below the Twin Cities. When the tests were repeated with only the samples from above the Twin Cities, the relationship was still significant but not as strong ($p = 0.012$, R^2 0.24). Although numerous dams now block upstream movements of fish, and stocking has occurred in systems that connect with the river, walleye still show genetic patterns consistent with historical population connectivity throughout the upper Mississippi River above St. Anthony Falls.

Figure 5 Genetic distance (Fst/[1-Fst]) versus river mile distance between sample sites. The symbols indicate comparison within and between sites above (A) and below (B) the Twin Cities. The top line is the linear regression (i.e. statistical relationship between genetic and geographic distances) for all pairs of sites while the bottom line is for comparisons of above Twin Cities sites only (A-A).



We found no detectable relationship between genetic diversity and levels of endocrine disruptor effects in Mississippi River walleye. In one test known as analysis of molecular variance, samples were grouped to determine if they shared common patterns of genetic variation. An analysis run with samples grouped by EDC levels (hot spots versus reference sites) showed that this factor explained little of the genetic structure of populations but geography (above and below the Twin Cities) had significant effects (Table 4). Because geography had a strong effect, we repeated the analysis with only populations above the Twin Cities but still found no patterns of genetic variation that differentiated EDC hot spots from references sites.

Table 4. Analysis of molecular variation (percentage of the genetic variation explained by the grouping factor) for samples of Mississippi River walleye grouped according to EDC level (hot spot v. reference) or geography (above or below Twin Cities).

Samples included	Grouping factor	% variation	Statistically significant?
All sites	Hot spot v. reference	0.15	No
Above Twin Cities only	Hot spot v. reference	0.65	No
All sites	Above v. below Twin Cities	2.78	Yes

A second test directly compared measures of genetic diversity between EDC hot spots and references sites. Allelic richness and heterozygosity would both be expected to decline if EDCs were affecting reproduction but allelic richness is a more sensitive indicator because it is expected to decline more quickly following a population bottleneck (Allendorf 1986). Neither measure of diversity showed reductions at EDC hot spot locations. Instead, there was a trend of increased diversity from upstream to downstream, especially for heterozygosity, with a significant increase in samples below the Twin Cities (Figure 6). Above the Twin Cities, the three hot spots did not differ significantly from any reference site for allelic richness or from Rice L. or Aitkin for heterozygosity. Below the Twin Cities, the two hot spots did not differ from the reference site. We also compared the diversity of Mississippi River populations to that in other lakes and rivers to determine if there were any broad river-wide patterns (Figure 7). Sample sites below the Twin Cities have the highest levels of genetic diversity of all Minnesota walleye populations studied while sites above the Twin Cities are similar to other locations within and outside the Mississippi River basin. Our results show the importance of sampling many sites when evaluating possible population-level genetic effects of pollutants to account for other patterns of genetic diversity. In our case, the decreased genetic diversity above the Twin Cities may be a natural pattern related to the isolation of walleye populations as St. Anthony Falls was formed.

Figure 6. Measures of genetic diversity, average allelic richness (above) and average expected heterozygosity (below), for 10 microsatellite DNA markers in walleye samples from 12 sites in the Mississippi River. Sites are in order going downstream from Bemidji to Lake Pepin.

