Upper Mississippi River Ichthyoplankton Sample qPCR Screening for Invasive Carp

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FINAL REPORT

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Background

Invasive carp (Bighead, Silver, Black, and Grass carp) threaten native ecosystems and recreation in Minnesota waters. These invasive species have been spreading upstream in the Mississippi River basin since their escape in the 1970s (Henderson 1976). With high fecundity and the ability to disperse great distances, invasive carp can reach substantial populations, sometimes comprising most of the fish biomass in certain systems (MICRA 2002). At high densities, invasive carp can impact native fish condition, injure boaters with their jumping behavior, and disrupt tourism and commercial fishing economies (Irons et al. 2007, Kolar et al. 2007).

The Minnesota Department of Natural Resources (MNDNR) conducts sampling for all life stages of invasive carp as part of monitoring and response efforts. Bighead, Silver, and Grass carp have been detected in Minnesota and have reached as far upstream in the Mississippi River as the Lower St. Croix River (Mississippi River Pool 2), and in the Minnesota River to Granite Falls (MNDNR 2022). Invasive carp are still present at relatively low densities in Minnesota, although catches have increased in recent years. Over 100 invasive carp have been captured in Pool 8 of the Mississippi River, raising concerns about the potential for reproduction. However, no reproduction has been detected in Minnesota waters to date.

MNDNR conducts ichthyoplankton sampling annually to monitor the population and inform management efforts. Larval trawls are performed during spring and summer at locations where spawning habitat is suitable for invasive carp. Light traps were added in 2022 to sample nursery habitat for juvenile fish. These samples are preserved in 90% ethanol, and visually identified to species by experts at an external laboratory.

Visual identification of larval fish from tows and light traps can be time consuming, requires funding an external laboratory, and may not detect any invasive carp at the end of sample analysis. Recently, Fritts et al. (2019) identified a method to use quantitative PCR (qPCR) markers developed for invasive carp eDNA surveys to pre-screen preserved samples for the presence of larval fish and eggs. These authors found that when tissue samples from larval invasive carp and carp eggs were stored in ethanol, qPCR could be used to detect invasive carp in aliquots of the ethanol. These results suggest that qPCR can be a useful tool to pre-screen larval samples collected by MNDNR and identify a subset of samples which could then be visually screened for species confirmation.

The objective of this project is to use existing quantitative PCR (qPCR) methods to screen larval tow and light trap samples collected by MNDNR in the upper Mississippi River (UMR) in 2022 for the presence of invasive Silver, Bighead, and Grass carp DNA. Samples that show positive qPCR results will be screened visually by an external laboratory to confirm the presence of larval invasive carp and eggs.

Methods

Sample collection

Larval trawling was conducted during the 2022 field season. Sampling was conducted in Pools 5A and 8 of the Mississippi River (Figure 1). Trawls were conducted on a weekly basis from June to August. Trawling occurred when there was a spike in the hydrograph at the immediate upstream lock and dam and when water temperatures were above 17°C (conditions presumed to be favorable for spawning).

