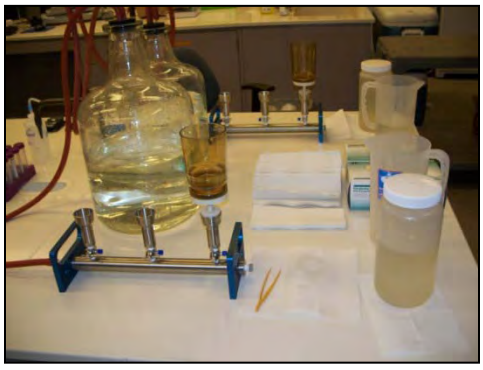
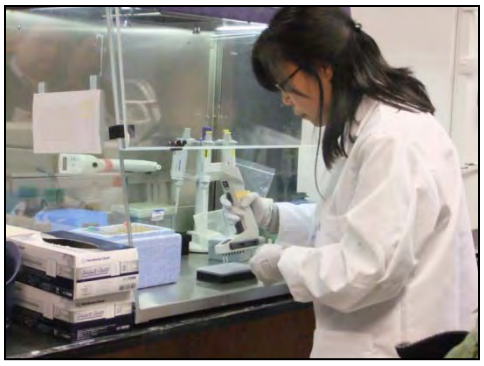
Environmental DNA Calibration Study

Interim Technical Review Report

February 2013





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Preface

This report contains technical results through December 2012 from the Environmental DNA Calibration Study (ECALS), and represents an update to the March 2012 interim technical review report. Due to the schedule of work for ECALS, there will be several sections in the document that will have no results to report until later in the study. The final report will provide greater detail and more complete citation list. The following have contributed to the ECALS project and the results in this report:

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Glossary

Allele	an alternative form of a gene that is located at a specific position on a specific chromosome
Amplicon	a piece of DNA (e.g., a marker) that is amplified greatly in numbers during the PCR process
Asian carp	silver carp and/or bighead carp (for the purposes of this report)
CNT	carbon nanotubes; positively charged nanotubes can significantly enhance the specificity and efficiency of PCR
cPCR	conventional PCR; an analytical technique that produces multiple copies of a target DNA sequence (marker) for detection using gel electrophoresis. This technique can only determine the presence or absence of DNA; it cannot quantify the amount of DNA in the sample.
DNA	deoxyribonucleic acid
eDNA	environmental DNA. In this report eDNA pertains to Asian carp DNA that originates in a waterbody
Fomite	any inanimate object or substance capable of carrying and transferring a substance like eDNA from one place to another
gel electrophoresis	a technique for separation and analysis of DNA fragments based on size and electrical charge
Haplotype	any set of closely linked markers which travel together when they are passed on to the next generation
Marker	a DNA sequence at a known location on a chromosome that can be used to identify a species

microsatellite	short, tandem repeats of DNA sequence consisting typically of 2 to 6 base pairs
mitochondria	organelles within cell cytoplasm that are the sites of cellular respiration, which generate fuel for cellular activities
naked DNA	in the aquatic environment, free-floating DNA no longer contained in a cell
nucleotide	one of four base that comprise the links between the two primary DNA strands. These consist of adenine (A), cytosine (C), guanine (G), and thymine (T)
parallel tagged amplicon sequencing	a next-generation sequencing technology that allows the sequencing of multiple amplicons simultaneously
PCR	polymerase chain reaction. A technique in which primers specific to the DNA marker sought are added to the genetic sample. Through a series of steps, the number of copies of the DNA marker strands are amplified many times to the point at which they can be detected by gel electrophoresis (cPCR) or real-time quantitative PCR (qPCR).
Piscivore	an animal that eats fish
polymerase	an enzyme used to synthesize polymers of nucleic acids, typically by copying a template using base-pairing interactions
positive detection	A confirmed and sequenced positive for Asian carp eDNA using QAPP methodology
Primer	a short strand (around 20 bases) of nucleic acid used to catalyze the PCR process, and can be chemically synthesized in the laboratory for a specific marker

QAPP	Quality Assurance Project Plan for the Environmental DNA (eDNA) Monitoring of Invasive Asian Carp in the Chicago Area Waterway System (CAWS); outlines the detailed procedures for the planning, collection, filtering, processing and reporting of eDNA samples
qPCR	quantitative PCR or real-time PCR; a technique to amplify and concurrently quantify a targeted DNA molecule
sequencing	the process of determining the precise order of nucleotides within a DNA molecule
single nucleotide polymorphism	aka SNP; a single nucleotide difference between 2 DNA strands

List of Abbreviations

ACRCC	Asian Carp Regional Coordinating Committee
BMP	Best Management Practice
CAWS	Chicago Area Waterway System
CERC	Columbia Environmental Research Center (USGS; Columbia, MO)
CNT	Carbon nanotubes
CSO	Combined Sewer Overflow
ECALS	Environmental DNA Calibration Study
eDNA	Environmental DNA
ERDC	Engineer Research and Development Center (USACE; Vicksburg, MS)
mtDNA	Mitochondrial DNA
nDNA	Nuclear DNA
PCR	Polymerase Chain Reaction
PDT	Project Delivery Team
QAPP	Quality Assurance Project Plan
RNA	Ribonucleic Acid
SNP	single nucleotide polymorphism
UMESC	Upper Mississippi Environmental Research Center (USGS; La Crosse, WI)
USACE	United States Army Corps of Engineers
USFWS	United States Fish and Wildlife Service
USGS	United States Geological Survey
WBS	Work Breakout Structure

Executive Summary

The Environmental DNA Calibration Study (ECALS) is a three-year study to improve the understanding and interpretation of the detection of Asian carp DNA in environmental samples (eDNA). eDNA surveillance programs seek to detect the presence of genetic material (DNA in cells sloughed off in slime, feces, urine, etc.) extracted from water samples; the detection of genetic material is linked to the possible presence of Asian carp. The study involves collaboration between the U.S. Army Corps of Engineers, the U.S. Geological Survey, and the U.S. Fish and Wildlife Service. ECALS addresses three major Action Items from the Asian Carp Regional Coordinating Committee (ACRCC) Asian Carp Control Strategy Framework, of which results to date are presented below. Initial ECALS efforts focused on eDNA vectors whereas marker development and calibration experiments will receive greater attention in 2013.

Asian Carp eDNA Vectors

In addition to DNA shed by live Asian carp, vectors of Asian carp eDNA could transfer eDNA into the Chicago Area Waterway System (CAWS). To integrate what has been learned through ECALS and other ACRCC studies, a conceptual model is being developed to provide a structured visualization of the potential eDNA inputs (e.g. presence of a live fish vs. vectors of eDNA) as well as the factors or variables that influence release, transport, persistence, and detection of eDNA in the CAWS. ECALS is investigating several potential eDNA vectors:

Storm Sewers. Asian carp carcasses are transported on ice brought to Chicago-area fish markets. That ice and associated body fluids are dumped into storm gutters and street drains. Because fish may be displayed on ice at these markets during the day, change out of melting ice (potentially multiple times during the day) may supply additional amounts of ice/ice water containing Asian carp fluid/tissue into the storm sewer system. The ECALS Team executed trials in fall 2011, summer 2012, and fall 2012 to demonstrate that ice from ice chests holding Asian carp carcasses could be a source of eDNA in the CAWS.

- Two points of particular interest have been observed – One, Asian carp eDNA was detected in sewers prior to our trials (perhaps originating in fish markets) and two, eDNA that was deposited into storm sewers during experimental trials largely dissipated in receiving waters (CAWS) within a day. Whether the eDNA signal was lost due to degradation, dilution, or downstream flow is unclear.

Fertilizers. Wild-caught Asian carp are used to develop fertilizer for commercial and residential uses. The team tested two brands of fertilizer that contain Asian carp as part of the fertilizer formula for the presence of detectable eDNA (using current markers).

- There were no eDNA detections in assays of small volumes of either brand of fertilizer. Tests of larger volumes or more samples of fertilizer may be needed to completely rule out this potential vector. Currently, the team is searching for more information on the role of Asian carp in producing these fertilizers and for additional brands of fertilizer that may contain Asian carp DNA.

Fisheries gear. Gear (boats, nets) used by natural resources agencies, contract fishermen and/ or recreational anglers may be exposed to Asian carp DNA in waters where carp are present then moved into the CAWS where some Asian carp DNA could be sloughed off into the water. The potential for these sources to harbor eDNA and result in a positive eDNA detection was evaluated in fall 2012.

- Vessel hulls have considerable amounts of adhering DNA, which can persist for days and is not removed by overland transport.
- Adhering DNA also does not appear to be completely or quickly washed off of boats moving through the water. Thus, vessel hulls can be vectors for DNA movement.
- Nets appear to be sources of very large amounts of eDNA but require confirmation and quantitation of DNA associated with nets through an additional sampling trial.

Bird Transport and Deposition of eDNA. Given the assumption that eDNA is deposited by piscivorous (fish-eating) birds, ECALS has focused on the amount of eDNA in a bird fecal sample, degradation, and piscivorous bird feeding and movement patterns in the Chicago region.

- Piscivorous birds have the capacity to be a direct vector of Asian carp DNA or to contaminate fomites (e.g. barges, boats) with Asian carp DNA via fecal deposits.
- Silver carp DNA was detected in fecal samples collected from piscivorous birds offered one to three meals of silver carp.
- Silver carp DNA could be amplified from bird fecal samples collected up to 1 week following consumption of a silver carp meal.
- Silver carp DNA in fecal material deposited on metal sheets persisted for 30 days under ambient environmental conditions despite exposure to temperatures exceeding 60°C (140°F).
- Satellite-tagged double-crested cormorants exhibited large variation in daily and seasonal bird movement, with some birds staying close to tagging locations and others traveling as far as Canada or the Gulf Coast. Additional work will examine available records of the frequencies of observation for other piscivorous birds in the CAWS region.
- Throat and cloacal swabs taken at the time of satellite-tagging resulted in silver carp DNA detection in 13 of 15 cormorants (positive carp DNA from: 3 throat only, 4 cloaca only, and 6 both throat and cloaca) from a rookery near Peoria, IL and 7 of 15 cormorants (positive carp DNA from 6 throat only and 1 both throat and cloaca) from a rookery near Baker's Lake (within the CAWS), showing evidence of Asian carp consumption.

Fish Carcasses. Since biologists had reported the presence of dead Asian carp on decks of barges above the U.S. Army Corps of Engineers Electric Dispersal Barrier in the Chicago Sanitary and Shipping Canal (CSSC) and slime from those decaying carp trailing down the sides of barges to

the water line, concerns have existed regarding the capacity of fomites (objects that carry DNA) like barges to transport Asian carp DNA (in the form of carcasses or slime) from areas where Asian carp are present to areas where they are not present or abundant. The goal of this study was to assess whether Asian carp carcasses or residual slime on fomites such as barges or boats could be responsible for the presence of Asian carp DNA in waters where Asian carp are not present.

Silver carp eDNA can be detected for at least 18 days when the surfaces of carp carcasses or water that had flowed over those carcasses were sampled. Samples from Asian carp slime coat that had been placed on metal surfaces also showed intact Asian carp DNA, but disappeared by day 18. These trials indicate that carcasses, or rain or other run-off from surfaces where Asian carp carcasses or slime residue reside, can be a source of eDNA entering a system.

Barge Transport of Carcasses. Guidelines for vessel operators were developed in May 2012 by USACE, ILDNR, and USEPA for vessels that enter the CAWS through four lock and dams (Dresden Island, Brandon Road, Lockport, and TJ O'Brien). The guidelines outline the protocol for vessels that may be carrying dead silver or bighead carp carcasses (and potentially depositing them on the upstream side of the barrier) and require that lock staff document these occurrences, verify the species, and ensure removal before the vessel crosses.

- During the 2012 shipping season, there were three reported incidents concerning a total of five Asian carp carcasses on vessels. On 10 April, two silver carp were found on the deck of a tow at Lockport; on 12 April one silver carp was found on a barge at the mouth of the Calumet River; and on 8 June two separate barges locking upstream at Brandon Road each reported having one silver carp on deck.

Sediments. The potential for sediments both within the CAWS and outside of the CAWS to sequester and/or transport eDNA was investigated.

- Five of 13 stream bank samples taken approximately 105 km downstream of Lockport on the Illinois River tested positive for silver carp DNA; bighead carp DNA was not detected.
- Sediment samples (n=28) were collected from Lake Peoria dredged materials being off-loaded at the old US Steel site near Calumet Harbor. Eleven samples tested positive for silver carp DNA, and one sample tested positive for bighead carp DNA.
- Additional surface sediment samples were collected in November 2012 from Lake Calumet and Lockport Pool for eDNA sorption studies that are presently underway.

Asian Carp eDNA Genetic Marker Development

The current eDNA markers for both bighead and silver carp are comprised of short segments of the mitochondrial DNA control region (or "D-loop") and primarily provide information on presence/absence of that DNA in a sample. The team's aim is to develop a suite of different markers that provide different capabilities, including 1) improved detection probabilities by

increasing the number of markers simultaneously assayed, 2) more efficient processing by reducing background non-target PCR amplification, 3) real-time quantitative PCR estimates of DNA abundance (qPCR has added benefit of increased efficiency by eliminating gel electrophoresis and reducing or eliminating the need for sequencing), 4) data on allelic variability (or "polymorphism") to a degree that will allow at least broad estimation or corroboration of Asian carp abundance, and 5) some indication of the nature or time since deposition of an eDNA sample.