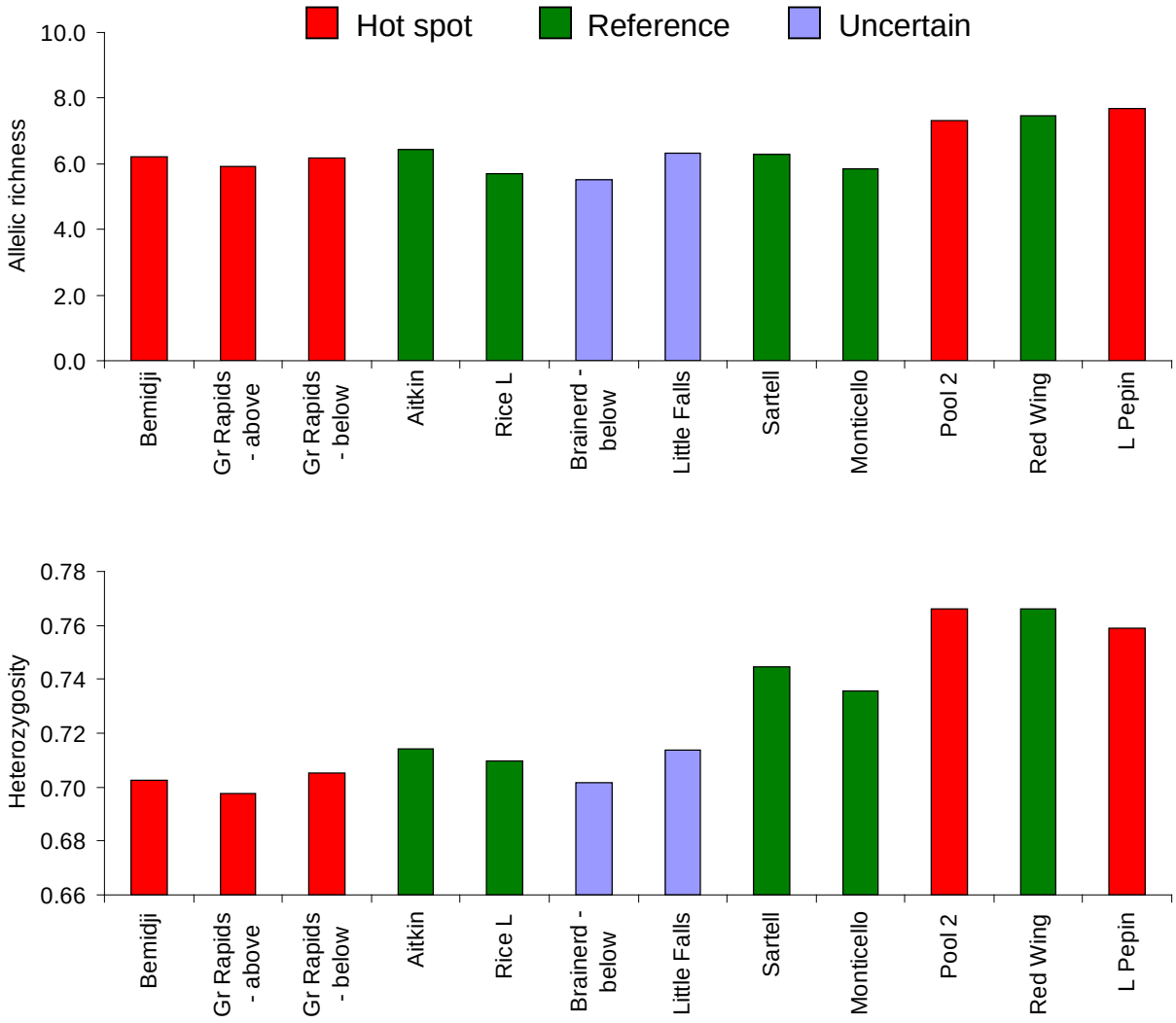
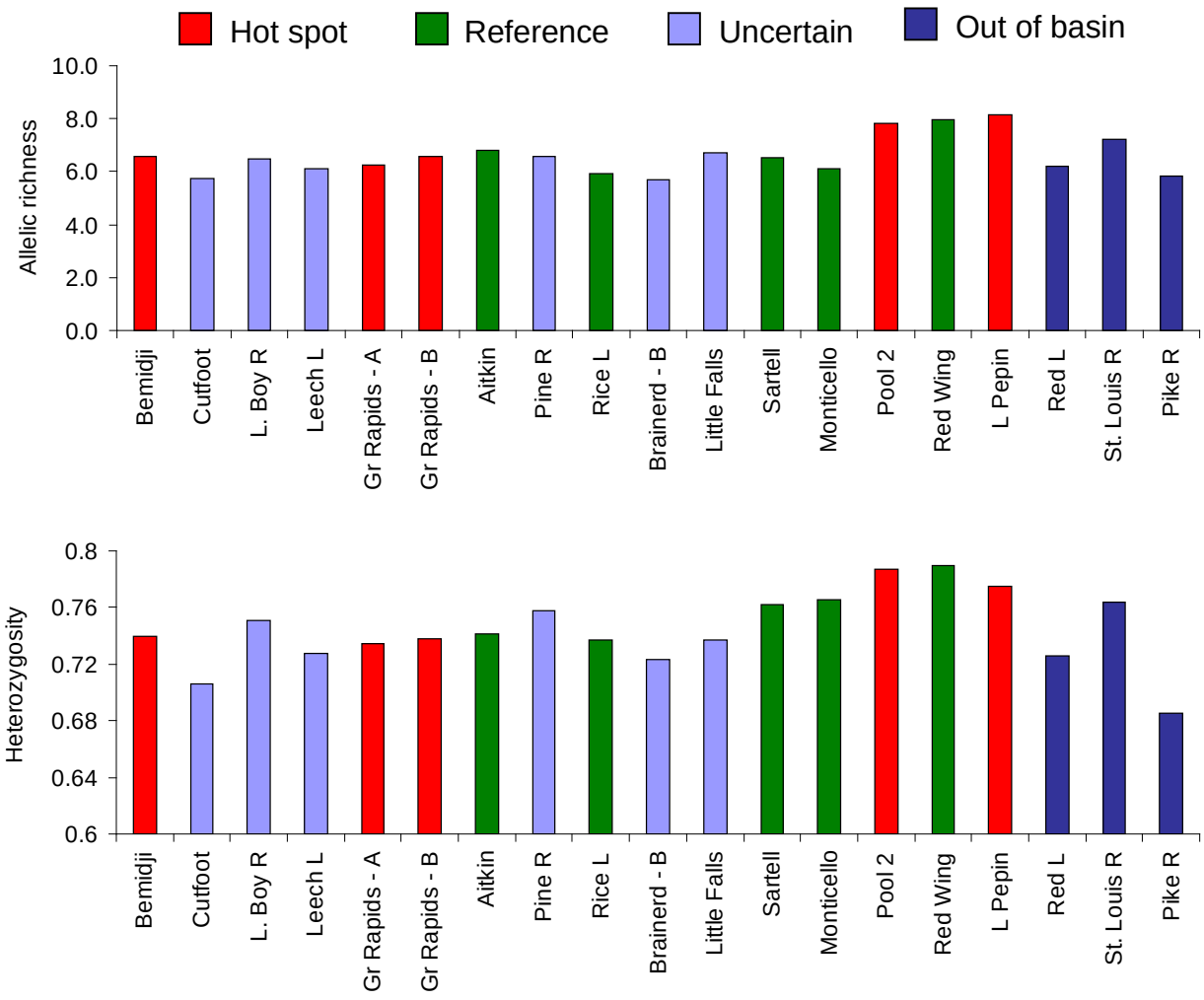