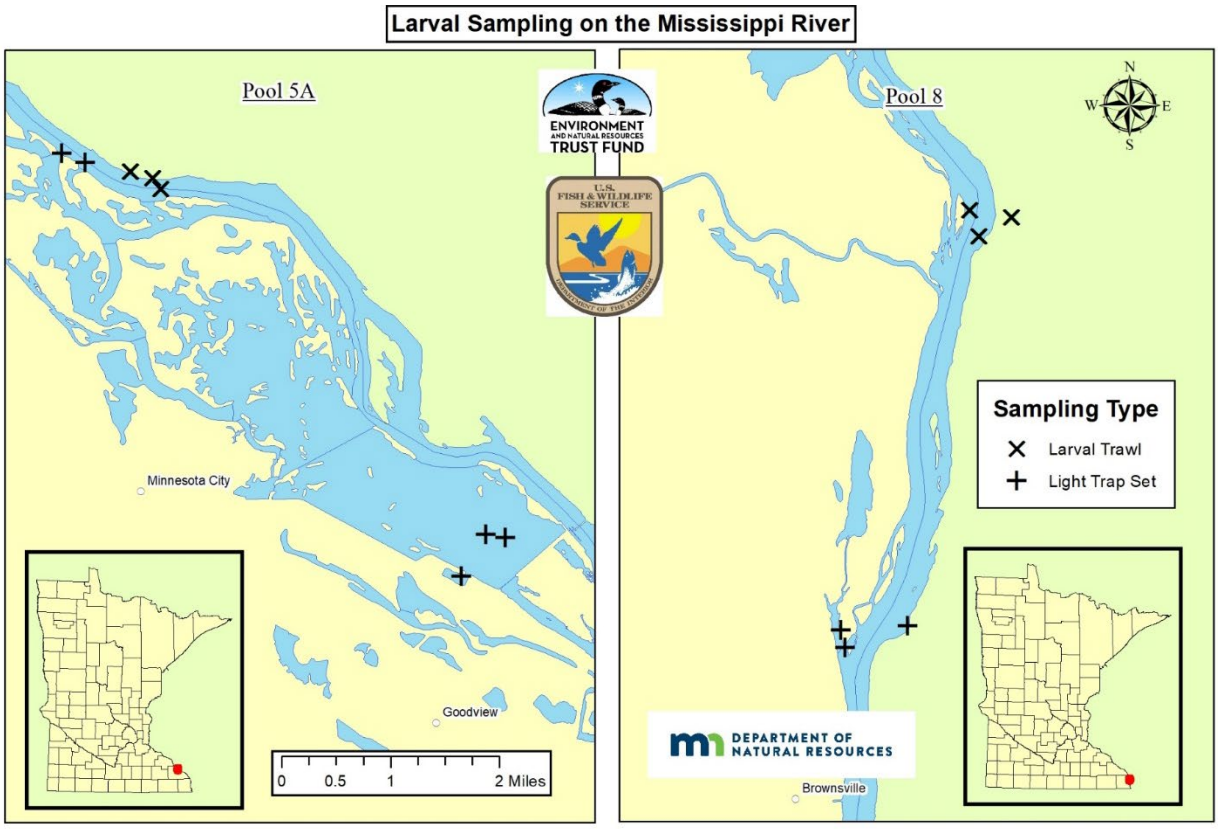

Figure 1. Standardized larval fish trawling (dark cross) and larval light trap (dark plus sign) locations on the Mississippi River.

Sampling was conducted by Minnesota DNR with the aid and support of the US Fish and Wildlife Service, La Crosse FWCO. Each day of sampling, 12 trawls were pulled for 5 minutes per trawl, 15-20 minutes apart. The samples were then put into plastic containers and transported back to the MNDNR lab at the East Metro Fisheries office. Fisheries biologists drained the water and preserved the samples in 90% ethanol. After 24-48 hours the ethanol was drained and replaced as per the protocol in Fritts et al. (2019). Samples were preserved at ambient temperatures until genetic laboratory analysis.

Larval light traps were deployed in conjunction with larval trawling. Traps were set overnight in Pool 8 and Pool 5A backwaters. Sites were chosen at random based on accessibility by boat and water current conditions in backwaters. Traps were set in backwaters at the lower ends of the pools. Each light trap set consisted of five floating traps, each equipped with a flashing green light, attached five feet apart and anchored on both ends. Orientation line, current, depth, set/pull time, and water temp were recorded. Traps were set out overnight in a line of five traps. The samples were then collected the following morning and placed in plastic containers to be transported back to the MNDNR East Metro Fisheries office where the water was drained and replaced with 90% ethanol for preservation. After 24-48 hours the ethanol was then drained from the sample and replaced with 90% ethanol. The samples were then stored at ambient temperatures for the remainder of the field season.

Laboratory Methods

DNA Extraction

After samples were received at the lab, we took three separate 1-mL aliquots/subsamples for extraction and qPCR. A 1.0 ml pipette was used to transfer each subsample into a separate 1.5 ml screw top MCT (microcentrifuge tube). After subsamples were aliquoted, they were centrifuged at max speed (~18,000 rpm) to concentrate any cells and/or eDNA at the bottom of the MCT. The supernatant was then discarded, and the subsamples were set to dry in a 60°C bead bath long enough to evaporate any excess ethanol. As a quality control measure, a hood control (an empty 1.5 mL MCT) was placed in the bead bath with each set of subsamples to inform us of any cross contamination that may have occurred in this step. If any carp DNA was detected in this hood control, the subsamples in that range would be discarded from analysis. Once the subsamples were dry, they were removed from the drying hoods and stored in a -20°C freezer until we were ready to extract DNA. DNA was extracted using the commercially available gMax mini genomic DNA extraction kit (IBI scientific) following the manufacturer's instructions and final extracts contained a volume of 200 µL of elution buffer. An extraction positive control sample (brook trout cells) and negative control sample (DI water only) was added to each extraction set to ensure two things: 1) the extraction worked properly and 2) the negative was void of any carp DNA contamination. All DNA extractions were conducted in rooms dedicated to invasive carp eDNA extractions where no other laboratory procedures were performed.