- Asian carp specimens from across North America (10 silver carp populations, 12 bighead carp populations) and Asia (3 populations each, silver and bighead carp) were acquired, and DNA sequencing was performed using a next-generation DNA sequencer. As a result, complete mitochondrial DNA sequences for 33 bighead and 25 silver carp from 9 North American locations were obtained.
- Genetic material for numerous non-target fish species occurring in the CAWS was procured and, along with existing data on aquatic species DNA residing in GenBank, is being used to test new markers to ensure that they are specific to silver and/or bighead carp.
- For presence/absence markers, the team is testing 12 trial markers that potentially could be used to selectively detect silver carp, testing 11 for bighead carp, and testing 17 that could potentially amplify both Asian carp species to the exclusion of all other species. Expectations are that most will be eliminated, as DNA segments that correspond to eDNA markers that would have both absolute specificity for a target species and high detectability (in large part a function of being relatively more numerous in cells than other DNA segments, like mitochondrial DNA) are rare.

Asian Carp eDNA Increased Efficiency and Calibration Studies

Increasing Efficiency

Presently, the time from field sampling to analytical results for eDNA can take as long as two weeks. Even before laboratory analysis, several hours of very intensive fieldwork followed by laborious sample filtering is required. ECALS is evaluating ways to reduce time and effort for this process. Identification of the most cost and time-efficient extraction approach and most robust cross-platform quantitative PCR (qPCR) approach will benefit future monitoring efforts.

- Tissue grinding using a higher-throughput bead-beater instrument demonstrated no significant difference in apparent DNA yield or quality compared to the Quality Assurance Project Plan (QAPP) method, and could replace the longer vortexer-based step in the existing protocol.
- Comparison of different DNA extraction kits suggests that different extraction kits may yield different quantities of amplifiable DNA, and that different extraction kits may have varying susceptibilities to environmental inhibitors.

- A comparison of different sampling methods (filtration, centrifugation, sieve cloth) has been hampered by difficult field conditions and equipment contamination. Refined protocols and additional fieldwork are planned for 2013.
- A comparison of sampling from different depths in the CAWS water column yielded more positive eDNA hits (7 of 15 samples) for surface samples than for mid-column samples (0 of 15 samples) or bottom-depth samples (1 of 15 samples).

Calibration Studies

Calibration studies seek to examine eDNA release (i.e. shedding) rates and degradation rates under laboratory conditions to inform hydrodynamic modeling of how deposited eDNA may be distributed by water flow in the CAWS. The team has designed experiments to determine how fish size, number, behavior, as well as water temperature and diet influence eDNA loading (or shedding) by Asian carp. We will also investigate sperm as a source of eDNA over time in static water conditions.

Loading Studies

- Preliminary studies show that eDNA shedding rates are consistent over different water-flow rates. Currently, one experiment assessing effects of temperature on shedding rates of silver carp sub-adults has been completed. The team found no effect of temperature on shedding rates.
- Preliminary studies of eDNA from sperm in water showed that eDNA was detectable for at least 17 days.
- Results of these studies will provide information necessary to determine the degree to which qPCR can be used to determine abundance or biomass of bighead and silver carp from eDNA samples. Eight of the 12 designed studies (examining effects of temperature, biomass and diet; and sperm degradation) have been completed.
- However, some trials are incomplete because PCR inhibitors have prevented DNA quantification using qPCR. The team observed that the greatest PCR inhibition is associated with samples from tanks that were fed algae and is currently working to find ways to reduce PCR inhibitors without reducing the sensitivity of the qPCR assay for these lab-based samples. However, it has become apparent that a more accurate measurement of inhibition and a more extensive survey of methods for avoiding or removing inhibitors will be necessary, especially for the processing of field samples that are likely to have many more (and more diverse) PCR inhibitors.

Hydrodynamic Model

- The hydrodynamic grid for the area to be modeled has been completed, and protocols have been established to enable passing of information back and forth between the hydrodynamic and eDNA transport (i.e. water quality) parts of the model. Results from other ECALS investigations (e.g. eDNA degradation studies) will be incorporated into the eDNA transport model when available. 3D simulations of the hydrodynamics of the barrier area are also underway, and the electrical field modeling is in preliminary development at present.

1 Introduction

1.1 Project Background

Invasive aquatic nuisance species pose a major threat to aquatic ecosystems worldwide. Invasive Asian carps, including bighead carp (*Hypophthalmichthys nobilis*) and silver carp (*H. molitrix*) have been steadily dispersing upstream through the Mississippi, Illinois, and Des Plaines Rivers since the 1990s. To prevent further movement up the Illinois River into the Chicago Area Waterway System (CAWS, see Figures 1.1 and 1.2) and possibly Lake Michigan and the Great Lakes ecosystem, an electrical barrier has been operating near Lockport to deter the advance of Asian carp. Although a few individuals have been detected in Lockport pool of the Illinois Waterway, the leading edge of the invasion of bighead and silver carp is considered to be at RM 278 in Dresden Island Pool, 18 miles downstream from the barrier and 55 miles from Lake Michigan, and that front has not progressed upstream since 2006.

Should a sustainable Asian carp population become established in the Great Lakes, native fish populations, as well as many threatened or endangered plant/animal species populations, could be impacted. In response to this threat, the Asian Carp Regional Control Committee (ACRCC) was formed in part to coordinate efforts to understand and organize against the Asian carp threat. The Asian Carp Control Strategy Framework (2012a) outlined major tasks to be completed for a better understanding of factors related to the advance of Asian carp populations towards the Great Lakes. In addition, the ACRCC formed the Monitoring and Rapid Response Workgroup to address Asian carp monitoring and removal (ACRCC 2012b).

Since 2009 environmental DNA (eDNA) has been used to monitor for the genetic presence of Asian carp DNA throughout the CAWS, Des Plaines River, and near shore waters of Lake Michigan. This technique is potentially useful for early Asian carp DNA detection and to identify distribution patterns of DNA in the waterway because it may have potential to detect the presence of DNA in water when fish populations are at very low levels of abundance. A positive eDNA sample indicates the presence of Asian carp DNA and the possible presence of live fish. At present, eDNA evidence cannot verify whether live Asian carp are present, whether the DNA may have come from a dead fish, the number of Asian carp in an area, or whether water containing Asian carp DNA may have been transported from other sources (e.g., translocation by vessels or birds). Furthermore, eDNA cannot at present provide precise, real-time information on where

Figure 1.1. Map of the Illinois Waterway, with lock and dam structures labeled.

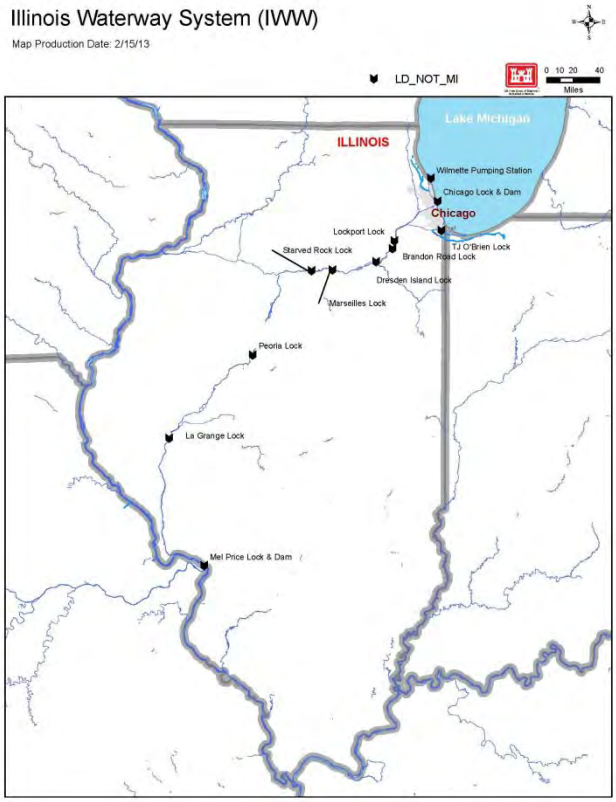
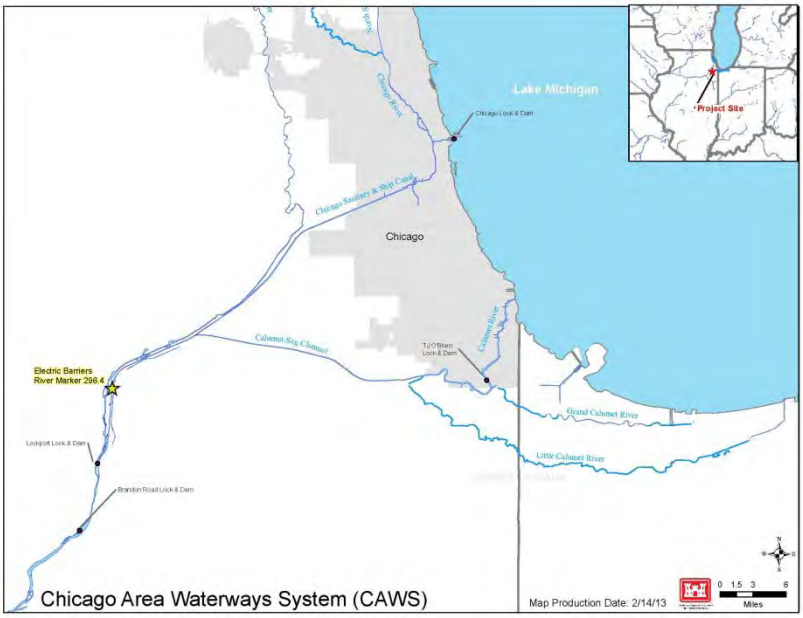


Figure 1.2. Map of the Chicago Area Waterway System. The USACE electrical barriers (depicted by the star) are approximately 37 miles from Lake Michigan.



Asian carp might be due to currently undetermined and likely variable eDNA residence times in aquatic systems, as well as the approximately nine day period required to process samples.

The ACRCC 2011 Asian Carp Control Strategy Framework identifies three specific Action Items relevant to the use of eDNA, including Action Item 2.5.3 (Research on the Impacts of Potential Asian Carp Vectors Being a Source of Fish or eDNA Movement in the CAWS), Action Item 2.6.3 (eDNA Calibration and Increased Efficiency), and Action Item 2.6.5 (eDNA Genetic Marker Development).

The Environmental DNA Calibration Study (ECALS) was developed by a Federal interagency team (US Army Corps of Engineers, US Fish and Wildlife Service, US Geological Survey) and represents a true collaboration between several partners. ECALS will address the three aforementioned Action Items, which represent Goals 1, 2, and 3 of the present study, respectively.

1.2 Project Goals, Objectives, and Products

Goal 1 is to determine the impacts of potential Asian carp vectors being a source of fish or eDNA movement in the CAWS (ACRCC Framework Item 2.5.3). The product of Goal 1 is a report and graphical representation of potential sources and vectors of eDNA within the CAWS and factors that influence eDNA occurrence and transport. This conceptual model should facilitate insights and general qualitative conclusions to help inform discussions about the causes of occasional positive eDNA detections within the CAWS other than live fish having passed upstream of barriers. Based on the ECALS work breakout structure (WBS), the ECALS objectives under Goal 1 are:

- Objective 1.1: Develop conceptual model of most likely possible avenues, aside from actual fish passage of barriers in CAWS, for Asian carp eDNA to be deposited upstream of barriers
- Objective 1.2: Assess Asian carp eDNA prevalence in storm sewers, etc.
- Objective 1.3: Assess the potential for detectable Asian carp eDNA to be transported/deposited via piscivorous bird excrement
- Objective 1.4: Assess the likelihood of eDNA positive hits resulting from the trans-barrier transport of Asian carp carcasses on barges
- Objective 1.5: Assess the role of sediments in eDNA transport.

Goal 2 is to develop high-fidelity, sensitive genetic markers for detecting the presence of Asian carp DNA in filtered water samples based on quantitative real time polymerase

chain reaction (qPCR; ACRCC Framework 2.6.5) or other approaches, such as digital PCR or parallel tagged amplicon sequencing. The current marker used for Asian carp detection gives presence/absence data only using the original assay method. Additional markers will provide the basis for new assay techniques such as qPCR, provide additional supporting evidence for carp presence through testing for multiple markers, and provide additional information about the DNA source (i.e. carp abundance, time since DNA deposition, etc.). The product of Goal 2 is a report describing a set of highly polymorphic mitochondrial DNA (mtDNA) or nuclear DNA (nDNA) markers that provide some degree of inference as to minimum numbers of individual Asian carp responsible for an eDNA sample. Based on the ECALS WBS, the ECALS objectives under Goal 2 are:

- Objective 2.1: Sequence multiple mitochondrial genomes from both bighead and silver carp
- Objective 2.2: Design and test new markers
- Objective 2.3: Develop approach for detection of multiple alleles.

Goal 3 is to better understand the relationship between the number and distribution of positive Asian carp eDNA detections and the density of Asian carp at a location (ACRCC Framework 2.6.3). The products of Goal 3 include a robust protocol for rapid extraction and analysis of eDNA samples; detailed conversion of the current PCR band-based (i.e., presence-absence) assay to more informative and efficient assays; an optimized water sampling protocol; a series of relationships between Asian carp biomass, number, and behavior and eDNA detection using PCR including rate and extent of dispersion of Asian carp eDNA in both non-flowing and flowing waters; the relationship between environmental factors (water temperature, light exposure, planktonic/microbial biomass, water turbulence, dissolved oxygen, total organic carbon, and pH) on eDNA degradation rates systems; a set of experimentally validated expectations for detection of carp DNA from point sources, such that sampling efforts can be planned with reasonable expectation of obtaining independent samples (not from same eDNA plume); complete description of demographic characteristics (size, biomass, sexual maturity), collecting techniques, housing, and feeding of the fish for use in the methods and materials of all tests completed (including a protocol for procedures using live fish in laboratory and pond settings, which will be submitted to the Institutional Animal Care and Use Committee for approval or modification); an updated/expanded hydrodynamic model of the CAWS for use as the basis to transport eDNA in the system, including influence of barges and the electrical barrier; and a model to estimate the probability that each of the potential sources of eDNA in a water body is, in fact, an

actual source of eDNA in that water body, and derive the probability that an Asian carp population is present in that water body above the monitoring location. Based on the ECALS WBS, the ECALS objectives under Goal 3 are:

- Objective 3.1: Increase the efficiency and throughput of eDNA processing
- Objective 3.2: eDNA calibration guidance studies
- Objective 3.3: Fish supply
- Objective 3.4: Hydrodynamic model
- Objective 3.5: Probabilistic model.