Figure 7. Measures of genetic diversity, average allelic richness (above) and average expected heterozygosity (below), for 8 microsatellite DNA markers in walleye samples from 19 sites. Samples from previous studies were added, including Pine River, Little Boy River, Leech Lake and Cutfoot Sioux Lake (Winnibigoshish) in the Mississippi River basin and three sites on the right from other basins. Mississippi River basin sites are in order going downstream from Bemidji to Lake Pepin.



Summary of laboratory methods and data analysis applied:

Genotyping

Genotypes were determined for 10 published walleye microsatellite DNA loci (*Svi2, Svi4, Svi6, Svi16, Svi18, Svi20, Svi26, Svi33, SviL2 and SviL6*; Borer et al. 1999; Wirth et al. 1999; Eldridge et al. 2002). DNA was extracted from tissue samples by boiling a scale or sliver of tissue in a 250 µL 5% Chelex (Sigma Chemical, St. Louis, MO) solution. Polymerase chain reaction (PCR) amplification was performed in 15 µL reactions containing 1x polymerase buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100), 1.5 mM MgCl2, 0.2 mM each dNTP, 0.5 mM of each primer with the forward primer fluorescently-labeled, and 0.5 U *Taq* DNA polymerase (Promega, Madison, WI). Each run included a water blank as a negative control to detect possible contamination of PCR solutions.

Amplifications were conducted in a Hybaid Omn-E thermocycler (Thermo-Hybaid U.S., Franklin, MA) using 35 cycles and a 50°C annealing temperature. Products of PCR amplifications were submitted to a genetics core facility (Biomedical Genomic Center, University of Minnesota, St. Paul) for electrophoresis on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Allele scores were determined relative to an internal size standard in each lane using Genemapper v.4.1 (Applied Biosystems).