Quantitative PCR (qPCR)

DNA extracts were analyzed using qPCR to determine the presence of Silver, Bighead, or Grass carp DNA. For Bighead and Silver carp, we used a multiplex qPCR that contains six total loci. The qPCR multiplex includes two general invasive carp markers (i.e., can detect Silver and Bighead carp, but cannot differentiate between the two species) located on the cytochrome oxidase I gene of both species (ACTM 1/3), two markers specific to Silver Carp located on the ND6 and ND2 regions of the mitochondria (SCTM 4/5), and two markers specific to Bighead Carp located on the ND4 and ND6 regions of the mitochondria (BHTM1/2) (Farrington et. al 2016). qPCR master mix concentrations and thermal profiles followed standard USFWS lab protocols for these markers developed for eDNA monitoring (USFWS 2022). For Grass Carp, we used three separate qPCR assays: GCTM10 located on the ND2 gene region, GCTM22 located on the cytochrome oxidase II region, and GCTM32 located on the cytochrome oxidase III region (USFWS unpublished data). qPCR master mixes were set up in a room dedicated to reagent prep and then master mixes and template DNA were added to 384 well plates in a separate room dedicated to PCR using an automated liquid handling robot (Eppendorf EP motion 5075). Each subsample was analyzed in replicates of four. Any subsample that had at least one detection for any of the four replicates at a specific assay was considered a positive detection for that assay. All qPCR plates included both positive (invasive carp DNA extracted from cells) and negative (water only) controls. For Silver and Bighead carp we used a five-point standard curve consisting of 10 ng/µl, 50 ng/µl, 250 ng/µl, 1250 ng/µl, and 6250 ng/µl standards. For Grass carp we used a six-point standard curve consisting of 10 ng/µl, 50 ng/µl, 250 ng/µl, 1250 ng/µl, 6250 ng/µl and 31,250 ng/µl standards.

Results

A total of 72 larval trawl samples were taken during the 2022 field season; 42 samples were taken in UMR Pool 8 and 36 samples were taken in UMR Pool 5A. Sixty larval light traps

were set with 15 traps set in Pool 8 and the remaining 45 set in Pool 5A. In total, there were 132 samples and after subsampling in triplicate, we processed a total of 396 (132 x 3) subsamples and 24 control samples. All control samples showed the expected results (e.g., all extraction negatives showed no qPCR amplification) suggesting that there was no evidence of field or lab contamination.

We detected the presence of invasive carp DNA in three samples (MNDNR22-113, MNDNR22-114, MNDNR22-116; Table 1). All three positive detections were from larval tow samples taken on June 1, 2022 in Pool 8 and were positive for the ACTM 1/3 marker set only. Detections at this marker set indicate that DNA from Bighead or Silver carp was present, but not which species in particular since the marker set cannot distinguish the two. MNDNR22-113 had a single qPCR replicate with a positive detection in two of the three subsamples with starting copy numbers of 2.42 and 2.06, respectively. MNDNR22-114 also had a positive detection for a single qPCR replicate in two of the three subsamples with starting copy numbers of 0.72 and 1.13, respectively. MNDNR22-116 had two positive qPCR replicates in one of three subsamples. Starting copy numbers were 0.86 and 0.94, respectively. There were no positive detections for the Silver-specific or Bighead-specific markers and there were no detections for any of the three Grass Carp eDNA assays.

Table 1. Summary of detection data for samples with positive eDNA detections at the ACTM 1/3 marker set. There were no positive eDNA detections for any of the other markers we used in this study.

Subsample ID	Number of positive replicates	qPCR Cq value	Starting quantity (DNA copies)
MNDNR22-113B	1/4	38.07	2.42
MNDNR22-113C	1/4	38.29	2.06
MNDNR22-114A	1/4	39.76	0.72
MNDNR22-114B	1/4	39.12	1.13
MNDNR22-116A	2/4	39.44*	0.91*

*Mean values for the two positive replicates for sample MNDNR22-116

Discussion

The number of DNA copies present can be used to infer the likelihood that larval carp and/or eggs were present in the preserved samples. Fritts et al. (2019) showed that samples with > 10,000 copies of invasive carp DNA had larval carp or eggs present 100% of the time and that samples with less than 10 copies never contained carp eggs or larvae. It is important to note that these values should not be interpreted as absolute thresholds for the presence/absence of larval carp and/or eggs, rather they provide some idea of the relative number of DNA copies observed when carp are present in the sample. The positive subsamples in our study all had starting copy numbers less than 10, suggesting a low likelihood of carp eggs or larval fish being present in the samples. If samples were visually inspected for species ID, we would recommend any suspected larval carp and eggs should then be genetically confirmed since they can be difficult to distinguish based on morphology alone (Larson et al. 2016). Minnesota DNR is contracting with an external laboratory for visual identification of the positive samples.

It is important to point out that a positive detection does not indicate that carp eggs or larvae are present in the original sample. eDNA can originate from a number of sources in

addition to the target species including contaminated boats, predatory birds, and sampling gear (Merkes et al. 2014, Homel et al. 2021) Invasive carp are present in the areas where these samples were collected and its certainly possible that DNA from adult fish was present in the water column when these larval samples were taken and that DNA was collected by the sampling gears used and transferred to the preserved samples. Fritts et al. (2019) notes that exchanging the ethanol the samples were stored in and then conducting qPCR on the new ethanol (as was done in our study) should minimize the potential for these types of detections.

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