Goal 4 is project management, with products including progress updates, team workshops, technical reporting, project management plan development, and project communications.

The purpose of this second interim report is to provide results to date from the ECALS. It does not include details on the scope, schedule, or budget for the individual tasks that fall under the objectives above. Those details can be found in the Project Management Plan.

1.3 Genetic Marker and DNA Processing Terminology

In this report a number of genetic markers and analytical procedures are presented, which might be confusing to the reader; the following discussion is intended to provide clarity.

Prior to ECALS, individual markers were developed for silver and bighead carp by researchers at the University of Notre Dame (e.g. Jerde et al. 2011). Their methods were based on conventional PCR (cPCR) analysis in which the presence or absence of eDNA is determined by gel electrophoresis (i.e. the quantity of eDNA cannot be determined). These markers using cPCR have been used for eDNA monitoring in the CAWS since 2009, also known as the "QAPP method". The Quality Assurance Project Plan (QAPP) for the Environmental DNA (eDNA) Monitoring of Invasive Asian Carp in the Chicago Area Waterway System (CAWS) outlines the detailed procedures for the planning, collection, filtering, processing (with cPCR) and reporting of eDNA samples and will be refined periodically. This document (USACE 2012), which has undergone a technical review by scientists at Argonne National Laboratory (e.g. Battelle 2010), is the result of collaboration between USACE biologists and geneticists and builds upon the initial protocols developed by researchers at the University of Notre Dame.

During the course of the ECALS project, new approaches have been utilized with respect to eDNA collection, processing, and analysis (Table 1.1). Two new approaches utilized extensively in ECALS deserve attention here. First, the use of centrifugation (rather than filtration) to concentrate eDNA samples has been used in experimental trials to speed up the analytical process. Second, a new silver carp-specific marker was developed by ECALS researcher Dr. Jon Amberg (UMESC) specifically for use with real-time quantitative PCR (qPCR). Note that these approaches are not approved for the QAPP at this point in time.

In ECALS if a water sample is collected, processed by filtration, and analyzed for the Notre Dame marker using cPCR, the technique can be referred to as the "**QAPP method**". Otherwise for each experimental trial presented in this report, we will describe the 1) sample media, 2) processing method, 3) marker used, and 4) PCR method.

Table 1.1. ECALS approaches used for DNA analyses*

Sample Media	Sample Processing	Genetic Marker	Analysis
water; fish tissue, feces, scales, gametes, or slime; bird feces	filtering, centrifugation, sieving	Univ. of Notre Dame, UMESC	cPCR, qPCR

*This list is not comprehensive, but encompasses the majority of ECALS work

Finally, notice that there is a distinction made between eDNA and DNA in this report. We define eDNA as Asian carp DNA that originates in a waterbody, whereas we define DNA as any other media from which the Asian carp DNA originated. For example, if we took a water sample from an experimental trial Asian carp tank, we are sampling for eDNA. If we sample tissue directly from an Asian carp carcass, we are sampling for DNA. Restated, the media determines the terminology: eDNA from a water sample, DNA from any other media in Table 1.1.

2 Asian Carp eDNA Vectors

In addition to DNA shed by living Asian carp, there are alternative vectors that might transport Asian carp eDNA into and within the CAWS. These alternative vectors are the focus of ECALS Task 1. Initially, experts in various pertinent fields were tasked with identifying potential eDNA vectors. Laboratory and field trials have followed up on those vectors deemed most likely by experts. These trials will determine whether detectable eDNA[1] can actually be transported by the proposed vector and whether or not it seems likely that eDNA detected in monitoring samples could have been moved upstream of the electrical barrier by that vector.

2.1 Conceptual Model

The conceptual model will describe potential sources and vectors of eDNA within the CAWS and the processes that influence the transport and occurrence of eDNA in monitoring results. The conceptual model will be presented in the form of a graph (Koller and Friedman 2009). The graphical model structure provides a useful technique for decomposing complex systems. Therefore, the conceptual model will assist the ECALS project team to synthesize the results of the ECALS study, evaluate the role of Asian carp and other vectors as potential sources of eDNA detected in monitoring samples, and develop qualitative explanations for the pattern of eDNA detections in the CAWS. Graphical models are also a useful medium for communicating about complex systems to lay audiences. Therefore, the graphical model will also assist the ECALS team to communicate results of the ECALS study to the public and explain how Asian carp DNA may be released and transported within the CAWS. An expert workshop was held in November, 2011, to help inform development of the conceptual model.

2.1.1 Expert Workshop

Members of the ECALS project delivery team (PDT) convened a workshop of over 30 disciplinary experts and relevant stakeholders on November 17, 2011 in Chicago, IL to discuss alternative eDNA vectors. Areas of expertise included birds, DNA in aquatic environments, carp, barges, fish markets, forensics, lock and dam operations, as well as representatives from local, state, and federal agencies and the shipping industry.

[1] Detectable eDNA refers to eDNA that is detectable via polymerase chain reaction (PCR). Detection by PCR will largely be determined by the amount and strand integrity of the eDNA.

Facilitated morning and afternoon breakout sessions divided the participants into two groups and posed five questions, which will be discussed in turn in this report. At the end of the day all attendees convened in one room and breakout group representatives summarized their results, followed by group discussion.

The following text of this section represents feedback from the workshop participants and does not represent ECALS conclusions.

Question 1: What are the potential sources of eDNA in Chicago-area water bodies?

Four potential vectors were dismissed after discussion during the workshop. Barge ballast water was not deemed a significant source in the CAWS because barges in the CAWS do not typically intake and output ballast water (supported by results from a USGS study on the issue). The only potential location that might be considered is a low railroad bridge located well within the CAWS, but barges would likely (if needed) take on ballast prior to that bridge and release that ballast immediately after passing under that bridge. The only way eDNA in water could effectively enter a barge is if there was a large hole in the side of a barge; however, the barge would not be allowed to enter the CAWS with such a hole due to inspection by authorities at the barrier. The three other vectors dismissed were ceremonial prayer release (an intentional release of a live fish for religious purposes), overland boat transport from a water body containing Asian carp (unlikely), and flow reversal in the canal (would likely only have an influence about ½ mile above the electrical barrier). Note, however, that ECALS trials in 2012 (Section 2.1.3) demonstrated that overland boat transport has the potential to bring eDNA into the CAWS.

Fourteen additional major eDNA vector categories were identified during the workshop; a brief review of each follows (order does not indicate importance).

- **Animal Feed or Fish Meal**

The use of Asian carp in the production of animal feed or fish meal may occur, with DNA passing through animals prior to entering the CAWS via runoff and/or sewers. It was mentioned during the workshop that a very small percent of Asian carp is used at fish meal processing plants, and meal is not likely to end up in the CAWS. Cat food would not likely have Asian carp in it because carp have intramuscular bones which are known to pose choking hazards for cats. Use of Asian carp for livestock feed (e.g. pigs, chickens) and/or dog food may be possible but was unknown to workshop participants.

Additional Questions Posed: Can Asian carp DNA survive the manufacturing process? Which companies in the region use Asian carp and how much? What is the likelihood that feed/meal-derived DNA reaches the CAWS via livestock facilities or pet excrement? Would enough DNA enter the CAWS via this vector to be detected at monitoring points?

- **Asian Fish Markets**

The possibility exists that eDNA is entering storm drains in the CAWS near fish markets that sell Asian carp. Bighead carp is more common in markets than silver carp. Fish are often displayed and/or stored on ice, but during the day as the ice melts there is a need to replace the ice. The melted slushy ice may be dumped onto streets/parking lots and enter the storm sewer system which leads to the CAWS.

Additional Questions Posed: Where is the origin of the Asian carp in the fish markets? Are there any diagnostic genetic markers associated with potential source populations? Which storm sewers drain areas with fish markets? Is eDNA present within the sewers and how long can it remain detectable? Is there any detectable pattern of positive eDNA hits in the CAWS upstream or downstream from storm sewers draining fish markets or any pattern of water flow from storm sewers during/prior to the collection of samples with positive hits? Is the hit bighead or silver carp? Are relatively large fish parts being deposited into the sewer system?

- **Bait Trade**

Asian carp is popular for use as bait by trappers because it is inexpensive. A large amount of fish bait is needed for raccoons with lesser amounts for turtles. Anglers may also be using Asian carp for cut bait, with cleaning and disposing of cut bait directly into water bodies. Related pathways include bait shops (tested for eDNA by Illinois Department of Natural Resources and Notre Dame; no positive hits), live wells, contaminated trailers, and disposal of angler-caught fish. Trapper/angler surveys in the CAWS might be useful, as well as checking with Illinois Department of Natural Resources because trappers need to be licensed.

Additional Questions Posed: Where in the CAWS are people trapping/fishing? What bait are they using and where is the bait's origin? Is the CAWS a suitable fishing location?

- **Barges and Boats**

Barge-associated activities may transport Asian carp DNA across the electrical barrier via residue (slime) on sides or hulls (i.e. fish banging against the boat, leaving skin tissue), tires hanging off the sides of barges, carcasses on decks (i.e. live fish leaping

onto decks below the barrier; carcasses being kicked off into CAWS later), and entrainment in propeller wash. Open barge cargo (e.g. coal, wood chips, mulch) may get contaminated by leaping fish as well. Recreational boat traffic may possibly transport DNA in live wells, bilge water, or on hulls.

Additional Questions Posed: How often do any of these potential events occur? How much carp slime is on hulls? Where are the heavy barge traffic areas? Are there lots of eDNA detections in barge staging areas or other barge traffic areas? Where are the recreational fishing locations and during what periods?

- **Birds and Other Fish Predators and Scavengers**

Fish-eating birds and/or animals may be eating Asian carp and defecating or regurgitating in the CAWS, or birds might also carry fish and drop them or eat them within the CAWS, or transport water contaminated with DNA in their feathers. Many studies have shown that it is possible for DNA to pass through a mammal's digestive system and recent studies have used DNA to study bird diet. DNA is expected to be expressed in bird and other predator feces within 6 to 8 hours post consumption. Examples of mammal scavengers include raccoons, skunks, and feral cats. Domestic cats and dogs excreting in parks were not deemed likely sources. Noted piscivorous birds in the CAWS were cormorants, pelicans, terns, eagles, great blue herons, and osprey. Rookery locations, home ranges, migration periods and routes, and distance from Asian carp spawning areas are important considerations for consideration of bird vectors.

Additional Questions Posed: What are the mammal scavenger movement patterns, especially near Asian fish markets?

- **Des Plaines River**

The Des Plaines River and its tributaries have a small Asian carp population which may be a source of eDNA to the CAWS via flooding/overflow, pumping, or cracks in the bed-rock. Overbank flooding from the Des Plaines River to the CSSC occurs most years and may be a pathway for eDNA to the CAWS. The transfer of eDNA through fractures in the bedrock has been evaluated by a USGS study (report in review) in the area and is considered unlikely.

- **Bottom Sediments**

There is the possibility that a pre-existing reservoir of Asian carp DNA exists in the CAWS bottom sediments. The origin of any eDNA attached to these sediments may come from any of the other sources and vectors. Cold and anoxic conditions could

preserve DNA for a while. Disturbance of the sediments would move sediment-associated DNA into the water column. Suggested disturbances include barges stirring up the bottom, CSO events, and dredging. It was noted that minimal dredging occurs in the CAWS.

Additional Questions Posed: Is there information on the transfer of dredge spoils from Asian carp affected areas? What is condition of the sediment (settled DNA stirred up from turbulence may introduce a signal that is not representative of resent Asian carp presence)? What is rate of burial under sediments?

- **Fertilizer**

Asian carp may be used to manufacture fertilizers, but the extent was not known to workshop participants. The ability of DNA to remain detectable after processing into fertilizer is also unknown.

Additional Questions Posed: Are there fertilizer manufacturers using Asian carp in the region? Does DNA survive the manufacturing process? Where is Asian carp-based fertilizer being used in the CAWS region (e.g. golf courses, community gardens) and how much is being used? Can runoff from such locations effectively reach the CAWS?

- **Gear Contamination**

Fisheries gear (boats, nets) from natural resources agencies, contract fishermen, recreational anglers may be exposed to DNA and brought into the CAWS where some DNA could be sloughed off into the water. The extent to which these possible sources contribute to eDNA is unknown.

- **Human Transport**

Human transport of Asian carp (live or dead) into the CAWS may or may not be intentional. Intentional transport of live Asian carp into the CAWS with the intent of 1) establishing a population for personal consumption, or 2) having the fishes' presence prompt closing of the canal, is possible. While one could look at criminal records of environmental activists to explore this possibility, there is no information we can get to clarify an eco-terrorist as a source (i.e. connect them with a given fish).

- **Improper Fish Disposal**

Consumption of Asian carp in the CAWS region may occur in restaurants and/or private homes. Disposal of fish remains into dumpsters or landfills may be possible routes of

transport of DNA to the CAWS. The frequency of Asian carp consumption in CAWS-area homes and local restaurants is not known.

- **Live Fish**

The possibility exists that live Asian carp are bypassing the electrical barrier upstream of Lockport. Karst cracks through which small fish could pass are localized in the canal, making that pathway unlikely. If a positive eDNA hit occurred near the electrical barrier, one could sample for live fish.

Additional Questions Posed: Does suitable carp habitat exist in the area (including spawning habitat)? What is the range of larval Asian carp in the area?

- **Outfalls**

Outfalls other than those near Asian fish markets may also be sources of eDNA.