Data analysis

We assessed genetic structure, or patterns in the distribution of genetic variation, using several approaches. First, we estimated the measure of genetic differentiation F_{st} (the analog theta from Weir and Cockerham 1984) between all population pairs using Fstat (Goudet 1995) and 1,000 permutations to test significance. A neighbor-joining tree depicting genetic relationships between populations based on F_{st} distances was constructed in TreeFit (Kalinowski 2009) and visualized using TreeView. The program STRUCTURE (vers. 2.2.3; Pritchard et al. 2000; also refer to http://pritch.bsd.uchicago.edu) was used to estimate the number of genetically distinct populations contributing to our samples. STRUCTURE uses Bayesian clustering algorithms to divide individuals, based on their genotypes alone and not location information, into groups that maximize genetic equilibrium. Three replicates were run to test the likelihood of 1-5 distinct genetic clusters in the Mississippi River data. The burn-in period was 50,000 replications, which was followed by 150,000 Markov chain Monte Carlo (MCMC) simulations run under a model that assumed possible admixture and correlated allele frequencies. Finally, to examine the possibility of an isolation-by-distance pattern of genetic differentiation, we conducted Mantel tests to determine if genetic distances between pairs of populations (F_{st}/[1-F_{st}]) were correlated with geographic distances (river miles).

We estimated common measures of genetic diversity in each sample. Observed and expected heterozygosities were calculated, and conformance with Hardy-Weinberg expectations was confirmed, for each locus in each sample using exact test

procedures of Guo and Thompson (1992), as implemented by GENEPOP v4 (Raymond and Rousseau 1995). Allelic richness was estimated for each sample by the software HP-Rare (Kalinowski 2005), using rarefaction techniques to standardize to an equal sample size of 30 genes.

We assessed the relationships of genetic diversity and structure with EDCs in two ways. We first used analysis of molecular variance (AMOVA) in the software Arlequin (Excoffier et al. 2005) to partition genetic variation into differences among individuals within population, differences among populations, and differences among groups of populations. Statistical significance was tested with 16,000 permutations of the data. Differences in genetic diversity measures were compared between pairs of populations using Wilcoxon signed-rank tests and one-sided p-values to test the hypothesis that EDC hotspots had lower diversity than reference sites.

New marker development attempt

As part of this project, we attempted to identify additional genetic markers to increase our power to assess differences in genetic diversity. We used a technique known as AFLP (Vos et al 1995), which does not require cloning and DNA sequencing to develop new markers. AFLP has been used successfully with numerous species, including fish, but no literature could be found showing it has been attempted on walleye. We had moderate success producing markers (bands) but few were polymorphic so they were not useful for assaying genetic diversity. Continued pursuit of AFLP markers for future studies would require a reinvestment in time and costs associated with DNA purification and screening with additional primer combinations.

Table 5. Total number of bands and number of polymorphic bands for 10 AFLP primer combinations in 24 Mississippi River walleye.

Primer combination	Number of bands	Number polymorphic
EcoC – Mse1	33	17
EcoC – Mse2	34	2
EcoC – Mse3	64	5
EcoC – Mse4	30	2
EcoC – Mse5	16	3
EcoG – Mse1	19	4
EcoG – Mse2	16	1
EcoG – Mse3	33	5
EcoG – Mse4	15	3
EcoG – Mse5	5	1

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V. TOTAL TRUST FUND PROJECT BUDGET:

Staff or Contract Services:

Schoenfuss, Project Leader – 1 Month/year 100% time (incl. fringe) to supervise field study and lab analysis. ($15,635 +37% fringe) **$** 21,420

Graduate Students, St. Cloud State University – 9 months/year 50% time to conduct laboratory assessment of fish health and assist in field study **$** 19,000

Loren M. Miller, Population Geneticist – University of Minnesota – 3 months/year to conduct genetic analysis. ($21,789 +33% fringe) **$** 28,980

Undergraduate Assistant, U of MN (10 weeks/year 50% time) **$** 3,600

Equipment: Field supplies $1,800; expendable SCSU lab supplies $ 4,980; field site travel $ 800; expendable AquaGen Lab supplies $ 15,920; field site travel $ 500.
Development: $ 0
Restoration: $ 0
Acquisition, including easements: $ 0
TOTAL TRUST FUND PROJECT BUDGET: $ 97,000

Explanation of Capital Expenditures Greater Than $3,500: *n/a*

VI. OTHER FUNDS & PARTNERS:

A. Project Partners:

Loren M. Miller – Population Geneticist, AquaGen, University of Minnesota $49,000
Matthew L. Julius – Aquatic Ecologist, St. Cloud State University

B. Other Funds Proposed to be Spent during the Project Period: $0

C. Past Spending: $120,000 for longitudinal study of Mississippi River at 43 sites from Lake Itasca to Iowa border.

D. Time: Our recently completed longitudinal study of the Mississippi River provides the most exhaustive data set to date on the health of fish in the Mississippi River in correlation with the presence of emerging contaminants in the water and sediment. The transient nature of this information in the context of future studies requires any approach that uses this data set to occur in the very near future, i.e., the next two years. Beyond such time, a complete re-sampling of water and sediment would have to be conducted, more then doubling the overall cost of the project.