Combined Sewer Overflows (CSOs): CSO events may flush out DNA already present in storm sewers. Is there residual DNA present within the sewers which might be washed out during outfall events? What areas drain to what sewers? How much water does it take to make storm sewers flow into the River? When did last CSO event occur? Did something unusual cause the CSO event? How much water does it take to cause a CSO event?

Processing Plants: Storage areas, wash-off areas, and waste operation areas of manufacturers who utilize Asian carp may be a source of eDNA in the CAWS.

Wastewater Treatment Plants: There are three wastewater treatment plants with outfalls to the CAWS, none with tertiary treatment. The possibility exists that DNA may enter a wastewater treatment plant after a CSO event. It is unknown if DNA can remain intact after passing through a wastewater treatment plant. Previous work by Notre Dame researchers detected no eDNA at the source. Targeted sampling may address questions related to this potential pathway.

- **Stock Ponds**

Ponds for recreational fishing have been stocked in the past with catfish, but may have unintentionally included bighead carp. These fish are now typically very large suggesting they've been there a long time. Records, if any, would likely be poor. Grass carp have been and continue to be stocked in golf courses; Asian carp may be unintentionally

stocked here as well. Runoff from stock or golf course ponds may occur during flood conditions, transporting eDNA to the CAWS.

Additional Questions Posed: Where are stock ponds in the CAWS? What is their drainage connection to the CAWS? When are runoff events occurring from these areas?

Question 2: What factors might influence the persistence of eDNA in the water column?

Responses generally fell into 4 categories: degradation due to environmental conditions, transport-related issues, concentration of the DNA source, and seasonal effects.

- **Environmental Degradation Factors**

Factors associated with eDNA degradation are quite numerous in the CAWS, but a number of major categories emerged from the workshop including temperature, ultraviolet radiation exposure (and influence of turbidity), cell disrupting factors (e.g. soaps or detergents, enzymes, toxics, reactive chemicals), buffering capacity (pH, alkalinity), thermal stratification, dissolved oxygen (e.g. aerobic vs. anaerobic, biochemical oxygen demand), biological activity (DNA bioavailability, microbial community), chloride and conductivity, pharmaceuticals, DNA binding (e.g. sediments, DNA-masking chemicals, organic content), and release from sediments (methane and other gas releases, microbial community).

- **Transport-Related Factors**

Movement of eDNA into and throughout the CAWS can be influenced by wind (blowing, dispersing, aggregating surface films) and water (flow rate, direction, turbulence due to flow rate and boats).

- **DNA Source Concentration**

The quantity of DNA released depends in part on the form released, and includes digestive tract lining, blood, slime, scales, milt, urine, feces, and larger tissue pieces. These sources of DNA are size-related, ranging from naked DNA to cells to larger chunks of tissue. An additional consideration is the release location of the DNA which may include the water surface film (e.g. organic floatables), material suspended in the water column (free-floating fish parts, attached to sediments), and material that sinks to the bottom.

- **Seasonal Effects**

The rate of eDNA input and detection to the CAWS depends in part on factors that vary temporally. Examples include source input (e.g. barge traffic), seasonal changes in ability to collect samples, sewer overflow event variation, fish behavioral differences, and piscivorous bird migration periods.

Question 3: What factors might influence the ability to detect eDNA at a particular sampling location?

This question was addressed in both field and laboratory contexts.

- **Field-Related Issues**

Items noted were sampling location (water, sediment, river banks) and frequency; sampling protocol including time of day and skill of the field technician; weather and flow conditions; fish behavior (e.g. spawning season); and water quality conditions.

- **Laboratory-Related Issues**

Upon field collection, many factors can influence the ability to detect eDNA in the laboratory:

Post-sampling/pre-analysis. Field handling, processing, and transport to analytical laboratory may cause sample contamination or decrease detection ability in the laboratory (e.g. improper filter paper handling, temperature, and storage; delays in transport).

Initial quantity of DNA in the sample. Excessive quantities of DNA in the sample, such as that potentially associated with abundant plankton loads, might inhibit the extraction process (e.g. massive amounts of DNA at a sewage plant, big tissue mass vs. filtered sample, interferences due to the presence of PCR-inhibiting secondary compounds associated with algae (e.g. chlorophyll)). The presence of very low concentrations of eDNA presents an issue of the PCR method's ability to simply detect the eDNA. Large numbers of samples to be analyzed may be an issue because of longer storage times and associated potential sample degradation.

PCR methodology in the laboratory. Different laboratory protocols may result in different abilities to detect eDNA.

Presence/absence of various inhibitors. Examples of inhibitors include lignins, tannins, humic acids, sewage, gut and fecal materials (e.g. bile salts), chlorophyll, and just about anything that binds to DNA.

Issues related to eDNA markers. The use of eDNA markers presents additional challenges in laboratory analysis, including whether the DNA is nuclear or mitochondrial, base pair length, cross-species reactivity, and method sensitivity and specificity.

Question 4: Given a positive eDNA detection result, what information would you seek to influence your belief that any one potential source of eDNA is the actual source of eDNA?

High Importance

- Ability of DNA to exist in a potential vector source
- Persistence of DNA in vector
- Quantity of DNA present within vector
- Documented observation of Asian carp at sample location (from reliable sources)
- Actual capture of Asian carp at location
- Genotype information on potential source population (if such diagnostic capabilities emerge).

Moderate Importance

- Distance of potential sources from point of detection
- Frequency of potential releases of DNA by potential vectors
- Environmental conditions (e.g. water chemistry, hydrology and hydraulics, ultraviolet radiation, rain events).

Low Importance

- Sediment dynamics and potential influencing variables
- Information on past sampling events.

Question 5: Consider each potential source of eDNA separately. Explain why this information might influence your beliefs about the source of eDNA. Explain how your beliefs might change in response to the range of potential results of an investigation.

- There could be multiple sources of Asian carp influence at each site, and each site should be considered dynamic.
- Need to consider data on many different vectors.

- Multiple vectors can contribute to the presence of eDNA and there is a probability for the presence of eDNA in sites normally inaccessible to the Asian carp.
- Might want to consider more sampling, even in areas that might not have had carp presence in the past.
- May be most logical to assess the top contributing vectors and based on their presence, and attach a probability to the detection of Asian carp at particular sites.
- May want to consider the use of RNA in future assays.
- Sampling method may be important.

2.1.2 Current Efforts

The ECALS project team is currently evaluating and synthesizing the information that has been learned through the ECALS studies and other ACRCC research initiatives. New lines of evidence that have the potential to inform the interpretation of eDNA monitoring results are also being identified based on insights that have been gained during the course of ECALS studies. This information will be integrated in a conceptual model that is scheduled for completion in August 2013. The methods being used to develop the conceptual model are described by Pearl (1988), Pearl (2000), and Koller and Friedman (2009). These methods have been widely used in the field of artificial intelligence and have also been used to analyze a wide variety of different problems in environmental science (Schultz et al. 2011). The conceptual model is a graph in which the nodes represent random variables and the edges denote influence of one random variable on the state of other random variables. This graph will provide a structured way of thinking about the potential sources and vectors of eDNA as well as the factors or variables that influence release, transport, persistence, and detection of eDNA in the CAWS. In particular, the graph will reveal how knowledge about observable variables or characteristics within the system might influence beliefs or knowledge about unobservable variables within the system. A report will describe each random variable, the topology of the graph, and the information that would be needed to parameterize each node. Parameterization is a process by which the conceptual model is converted to a quantitative model.

2.2 Alternative Pathways

Activities focused on four alternative pathways for eDNA; storm sewers, fertilizers, boat hulls, and sampling gear. These four pathways were chosen in part as a result of the November 2011 Vectors workshop (see section 2.1 above).

2.1.1 Storm Sewers

In October 2011 and June 2012 we executed trials to demonstrate that ice from ice chests holding Asian carp carcasses could be a source of eDNA in the CAWS. Asian carp that are transported to Chicago area fish markets are transported as carcasses on ice and the ice (and ice water) is dumped into storm gutters and down drains in the street. Because fish may be displayed on ice at these markets during the day, change-out of melting ice (potentially multiple times during the day) may supply additional amounts of ice/ice water to the storm sewer system.

Methods

In an October 2011 laboratory trial, 10 ice chests (volume) were half-filled with ice from ice machines at ERDC. One Asian carp head (either silver or bighead carp) was placed in each chest. Chests were set outside and moderately shaken once every hour until roughly half the ice appeared to be melted. One liter of the "fishy ice" water was collected, and 500ml were filtered through a 934-AH glass microfiber filters following the QAPP method. DNA was extracted by following the manufacturer's protocol for the MoBio PowerWater™ DNA Isolation Kit. The resulting water was sampled and assayed for Asian carp eDNA using cPCR. The non-quantitative PCR assay is based on Jerde et al. (2011) and is described in the Asian Carp monitoring QAPP (USACE 2012). This PCR assay produces an amplicon (or PCR product; also referred to as the "marker") that is observed as a band on an agarose gel and then, if putatively detected, is confirmed as Asian carp DNA using DNA sequencing. Separate PCR assays are run for the bighead and silver carp markers.

In a June 2012 field trial, carp fillets were placed on ice in a chest cooler (Figure 2.1). Once the ice had partially melted (~ 50% melted), the carp fillets and any discernible tissue chunks were removed and the ice was poured through a manhole into a storm sewer at a location approximately 75 m from a sewer outfall into the CAWS. Water from a fire hose was then used to flush the water and ice out of the sewer outfall. *Prior* to the ice dump, water from the fire hydrant was used to flush the sewer, with hydrant water being run until that water had run from the outfall for several minutes. Two-liter eDNA samples were taken from the CAWS from within small area near the outfall pipe before any activities, directly from the sewer outflow during the initial system flush, directly from the sewer outflow during the ice flush, and from the same area one day, one week, and two-weeks after the ice flush. Samples were assayed at ERDC. The resulting water samples were assayed for Asian carp eDNA using the QAPP method; extra sample material has been saved for additional assays with qPCR and stair-step markers.

Figure 2.1. Storm sewer experiment trials (top row, left to right): Asian carp tissue chunks in ice (tissue removed prior to deposition in sewer); ice slurry deposited into sewer; (bottom row, left to right) fire hose used to flush system before and after slurry deposition; water collected from outflow at entrance to river from boat.

Results

For the October 2011 laboratory trial, all 10 samples exhibited very strong DNA bands and were confirmed via DNA sequencing. All control samples were negative for Asian carp DNA. Results from the June 2012 field trials are presented in Table 2.1. The most interesting outcomes were that Asian carp DNA was apparently already found in the sewers in considerable abundance and that the "signal" in the receiving waters appears to have dissipated within a week. Whether the signal was lost due to degradation, dilution, or flow is unclear. Because other potential sources of Asian carp eDNA were not under study control, it should be noted that also it is possible that the 5 positive hits

detected one day after the flush could have originated from a different source than the "fishy ice" we flushed through the sewer.

Table 2.1. Results from June 2012 storm sewer trials

Sample	**June 2012**
Pre-Activities	1 of 40 samples positive for Asian carp
Initial Flush	10 of 40 samples positive for Asian carp
Ice Flush	29 of 40 samples positive for Asian carp
1-hr Post-flush	2 of 40 samples positive for Asian carp
1-day Post-flush	5 of 40 samples positive for Asian carp
1-week Post-flush	0 of 40 samples positive for Asian carp

It has been demonstrated that ice associated with transport and sale of Asian carp could contain large amounts of DNA and that it can travel through the sewers. The detection of silver carp DNA in water flushed through the storm sewer before any DNA was added by the study team indicates that other sources, potentially fish markets, can be sources of eDNA in receiving waters. The prevalence of Asian carp DNA in storm sewers emptying in the CAWS is unknown. The frequency with which storm sewers deposit material (largely in conjunction with heavy precipitation), has not been quantified.

2.1.2 Fertilizers

In October 2011, two brands of fertilizer based on liquefied Asian carp tissues were tested for the presence of detectable DNA. The two brands were:

- Schafer Liquid Fish Fertilizer (Schafer Fisheries, Thomson, IL), http://www.schaferliquidfish.com/

- New Life Super Soil Booster (New Life, Bristol, IN), http://www.newlifesoil.com/index.php

We were able to filter and test volumes of both fertilizers ranging from 4.2 – 7.5 ml. Protocols for assaying the fertilizer for DNA followed the QAPP method, including filtering of diluted fertilizer (1.5 μm glass-fiber filters). No positive Asian carp detections resulted from these assays. However, the volume of fertilizer we tested was very small – for example, we tested the same volume of the New Life Super Soil Booster that would be applied to only 39 ft^2 of lawn. Significantly larger volumes could not be filtered within reasonable time frames (8 hours required to filter 7.5 ml of soil booster) and we are currently unaware of any protocols or kits that allow for efficient DNA extraction from very large volumes of viscous liquid.

It is apparent that, based on the batches tested, neither brand of fertilizer contained high concentrations of detectable DNA. If Asian carp material were used in the production of the tested batches, either the DNA was degraded during processing or inhibitory substances prevented PCR detection. We are currently requesting information from both producers regarding the inter-batch prevalence of Asian carp in the fertilizers and testing for the effects of PCR inhibitors on samples assayed using our protocols. Tests of larger volumes of fertilizer would also provide stronger evidence for or against these fertilizers being vectors for eDNA, but, as stated above, feasible methods for extracting and purifying DNA from significantly larger volumes of fertilizer have not been identified.

2.1.3 Boat Hulls and Fishing Gear

Fisheries gear (boats, nets) from natural resources agencies, contract fishermen, recreational anglers may be exposed to DNA and brought into the CAWS where some DNA could be sloughed off into the water. The potential for these sources to enter the CAWS and result in a positive eDNA detection was evaluated by ERDC personnel in October 2012.

Methods

To determine if DNA can attach to and be spread by vessel hulls and fishing gear (e.g. nets) ERDC personnel collected 16 sets of samples in the CAWS from commercial fishing boats and government boats (Figure 2.4). Each sample consisted of 10 filter paper swabs of boat hulls (bottom half of hull, typically). Boats had a varied history of having been in waters with Asian carp, from boats that had been in such waters on the

previous day to not having been in such waters for 2 weeks. Some boats had been steam cleaned prior to being sampled. Some boats were sampled on consecutive days – these were involved in daily fishing or other activities in waters containing Asian carp. Also, at the end of the week, a sample (10 swabs) was taken from a boat that had traveled from waters with Asian carp for 9.5 miles distance in waters believed to free of living Asian carp (confirmation needed). Swab samples were then shipped to ERDC for cPCR DNA assays using the QAPP markers.

Additionally, ERDC personnel took 9 eDNA samples from 5 gallons of distilled water in which portions of nets used to capture Asian carp were rinsed. Two liters of water were "grabbed" from each tub and the QAPP method was followed.

Silver carp DNA was detected in 14 of 16 samples from boats and bighead carp eDNA was detected in 11 of 16 samples. The sample from the boat driven in putatively Asian carp-free waters was also positive for silver and bighead carp. All net samples showed very strong positive results, but several negative controls from the net sampling (2 L water grabs from tubs prior to net rinsing) also exhibited positive results, indicating some field contamination of samples. However, as observed on gels and measured using the UMESC qPCR marker, the levels of DNA detected in controls was very minor compared to large amounts of DNA associated with net samples (Figures 2.2 and 2.3). In 2013, new sampling will be conducted in order to provide additional results with a greater emphasis on quantifying DNA yields per area of net and change in amount of detectable DNA over time.

The results show that vessel hulls have considerable amounts of adhered DNA, that the DNA can persist for days, and that the DNA is not removed by overland transport. The DNA also does not appear to completely, quickly wash away as boats move through the water. Thus, vessel hulls can be vectors for DNA movement. Nets appear to be sources of very large amounts of DNA, but confirmation will require another set of samples and a clean trial.

Figure 2.2.Test results for bighead carp DNA on commercial fishing nets.

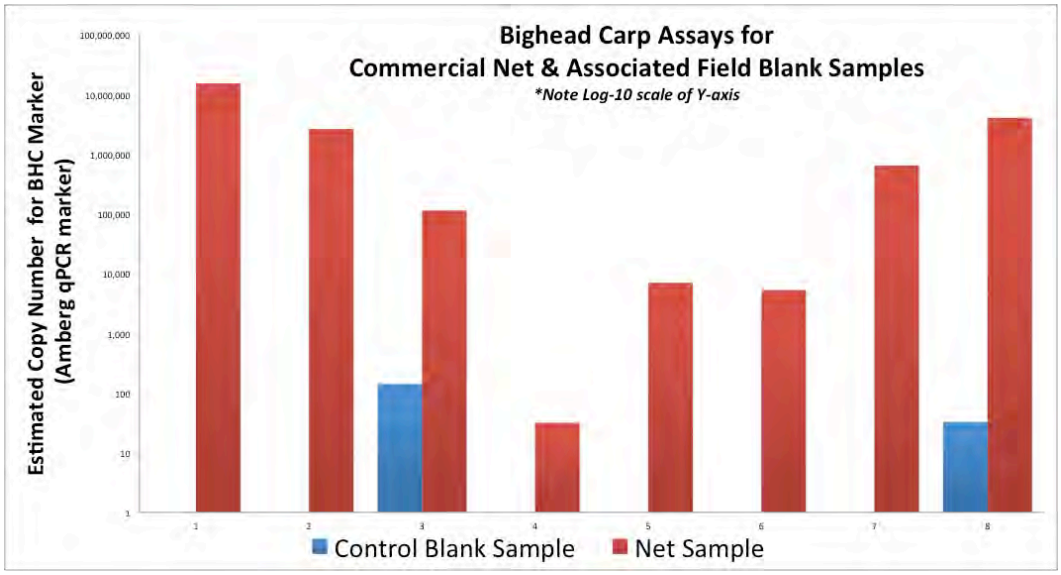


Figure 2.3. Test results for silver carp DNA on commercial fishing nets.

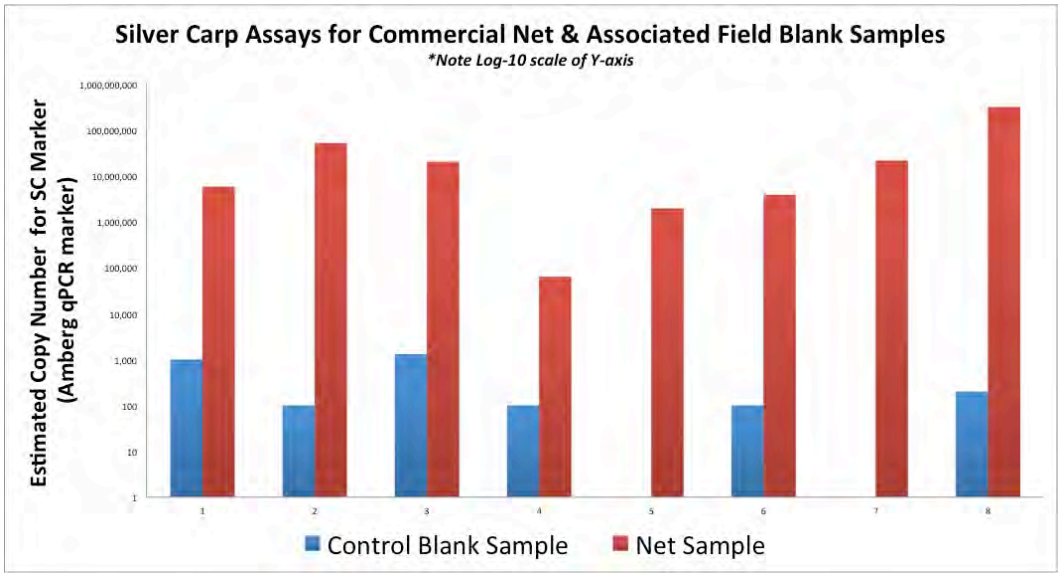


Figure 2.4 USACE field researchers testing commercial fishing boat hulls for the presence of Asian carp DNA

2.3 Bird Transport and Deposition

Overview

Scientific papers demonstrating that eDNA can be detected in the excrement of birds were identified by the ECALS team (Deagle et al. 2010; Doehm et al. 2011; Sutherland 2000). The assumption has been that eDNA is deposited by piscivorous birds and the ECALS subtasks are largely focused on the amount of eDNA in a bird fecal sample, its degradation properties, and piscivorous bird feeding and movement patterns in the Chicago region. The studies described in sections 2.3.1 and 2.3.2 confirm the capacity for piscivorous birds to be a direct vector of Asian carp DNA or to contaminate fomites (e.g. barges, boats) with Asian carp DNA in their fecal deposits. Silver carp DNA was detected in fecal samples collected from piscivorous birds offered one to three meals of silver carp. Silver carp DNA could be amplified from bird fecal samples collected up to 1

week following consumption of a silver carp meal. Silver carp DNA in fecal material deposited on metal sheets persisted for 30 days under ambient environmental conditions despite exposure to temperatures exceeding 60°C. Taken together, these findings suggest that the potential exists for Asian carp DNA to be distributed from areas where Asian carp are abundant to areas where Asian carp are not present or abundant through direct (e.g. direct deposition of feces into the water by piscivorous birds after consuming a meal of Asian carp) or indirect transfer (e.g. deposition of bird feces containing Asian carp DNA on a fomite such as a barge or boat).

2.3.1 Passage of DNA through Piscivorous Birds

During the summer of 2012, three trials were performed by UMESC personnel to assess the passage and persistence of Asian carp DNA in piscivorous birds after consuming silver carp. The first objective of these trials was to determine if silver carp DNA can be detected in fecal material from piscivorous birds following a meal of silver carp. Silver carp were used as a food for the birds rather than bighead carp since the current silver carp marker is thought to be more sensitive than the marker for bighead carp. Additionally, silver carp DNA has been detected using eDNA but no live fish have been captured at date, while little to no bighead carp DNA has been detected, but a live fish has been captured. The second objective, assuming DNA is detected, was to determine the number of days after consuming a silver carp that DNA could be detected in the feces from the bird.

Methods

In the first trial, four bald eagles (*Haliaeetus leucocephalus*) at the National Eagle Center (Wabasha, MN) were offered only silver carp until apparent satiation on a single day. Eagles in the study then returned to their normal diet consisting of freshwater drum, channel catfish and rats. Each day, plastic mats were placed under each bird's perch. Birds were allowed to defecate on these mats for approximately 24 hours. Fecal material for each bird was collected from these mats and placed into 50-mL centrifuge tubes. Tubes containing fecal material were immediately placed on wet-ice during transport (~2 hours) to the Upper Midwest Environmental Sciences Center (La Crosse, WI) where they were stored at -80°C. Fecal material from each bird was collected once daily for 7 days after the bird consumed silver carp. Fecal samples collected from each bird in the study during the 24 hours prior to feeding silver carp served as control material.

Trials 2 and 3 were performed in collaboration with the Brookfield Zoo (Brookfield, IL). In trial 2, five white pelicans (*Pelecanus erythrorhynchos*) were fed increasing amounts of silver carp over three days and then returned to a diet of marine fishes on the fourth and subsequent days of the study. Pelicans were cohabitated with unrestricted movement in a single outdoor habitat. The outdoor habitat contained a pond supplied with municipal water. Non-zoo animals (e.g. Canada geese, gulls, etc.) had access to the pelican habitat, including the pond.

In trial 3, two double-crested cormorants (*Phalacrocorax auritus*) and five white ibis (*Eudocimus albus*) were fed silver carp in increasing amounts for three days and then returned to a diet of marine fishes on the fourth and subsequent days of the study. Cormorants and ibises were cohabitated in an indoor habitat; other wildlife was excluded from the habitat. One cormorant was held in quarantine from days 2 through 7 of the study due to an illness unrelated to the study. Prior to quarantine, this bird had consumed one meal of silver carp. The pond in the cormorant/ibis habitat was drained and bleached on Day 8 as part of routine maintenance and not part of the study design. Water samples collected on all days prior to draining, then immediately before draining on day 8 and then again on day 9.

Fecal (n=15 for trials 2 and 3) and water samples (n=15 for trials 2 and 3) were collected daily for nine days post-feeding. Fecal samples were collected by passing a buccal swab through a <24-h old fecal deposit; each swab was in contact with a single fecal deposit though it is not known whether the fecal deposit was the result of a single bird. After contact with a fecal deposit, each swab was placed in an individual 2.0-mL centrifuge tube and stored at -80°C. Samples were centrifuged rather than filtered because of the extremely high algal and suspended solids content of the water. It was determined during methods development that a 2 L water sample from these ponds would require 20 – 40 filters and more than 1 h per sample processing. Fecal samples from the cormorant held in quarantine were collected daily and immediately stored at -80°C. Fecal samples from this cormorant were processed as described for Trial 1.

DNA from approximately 100 µL of liquid fecal material and water sample collected were extracted using the DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. The presence of silver carp DNA was determined by conventional PCR (cPCR, QAPP) using the current silver carp-specific markers (CNT forward-CCTGARAAAAGARKTRTTCCACTATAA; CNT reverse-GCCAAATGCAAGTAATAGTTCATTC) and the following thermocycling program: 94°C for 2 minutes followed by 45 cycles of 94°C for 1 minute, 50°C for 1 minute and 72°C for

1.5 minutes. Conventional PCR reactions (25 µL) were comprised of: 12.5 µL MangoMix (Bioline USA, Inc., Tauton, Massachusetts, USA), 0.3 µM of each primer and molecular grade water. Primers were synthesized by Invitrogen, Inc. (Carlsbad, CA USA). PCR products were then visualized by electrophoresis in a 2% agarose gel, stained with GelRed™ (Biotium Inc., Hayward, CA, USA) and presence of the appropriate band visualized under ultraviolet light. All samples were run in duplicate.

The presence of silver carp DNA was also determined using real-time quantitative PCR (qPCR) for all samples and as validation of cPCR results. Quantitative PCR was performed using TaqMan®-TAMRA technology using a novel qPCR marker. This probe-based qPCR provides extra specificity since binding of both primers and the species-specific probe are required from amplification. The novel qPCR marker was designed from alignment of annotated sequences for the D-loop region of the mitochondrial genome of silver carp retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/Genbank). Highly conserved regions were identified and used to design primers and probe. Asian carp-specific primers and probe were designed using Primer 3.0 v.0.4.0 (http://frodo.wi.mit.edu) and specificity for each primer was verified by BLAST analysis. Primers (SVC3 forward-GGTGGCGCAGAATGAACTA, and SVC3 reverse TCACATCATTTAACCAGATGCC) were synthesized by Invitrogen, Inc. (Carlsbad, CA USA) and the TaqMan®-TAMRA probe (SCV3-CCATGTCCGTGAGATTCCAAGCC) was synthesized by Applied Biosystems (Foster City, CA, USA). Quantitative PCR reactions (20 µL total volume) were comprised of 10.0 µL 2X fast master mix (Applied Biosystems, Foster City, CA, USA), 500 nM of each primer, 125 nM of probe, 2 µL DNA and molecular grade water. All assays were carried out in duplicate using a Mastercycler® ep realplex real-time platform (Eppendorf Inc., Westbury, NY, USA). PCR conditions were as follows: 94°C for 2 minutes for initial denaturation of the DNA, then 45 cycles of 94°C for 15 seconds and 56°C for 15 seconds and 72°C for 20 seconds.