VII. DISSEMINATION:

Dissemination of the results of this study will be made available to the funding agency and interested parties in quick and efficient fashion through the use of multiple dissemination tools:

(1) periodic progress reports to LCCMR
(2) final report to LCCMR

(3) reports to the MN Department of Natural Resources (related projects)
(4) presentations at regional, national, and international research conferences.
(5) press releases of significant findings through the St. Cloud State University Public Relations office.
(6) submission of manuscripts on the studies results to peer reviewed scientific journals.
(7) updates on the aquatic toxicology web site at St. Cloud State University (web.stcloudstate.edu/aquatictox)

VIII. REPORTING REQUIREMENTS:

Periodic work program progress reports will be submitted not later than Dec 31, 3007; June 30, 2008; Dec. 31, 2008; June 30, 2009; December 31, 2009. A final work program report and associated products will be submitted between June 30 and August 1, 2010 as requested by the LCCMR

IX. RESEARCH PROJECTS:

Attachment A: Budget Detail for 2007 Projects - Summary and a Budget page for each partner (if applicable)

Project Title: Threat of Emerging Contaminants to Upper Mississippi Walleye

Project Manager Name: *Project Partner - Miller*

Trust Fund Appropriation: $ *97,000*

1) See list of non-eligible expenses, do not include any of these items in your budget sheet

2) Remove any budget item lines not applicable

2007 Trust Fund Budget	**Result 1 Budget:**	**Amount Spent** *(date)*	**Balance** *(date)*	**Result 2 Budget:**	**Amount Spent** *(date)*	**Balance** *(date)*	**Result 3 Budget:**	**Amount Spent** *(7/23/2010)*	**Balance** *(7/31/2010)*	**TOTAL BUDGET**	**TOTAL BALANCE**
	Field Collection of Feminized Fish.			*Laboratory Assessment of Fish Health*			*Emerging Contaminant Population-Level Genetic Effects*				
BUDGET ITEM			0			0			0	0	0
PERSONNEL: wages and benefits	0		0			0	32,580	33,687	-1,107	32,580	-1,107
Other direct operating costs *(expendable laboratory supplies for DNA analysis)*			0			0	15,920	15,017	903	15,920	903
Travel expenses in Minnesota			0			0	500	296	204	500	204
COLUMN TOTAL	**$0**	**$0**	**$0**	**$0**	**$0**	**$0**	**$49,000**	**$49,000**	**$0**	**$49,000**	**$0**

Attachment A: Budget Detail for 2007 Projects - Summary and a Budget page for each partner (if applicable)

Project Title: Threat of Emerging Contaminants to Upper Mississippi Walleye

Project Manager Name: *Dr. Heiko L. Schoenfuss*

Trust Fund Appropriation: $ *97,000*

1) See list of non-eligible expenses, do not include any of these items in your budget sheet

2) Remove any budget item lines not applicable

2007 Trust Fund Budget	**Result 1 Budget:**	**Amount Spent** *(7/23/2010)*	**Balance** *(7/31/2010)*	**Result 2 Budget:**	**Amount Spent** *(7/23/2010)*	**Balance** *(7/31/2010)*	**Result 3 Budget:**	**Amount Spent** *(date)*	**Balance** *(date)*	**TOTAL BUDGET**	**TOTAL BALANCE**
	Field Collection of Feminized Fish.			*Laboratory Assessment of Fish Health*			*Emerging Contaminant Population-Level Genetic Effects*				
BUDGET ITEM			0			0			0	0	0
PERSONNEL: wages and benefits	16,400	16,400	0	24,020	24,020	0	0		0	40,420	0
Other direct operating costs *(expendable laboratory supplies for DNA analysis)*	1,800	1,800	0	4,980	4,980	0	0		0	6,780	0
Travel expenses in Minnesota	800	800	0	0		0	0		0	800	0
COLUMN TOTAL	**$19,000**	**$19,000**	**$0**	**$29,000**	**$29,000**	**$0**	**$0**	**$0**	**$0**	**$48,000**	**$0**