Because all birds were offered a silver carp meal on the same day, we assumed that if silver carp DNA was detected in the feces of one bird that all other bird feces were suspect positive for silver carp DNA even if silver carp DNA was not detected. We applied a standard to water samples similar to that of fecal samples in that all water samples collected from a habitat were considered positive even if silver carp DNA was detected in only one sample. Fecal samples and water samples for a given day post silver carp consumption were considered negative only if each fecal sample from that day was negative for silver carp DNA. We chose this classification scheme to provide a conservative estimate of the number of days that a fish-eating bird may be a vector of

silver carp DNA to locations where silver carp are absent. Thus, the vector duration was considered to be the number of days between consumption of a silver carp meal and the last day silver carp DNA was detected in any fecal sample or water sample.

Fecal samples

All fecal samples collected from eagles within 24 h of consuming a meal of silver carp were found to contain silver carp DNA whether processed with the cPCR or qPCR. The agreement between cPCR and qPCR diverged beginning on Day 3 post silver carp consumption. Silver carp DNA was detected in fecal samples from 4 of 4 eagles when samples were processed with cPCR and the CNT marker on Days 4 through 7 post feeding. When these same samples were processed with qPCR and the SVC3 marker, silver carp DNA was detected in fecal samples of 2 of 4 eagles on Days 4 through 6 and 0 of 4 on Day 7 post feeding.

Silver carp DNA was detected in pelican feces on Day 4, the last day the pelicans consumed silver carp. Feces from pelicans on Day 5 were also positive for silver carp DNA. Samples collected prior to Day 4 and later than Day 5 have not been analyzed at date. Silver carp DNA was detected by cPCR in cormorant/ibis fecal swabs collected on Day 1 and was last detected on Day 7, 4 days after the last meal of silver carp had been offered to these birds. Silver carp DNA was detected in fecal samples collected from the quarantined cormorant from Day 1 through Day 5, following a similar trend observed in eagles.

Water samples

Silver carp DNA was not detected by either cPCR or qPCR in water samples collected from the pelican habitat before the pelicans were offered silver carp. Silver carp DNA was detected with both cPCR and qPCR in water samples collected on Day 4 (5 of 10 samples), the last day that pelicans were offered silver carp, and on Day 5 (4 of 8 samples). Silver carp DNA was not detected in any water samples collected on Day 6 or 8, two and four days after pelicans consumed their last meal of silver carp.

Silver carp DNA was not detected in water samples taken from the cormorant/ibis habitat before those birds were offered a meal of silver carp. Silver carp DNA was detected with cPCR until water samples collected on Day 7, 3 days after the last meal of silver carp was offered to the birds. Silver carp DNA was detected by cPCR in water samples collected on Day 9, 5 days after the birds were offered a meal of silver carp and

1 day after the pond was drained and bleached. To date, samples have not been analyzed using qPCR.

Discussion

These results suggest piscivorous birds may be a vector of Asian carp DNA into systems without live Asian carp. As expected, silver carp DNA was detected in fecal samples collected in each trial within 24 h after the birds in those trials had consumed a meal of silver carp. What was less expected was continued detection of silver carp DNA in the fecal samples collected. In eagles, for example, silver carp DNA was detected using cPCR in fecal samples collected as long as 7 d after those birds had consumed a silver carp. The purpose of this study was not to quantify the amount or concentration of silver carp DNA in fecal samples. However, it is likely that the concentration of amplifiable fragments in fecal samples decreased after birds consumed the meal of silver carp from both digestive processes and dilution with other foods consumed. This apparent decrease in DNA concentration is represented by the decrease in the frequency of detection as the period between consumption of silver carp and sample collection increased. Though the frequency of detection and the magnitude of the DNA response decreased with time, these results suggest that some species of piscivorous birds could be a vector of Asian carp DNA for at least 1 week after consuming a meal of silver carp. The relationship between meal size, feeding frequency and other variables that may affect the duration of that amplifiable silver carp DNA would be present in the digestive tract of a bird was beyond the scope of this study.

Collaboration with the Brookfield Zoo allowed us to incorporate the collection of water samples of bird habitats into the study design, albeit an artificial habitat containing animals purposefully fed a diet consisting of silver carp. Inclusion of a water sampling component into the study design did allow us to investigate whether bird feces containing silver carp DNA could be detected. The design of the study did not allow the determination of whether silver carp DNA fragments in the water samples collected were the result of free silver carp DNA solubilized from bird feces deposited in water or were the result of collection of fecal particles with adhering silver carp DNA. Regardless, the detection of silver carp DNA in these habitats, especially the highly eutrophic pelican habitat, suggests that silver carp DNA in bird feces could be detected if collected as part of water samples taken as part of an eDNA monitoring program. If a piscivorous bird consumes a silver carp, these results suggest that the bird will be a vector of Asian carp DNA in its feces for at least 1 week after consuming that meal and, depending on flight patterns, could move that DNA to locations where Asian carp are not present.

Figure 2.5. White pelicans preening; pelican feces on a concrete slab.

2.3.2 Bird-Processed eDNA

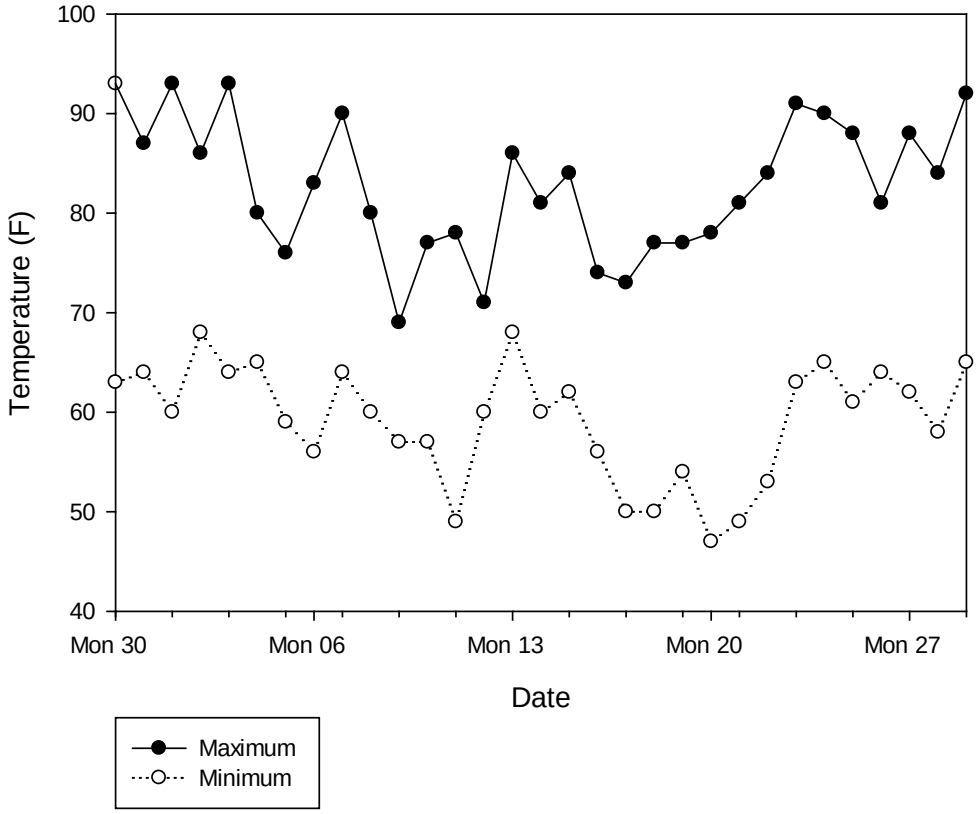
Understanding the degradation rate of Asian carp DNA within deposited bird feces is important because birds are known to feed in areas with an abundance of Asian carp then defecate on barges which may be transported through or into areas where Asian carp are not present or abundant (e.g. above the electric fish barriers in the Chicago Sanitary and Ship Canal). Fomites (e.g. barges, boats, etc.) on which bird feces containing Asian carp DNA are deposited have the potential to transfer Asian carp DNA to areas where Asian carp are not present or abundant. The persistence of Asian carp DNA in bird feces on simulated barge surfaces was evaluated in a controlled-access outdoor mesocosm at UMESC (La Crosse, WI).

Methods

Silver carp DNA-positive bald eagle fecal material from trial 1 were placed on sterile steel cooking sheets (4 sheets per bird; 1 sample (1.42 $\pm$ 0.31 g) per sheet). The sheets were assigned to one of four mesocosms (one sheet per bird per mesocosm). Two mesocosms were supplied with UMESC well water whereas the other two mesocosms were supplied with water pumped from an adjacent fish rearing pond. Mesocosms were used to mimic an actual barge on water and two different water sources were used to determine if water productivity has an impact on the degradation of DNA in feces deposited on a barge. Fecal material on each cooking sheet was sampled in triplicate by touching a sterile cotton swab to the fecal material. Swabs were stored at -80°C in individual centrifuge tubes. Fecal samples were collected on Day 1 then every other day through Day 21 We assumed that the silver carp present in the feces placed on the cooking sheets would degrade. We therefore planned to process samples in reverse collection order, except that the samples collected on Day 1 were processed immediately to confirm the presence of silver carp DNA in the fecal material placed on the cooking

sheets. We further planned to stop processing once all fecal samples from a given collection day were positive (e.g. if silver carp DNA was detected in 75% of samples collected on Day 21, then the preceding sampling period would be processed – this would be repeated until a 100% positive rate was achieved or all samples were processed). Because of concerns that silver carp DNA in eagle samples might persist beyond the 21-d sampling period, we chose to process the samples collected on Days 15 and 18 prior to Day 21 to determine whether the trial should be extended. The trial was extended to 30 days because silver carp DNA was detected in all Day 18 samples. The temperature of each cooking sheet was recorded daily (Figure 2.6).

Figure 2.6. Maximum (solid line) and minimum (dotted line) daily temperatures during trial evaluating the persistence of DNA in piscivorous bird feces.



DNA was extracted from individual swabs using the DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. The presence of silver carp DNA was determined by cPCR as described in section 2.3.1. All samples will be also analyzed with qPCR as described in section of 2.3.1.

Fecal samples for a given day post deposition were considered negative only if each fecal sample from that day was negative for silver carp DNA. We chose this classification scheme to provide a conservative estimate of the number of days that a fish-eating bird may be a vector of silver carp DNA to locations where silver carp are absent. Thus, the vector duration was considered to be the number of days between deposition of silver carp positive feces and the last day silver carp DNA was detected in any fecal sample.

Fecal samples collected on Day 1 confirmed the presence of silver carp DNA in the eagle feces placed on the cooking sheets (100% detection including cPCR process duplicates). Silver carp DNA was detected in all fecal samples collected on Days 15, 18 and 30. Silver carp DNA was detected in all cPCR process duplicates for samples processed from Days 15 and 18 but only 75% of the cPCR process duplicates were positive for samples processed from Day 30. Although silver carp DNA was detected in at least one duplicate cPCR of each Day 30 sample, the reduction in cPCR process duplicate agreement for Day 30 samples suggests that there was less amplifiable silver carp DNA present in those samples, potentially indicating that the silver carp DNA in the fecal material was beginning to degrade to sequences that could not be amplified by the cPCR marker used in this study. The average temperature (± SD) of the cooking sheets was 36.5 ± 13.5 °C. Temperatures in excess of 50°C were recorded on 10 days with a maximum recorded temperature of 62.2°C recorded on Day 5.

Discussion

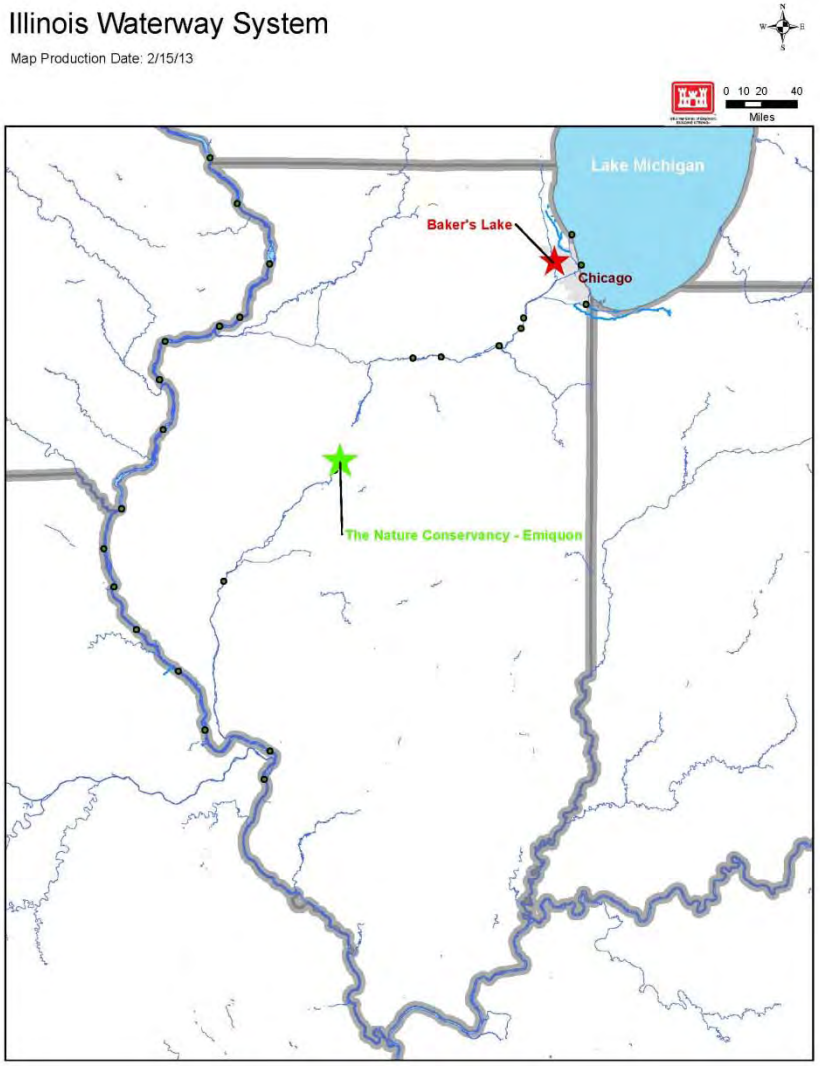
The results of this trial demonstrate that sequences of Asian carp DNA in bird feces deposited on metal surfaces can persist in an amplifiable state for several weeks after deposition, even when surface temperatures exceed 60°C. This suggests that if piscivorous birds consumed a meal of Asian carp then defecated on a barge that Asian carp DNA could easily persist during barge transit from areas of high Asian carp abundance to areas where Asian carp are not present or abundant.

2.3.3 Documenting Presence and Satellite Tracking of Piscivorous Birds

In this activity, we had 2 primary objectives; 1) Document the presence and relative abundance of piscivorous birds along the Des Plaines River, from The Nature

Conservancy (TNC) Emiquon Preserve (approx. 40 mi south of Peoria, IL) north to Romeoville, IL (location of the Dispersal Barrier System) and into the Chicago Area Water System (CAWS), and 2) Locate, capture, and affix satellite tags on 30 double-crested cormorants (*Phalacrorax auritus*) to document movement patterns and determine their potential role as vectors of Asian carp eDNA in the CAWS (Figure 2.7).

Figure 2.7. Map indicating the location of the Baker's Lake, Des Plaines River tagging site and the TNC Emiquon Preserve tagging site.



Methods

eBird Data

eBird (http://www.ebird.org) is an online checklist program developed and supported by the Cornell Lab of Ornithology and National Audubon Society. This program permits recreational and professional birders to upload and document bird observations and photographs while birding. These observations contribute to an extremely large database that will serve to provide constant, up-to-date data on the distribution and abundance of birds throughout North America. For this project, we downloaded all seasonal information on ten of the most common piscivorous birds utilizing the CAWS and surrounding region. These birds include the double-crested cormorant, bald eagle (*Haliaeetus leucocephalus*), American white pelican (*Pelecanus erythrorthynchos*), osprey (*Pandion haliaetus*), little blue heron (*Egretta caerula*), green heron (*Butorides virescens*), snowy egret (*Egretta thula*), great egret (*Ardea alba*), great blue heron (*Ardea herodias*), and black-crowned night-heron (*Nycticorax nycticorax*).

Recorded observations were downloaded from eBird for each season (Winter: December-February; Spring: March-May; Summer: June-August, and Fall: September-November) from 2005 until 2012. Only data from specific counties that border or include the Des Plaines River from Havana, IL, (just south of the TNC Emiquon Preserve) to the CAWS were included. These counties include Mason, Fulton, Tazewell, Peoria, Woodford, Marshall, Putnam, La Salle, Grundy, Will, Lake and Cook. Furthermore, these counties were grouped to provide information on piscivorous birds along 3 main sections of the Des Plaines River: 1) Havana to Hennepin (includes Mason, Fulton, Tazewell, Peoria, Woodford and Putnam Counties), 2) Hennepin to Romeoville (includes La Salle, Will and Grundy Counties) and 3) Romeoville to Chicago CAWS (includes Lake and Cook Counties).

Satellite Tracking of Double-crested Cormorants

From May 25 to May 29, 2012, we captured a total of 30 double-crested cormorants at 2 primary locations: 1) TNC Emiquon Preserve about 40 mi. south of Peoria, IL (Figure 2.8), and 2) Baker's Lake, part of the Cook County Forest Preserve (Figure 2.9). A third site, Lake Renwick (also part of the Cook County Forest Preserve) had to be dropped while in the field because permission to access this site could not be procured. Initially, we intended to capture and tag 10 birds at each site; since Lake Renwick had to be dropped, we captured and tagged 15 birds the TNC Emiquon Preserve and Baker's Lake,

respectively. The TNC Emiquon Preserve is located in an area with a large known Asian carp spawning population, while the Baker's Lake site is located in an area with few recorded Asian carp and no observed spawning activities (but as part of the CAWS, this site was located in an area with positive carp eDNA observations).

Figure 2.8. One of several small double-crested cormorant nesting sites found within The Nature Conservancy Emiquon Preserve, near Havana, IL (about 40 mi. south of Peoria, IL).

Figure 2.9. Artificial structure at the Baker's Lake rookery site in Barrington, IL, part of the CAWS.

Birds were captured at rookery sites during the breeding season. At both sites, the rookeries were either located in trees (TNC Emiquon Preserve) or artificial structures (Baker's Lake). We used a small boat to travel to the rookery site, where we used No. 3 Victor® Softcatch padded coil spring traps. These traps were modified with weaker springs to reduce the initial impacts of the padded jaws and minimize any potential harm to the birds. Traps were placed directly on an active nest, immediately adjacent to an active nest, or in the vicinity of an active nest. After trap placement, we moved away from the rookery (usually about 50-100 m) and observed the traps until we detected a capture (rarely more than 10 or 15 min after trap placement). After capture, we freed the bird from the trap and placed the bird in a burlap sack. We then took the bird to the shore and processed the bird by collecting specific data (total weight (kg), wing chord (mm), culmen length (mm) and tarsus length (mm)). We also used cotton swabs to collect DNA material from the throat and cloaca. These samples were used to test for the presence of Asian carp DNA. All samples were placed into vials that were marked with the date, time, cormorant ID number, and rookery location. After processing, all birds were released.

All captured cormorants were affixed with Sirtrack® Argos Satellite Platform Transmitting Terminal (PTT) Harness Transmitters (model: K3H 174A KiwiSat 303)

(Figure 2.9). These satellite tags use Doppler technology from orbiting satellites to estimate location of transmitting tags on the earth's surface. To maximize battery life, the satellite tags were programmed to transmit every other day for 14 hours. This permitted us to observe early morning and late evening foraging movements for up to 6 months. We used the data download capability available through CLS America, Inc., to regularly download all location data through an online website. After the last satellite tag ended transmission (on December 24, 2012) all downloaded data were uploaded into ARCGIS 9.2 database for spatial analysis and presentation purposes. Currently, we are using Hawth's Ecological Tools to examine frequency and direction of movements during the breeding (May-July), post-breeding (August-September) and fall migration/winter (October-December) seasons. These analyses are not yet complete, but we can provide detail on some outcomes of the tagged birds, the number of birds that can be analyzed for movement/foraging modeling, and some preliminary thoughts on the role of the double-crested cormorant as a potential vector of Asian carp eDNA in the CAWS.

Figure 2.10. A Sirtrack Argos Satellite PPT transmitter is affixed to a double-crested cormorant at the Baker's Lake rookery site.

Results

eBird Data

An estimated 16,701 observations of 189,807 piscivorous were observed along the Des Plaines from 2005 to 2012 (Table 2.2). The double-crested cormorant was the most common species with over 70 thousand observations, followed closely by the American white pelican with over 65 thousand observations. Greater than 20 thousand observations of the great blue heron and the great egret were recorded, followed by the black-crowned night-heron (4,809) and the bald eagle (2,904). Over 2,700 observations of the green heron were recorded, while much fewer observations were recorded for the Osprey (599) and the little blue heron (259); the snowy egret was the least common piscivorous bird with only 137 observations during the time period.

For most bird species, the spring and the summer seasons had the highest number of recorded observations. Only the bald eagle had the highest number of observations during the winter season; observation data were too low to determine seasonal patterns for the snowy egret and the little blue heron (Table 2.2). Six of 10 species (double-crested cormorant, great blue heron, great egret, little blue heron, green heron, black-crowned night-heron, and the osprey) has higher observations recorded in the Romeoville to Chicago section, while only the American white pelican and snowy egret had highest observations in the Havana to Hennepin section. The great blue heron, green heron, black-crowned night-heron, bald eagle, and the osprey had similar counts in the Havana to Hennepin and Hennepin to Romeoville sections relative to the higher counts in the Romeoville to Chicago section; the little blue heron and great egret had median counts in the Havana to Hennepin section and lowest counts in the Hennepin to Romeoville section.

Although the eBird data included observations throughout the entire counties, when the data are projected in Google Earth, most all distributions appear concentrated along the Des Plaines River and other open water habitats (Figures A-1 through A-10; Appendix A). Numerous small rivers, streams, ponds and lakes are distributed throughout the Chicago area, so this may explain why distributions appear much more dispersed in this area (Figures A-1 through A-10; Appendix 1).

Table 2.2. Data download from eBird showing the estimated[1] number of observations and number of birds (with mean and standard deviations) for the top ten most abundant piscivorous birds from 2005 to 2012 on three sections of the Des Plaines River (Havana to Hennepin, Hennepin to Romeoville, and Romeoville to Chicago).

River Section	American White Pelican (*Pelecanidae erythrorrhynchos*)			Double-crested Cormorant (*Phalacrocorax auritus*)			Great Blue Heron (*Ardea herodias*)			Great Egret (*Ardea alba*)		
	No. Obs.	Sum	Mean ±STD	No. Obs.	Sum	Mean ±STD	No. Obs.	Sum	Mean ±STD	No. Obs.	Sum	Mean ±STD
Havana to Hennepin: Spring	99	8,220	82.7±149.0	98	4,037	41.0±94.7	221	891	4.0±10.7	54	433	8.0±17.1
Summer	82	33,029	401.6±1065.7	111	2,929	26.3±57.7	253	2,268	9.0±16.4	166	6,255	37.7±88.0
Fall	97	15,729	162.8±448.7	72	1,832	25.3±78.8	169	735	4.4±6.5	89	876	9.8±21.0
Winter	27	123	13.5±27.2	5	14	2.9±3.5	47	193	4.1±7.2	n/a[2]	n/a	n/a
Sub-Total	**305**	**57,101**	**198.7±2798.4**	**287**	**8,812**	**30.7±77.3**	**690**	**4,087**	**5.9±12.4**	**309**	**7,564**	**24.5±67.4**
Hennepin to Romeoville: Spring	82	4,524	55.5±100.7	236	4,715	19.9±95.2	358	1,471	4.1±10.7	166	1,260	7.6±31.3
Summer	41	1,451	35.7±51.4	192	9,017	47.0±135.0	303	1,871	6.2±9.6	224	2,392	10.7±25.03
Fall	57	2,230	38.8±73.1	109	6,179	56.6±131.9	186	643	3.5±4.3	106	725	6.9±13.1
Winter	13	34	2.7±6.7	50	445	8.9±13.4	140	427	3.0±5.0	n/a	n/a	n/a
Sub-Total	**193**	**8,239**	**42.9±81.4**	**587**	**20,353**	**34.7±114.5**	**987**	**4,412**	**4.5±8.8**	**496**	**4,377**	**8.8±25.6**
Romeoville to Chicago: Spring	23	676	29.3±46.6	1,216	16,732	13.8±62.4	1,539	4,271	2.8±6.0	563	1,878	3.3±13.7
Summer	9	52	5.5±7.4	1,084	10,222	9.4±49.9	1,797	5,519	3.3±8.8	936	5,192	5.5±23.7
Fall	9	127	13.9±24.5	916	14,098	15.4±114.1	918	2,057	2.2±5.9	340	2,143	6.3±26.5
Winter	n/a	n/a	n/a	74	189	2.6±3.8	165	247	1.5±1.5	n/a	n/a	n/a
Sub-Total	**41**	**855**	**20.5±37.9**	**3,290**	**41,241**	**12.5±76.8**	**4,419**	**12,094**	**2.8±7.1**	**1,839**	**9,213**	**5.0±21.8**
TOTAL	**539**	**66,195**	**127.0±484.6**	**4,164**	**70,406**	**17.9±134.7**	**6,096**	**20,593**	**3.5±8.3**	**2,644**	**21,154**	**8.0±32.0**

Table 2.2. Continued.

River Section	Snowy Egret (*Egretta thula*)			Little Blue Heron (*Egretta caerulea*)			Green Heron (*Butorides virescens*)			Black-crowned Night-Heron (*Nycticorax nycticorax*)		
	No. Obs.	Sum	Mean ±STD	No. Obs.	Sum	Mean ±STD	No. Obs.	Sum	Mean ±STD	No. Obs.	Sum	Mean ±STD
Havana to Hennepin: Spring	N.S.	N.S.[2]	N.S.	N.S.	N.S.	N.S.	32	46	1.5±0.8	N.S.	N.S.	N.S.
Summer	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	67	118	1.8±1.7	N.S.	N.S.	N.S.
Fall	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	8	8	1.0±0.6	N.S.	N.S.	N.S.
Winter	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Sub-Total	**40**	**100**	**2.5±23.9**	**24**	**58**	**2.4±3.3**	**107**	**172**	**1.6±1.4**	**26**	**64**	**2.5±1.7**
Hennepin to Romeoville: Spring	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	24	26	1.1±0.6	13	41	3.2±4.9
Summer	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	92	168	1.8±0.6	42	88	2.1±1.8
Fall	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	14	21	1.5±0.9	21	58	2.8±1.9
Winter	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Sub-Total	**4**	**5**	**1.0±05**	**4**	**4**	**0.9±0.5**	**130**	**215**	**1.7±1.5**	**76**	**187**	**2.5±2.6**
Romeoville to Chicago: Spring	N.S.	N.S.	N.S.	33	45	1.4±0.7	584	881	1.5±1.3	491	2,540	5.2±20.5
Summer	N.S.	N.S.	N.S.	84	144	1.7±1.5	771	1,277	1.7±1.5	492	1,695	3.4±9.6
Fall	N.S.	N.S.	N.S.	n/a	n/a	n/a	164	206	1.3±1.2	170	291	1.7±2.1
Winter	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	20	32	1.6±0.9
Sub-Total	**33**	**39**	**1.2±0.7**	**122**	**197**	**1.6±1.3**	**1,519**	**2,364**	**1.6±1.4**	**1,173**	**4,558**	**3.9±14.7**
TOTAL	**77**	**137**	**1.9±1.7**	**150**	**259**	**1.7±1.8**	**1,756**	**2,751**	**1.6±1.4**	**1,275**	**4,809**	**3.8±14.1**

Table 2.2. Continued.

River Section	Bald Eagle (*Haliaeetus leucocephalus*)			Osprey (*Pandion haliaetus*)		
	No. Obs.	Sum	Mean ±STD	No. Obs.	Sum	Mean ±STD
Havana to Hennepin: Spring	90	283	3.1±4.4	10	9	1.0±0.4
Summer	41	88	2.1±2.4	9	11	1.2±0.6
Fall	71	208	2.9±3.4	9	11	1.2±0.5
Winter	114	639	5.6±10.6	n/a	n/a	n/a
Sub-Total	**316**	**1,218**	**3.8±7.1**	**28**	**31**	**1.1±0.5**
Hennepin to Romeoville: Spring	68	111	1.6±1.9	12	19	1.6±1.1
Summer	26	30	1.2±0.6	17	22	1.3±0.8
Fall	34	48	1.4±0.9	13	16	1.2±0.6
Winter	168	1,236	7.3±20.6	n/a	n/a	n/a
Sub-Total	**296**	**1,425**	**4.8±15.8**	**42**	**57**	**1.4±0.8**
Romeoville to Chicago: Spring	50	66	1.3±0.9	144	175	1.2±0.6
Summer	10	10	1.0±0.2	130	212	1.6**±1.2**
Fall	65	90	1.4±1.0	81	124	1.5±1.6
Winter	73	95	1.3±0.6	n/a	n/a	n/a
Sub-Total	**198**	**261**	**1.3±0.8**	**355**	**511**	1.4±1.1
TOTAL	**810**	**2,904**	**5.3±10.6**	**425**	**599**	**1.9±1.1**

[1] Total numbers and observations for the American white pelican and the bald eagle within the immediate river and adjacent floodplain varied between 20%-48% of the total numbers/observations for each section (based on total cumulative counts of all numbers/observations for each county comprising each section); therefore, estimated numbers and observations presented in this table are based on 35% of the total numbers/observation.

[2]N.S.: represents numbers/observations less than 50 per season for any specific section; n/a: represents seasons when no observation of a species is recorded.

Throat and Cloacal Swabs

Of the cormorants captured at the TNC Emiquon Preserve, 13 birds had positive results for carp eDNA from the throat and/or cloacal swabs: 6 birds had positive carps eDNA from both the throat and cloacal swabs, 3 birds had positive results from only the throat swabs, and 4 birds had positive results from only the cloacal swabs (Table 2.3).

Of the cormorants captured at Baker's Lake, 7 birds had positive results for carp eDNA from the throat and/or cloacal swabs: 1 bird has positive carp eDNA from both the throat and cloacal swabs, 6 birds had positive results from only throat swabs, while no birds had positive results from only the cloacal swabs (Table 2.3).

Movements of Satellite Tagged Cormorants

Movements of tagged double-crested cormorants were monitored from the moment they were tagged until the moment the tag stopped transmitting. The battery life of the transmitter was estimated at approximately 180 days or 6 months. Therefore, those tags that ended transmitting in late October and later likely ended transmissions due to normal operating capabilities. Numerous tags ceased transmissions much earlier in the summer. Diagnostic signals from the tags are still being analyzed, but some birds likely perished during the study, perhaps due to the unusually hot months of June and July in 2012. Cormorants are notorious for behaviors that can cause significant damage to tags, including frequent submerged diving and scrapping the tags against rocks and other debris. Although the tags were largely waterproof, any seepage of water through the seals could have short-circuited some tags resulting in transmission failures.

For the purposes of this study, we were generally focused on the behaviors and movements of cormorants during the breeding season. Therefore, to address the question of whether cormorants may be acting as vectors of Asian carp DNA, we focused on those birds whose movement data are available at least until the end of July, although we provide movement results for all birds during the duration of the study (see Table 2.4). The requirement of these birds to meet both the nutritional needs of themselves and their young was thought to induce some birds (particularly, the birds in Baker's Lake) to travel distances sufficient to provide opportunities to forage on Asian carp, and then by returning back to the rookery, would thereby be acting as vectors of carp eDNA though decimation of carp tissues (e.g., while regurgitating food to young) or by the excretion of fecal material throughout the CAWS.

Table 2.3. Presence of Asian carp eDNA results from throat and cloacal swabs collected from captured double-crested cormorants on the TNC Emiquon Preserve and Baker's Lake rookery sites.

Rookery Site	Satellite Tag #	Cormorant ID #	Carp eDNA	
			Throat	Cloaca
TNC Emiquon	119192	276	Positive	Positive
TNC Emiquon	119198	273	NA	Positive
TNC Emiquon	119199	275	Positive	Positive
TNC Emiquon	119203	277	Positive	Positive
TNC Emiquon	119204	272	Positive	NS
TNC Emiquon	119205	271	Positive	Positive
TNC Emiquon	119209	279	NA	NA
TNC Emiquon	119210	278	Positive	Positive
TNC Emiquon	119211	274	NA	NA
TNC Emiquon	119213	269	Positive	NA
TNC Emiquon	119216	281	NA	Positive
TNC Emiquon	119217	270	NA	Positive
TNC Emiquon	119218	280	Positive	NA

TNC Emiquon	119219	268	Positive	Positive
TNC Emiquon	119220	282	NS	Positive
Baker's Lake	119191	289	NA	NA
Baker's Lake	119193	297	Positive	NA
Baker's Lake	119194	295	Positive	NA
Baker's Lake	119195	287	Positive	Positive
Baker's Lake	119196	296	NA	NA
Baker's Lake	119197	285	NA	NA
Baker's Lake	119200	300	NA	NA
Baker's Lake	119201	292	NS	NS
Baker's Lake	119202	286	Positive	NA
Baker's Lake	119206	299	NA	NA
Baker's Lake	119207	288	Positive	NA
Baker's Lake	119208	291	Positive	NA
Baker's Lake	119212	294	Positive	NA
Baker's Lake	119214	293	NA	NA
Baker's Lake	119215	290	NA	NA

Since all 30 birds were tagged, we have recorded over 24,000 satellite locations during the breeding (13,081 locations), post-breeding (6,745 locations), and fall migration/winter (4,249 locations) seasons (Table 2.4). Seven birds tagged at the TNC Emiquon Preserve and 8 birds tagged at Baker's Lake yielded movement data to the end of July or later; movements of these birds are shown in Figures 2.9 and 2.10. Table 2.4 provides a summary of movements of all tagged birds during the study for the breeding, post-breeding and fall migration/winter seasons. During this study, bird exhibited a wide range of behaviors, with many birds undertaking long flights, many hundreds, and even thousands of kilometers away from the capture/tagging location.

Birds from the TNC Emiquon Preserve tended to move northwest through Iowa and south to Missouri. Multiple birds spent time in the Davenport/Clinton, IA area, and 3 birds spent time near the Greenville/Greenwood, MS area (one bird on the LA side of the river). Several birds visited the Harry S. Truman Reservoir, MO, and Lake Eufaula, OK. Multiple birds tagged at Baker's Lake spent considerable time along the Lake Michigan Coast, with several of these birds following the coast north to Green Bay, WI, Chambers Island, WI, and one bird that spent a large portion of its time in Canada. Several birds from the TNC site wintered in the Mississippi Delta region of Mississippi and Louisiana, while birds from Baker's Lake wintered along the Louisiana coast; 2 birds from Baker's Lake passed through Mobile, AL. One bird from the TNC site (Tag 119199) wintered along the Louisiana Coast very close to 2 birds from Baker's Lake (Tags 119195 and 119202) (Table 2.4 and Figures 2.11 and 2.12).

Table 2.4. Summary of tagged double-crested cormorant movements.

Rookery Site	Satellite Tag #	Last Transmission Date	Number of Observed Locations			Total	Summary of Movements
			Breeding	Post-Breeding	Migration/Winter		
TNC Emiquon	119192	6/23	301	0	0	301	Movements ≤20 km within vicinity of TNC site.
TNC Emiquon	119198*	9/17	654	444	0	1098	Movements ≤25 km within vicinity of TNC site.
TNC Emiquon	119199*	12/24	667	630	693	1990	Large movements ≥150 km north through WI and MN; remained in MN for breeding and post-breeding season (6 weeks); fall migration/winter: large movements ≥200 km south to Cedar Rapids, MO; south to AR near Memphis, TN; ≥200 km south to LA just west of Greenville, MS; ≥200 km to coastal LA.
TNC Emiquon	119203	6/27	303	0	0	303	Large movements ≥200 km south IL, close to St. Louis, MO.
TNC Emiquon	119204	6/7	116	0	0	116	Movement >25 to ≤80 km; remained in vicinity of TNC site.
TNC Emiquon	119205	6/5	111	0	0	111	Movements ≤25 km; remained in vicinity of TNC site.
TNC Emiquon	119209*	11/8	606	541	235	1382	Large movements ≤200 km north to Davenport/Clinton, IA; then south ≤600 km to Lake Eufaula, OK; post-breeding: ≤25 km movements in vicinity of Lake Eufaula; fall/winter: movements 25-75 km in vicinity of Lake Eufaula.
TNC Emiquon	119210	7/21	532	0	0	532	Large movements ≤200km west to MO; then 5 weeks at Harry S. Truman Reservoir, MO.
TNC Emiquon	119211	6/25	310	0	0	310	Movements ≤25 km in vicinity of TNC site.

TNC Emiquon	119213*	12/14	682	574	583	1839	Movements ≥150 km north to Davenport/Clinton, IA; then southwest to MO; west beyond Kansas City, MO; southeast to Harry S. Truman, MO; return back to TNC site; post-breeding: remained in vicinity of TNC site; fall/winter: large movement ≥500 km south to Greenville/Greenwood, MS – remained for 8 weeks.
TNC Emiquon	119216*	12/4	606	544	361	1511	Movements between 25-50 km in vicinity of TNC site for breeding/post-breeding; fall/winter: large movements ≥300 km southwest to Harry S. Truman Reservoir, MO (4 weeks); ≥200 km to Grreat Lake O' the Cherokees, OK; >200 km south to Robert S. Kerr Reservoir, OK, then ≥350 km to Dallas, TX.
TNC Emiquon	119217*	12/12	619	540	525	1684	Large movements >300 km south to central LA and Greenville/Greenwood MS area; post-breeding-fall/winter: movements 25-60 km, remains around Greenville/Greenwood, MS (about 10 weeks).
TNC Emiquon	119218	6/7	96	0	0	96	Movements ≤15 km in vicinity of TNC site.
TNC Emiquon	119219	6/5	121	0	0	121	Movements ≤25 km in vicinity of TNC site.
TNC Emiquon	119220*	9/7	611	370	0	981	Large movements ≥100 km to Davenport/Clinton, IA, return to TNC site; return to Davenport, IA for 3 weeks; back to TNC site; post-breeding: movement ≥250 km to St. Louis, MO; to Memphis, TN and North, MS; returned north near Dyersburg, TN.
Subtotal			**6,335**	**3,643**	**2,397**		**12,375**
Baker's Lake	119191	5/30	18	0	0	18	Transmission ended within 48 hours after tagged; never left Baker's Lake area.
Baker's Lake	119193	6/7	121	0	0	121	Movements ≤40 km around vicinity of Baker's Lake/CAWS.
Baker's Lake	119194*	12/12	693	643	486	1822	Movement >350 km north along Lake Michigan coast to Naubinway, Canada (6 weeks); post-breeding: south >150 m to Manitowoc, WI; then south >150 km to Waukegan, IL (CAWS area); then inland >25 km to Fox Lake (6 weeks); fall/winter: south >400 km to Carlyle Reservoir, IL (4 weeks); south >70 km to Rend Lake, IL (4 weeks), then south >400 km to Tunica, MS (1 week); then south >450 km to Mobile, AL.
Baker's Lake	119195*	12/6	599	550	423	1572	Movements 15-40 km within Baker's Lake/CAWS area; post-breeding: east >400 km to Detroit, MI, along Lake Erie coast (8 weeks); fall/winter: another 3 weeks in Detroit, MI; then 1,300 km south to Mobile, AL; then >80 km west to Biloxi, MS; then >220 km west to coastal LA (20 mi. west of Theroit, LA) (5 weeks).

Baker's Lake	119196*	9/7	680	376	0	1056	Initial movements ≤50 km between Baker's Lake and Lake Forest along coast; then >100 km along Lake Michigan Coast to Milwaukee, WI; north along coast >100 km to Manitowoc, WI; then north >100 to Chambers Island area, WI (near Canada Border) for 3 weeks; post-breeding: movement >85 km south to Green Bay, WI (4 weeks); then south back to Busse Lake (within 15 km of Baker's Lake) in the CAWS.
Baker's Lake	119197	7/13	490	0	0	490	Movements 25-100 km in vicinity of Baker's Lake and Waukegan, IL; along coast of Lake Michigan.
Baker's Lake	119200	6/25	317	0	0	317	Movements 25-60 km in vicinity of Baker's Lake and Lake Forest, along coast of Lake Michigan
Baker's Lake	119201	6/25	281	0	0	281	Movements ≥50 km south to Yorkville and Plainfield, IL; last location at Lake Renwick.
Baker's Lake	119202*	12/10	672	577	563	1812	Movement >20 km from Baker's Lake to Lake Forest along the Lake Michigan coast; then north 200 km to Green Bay, WI; >100 km north to Chamber's Island, WI; return to Green Bay (5 weeks): post-breeding: 8 weeks in Green Bay area; fall/winter: 4 more weeks in Green Bay; then south >1,100 km to West Helena, AR; then south >220 km to Tallulah, LA (2 weeks); then >300 km south to coastal LA (around the Vermilion and West Cote Blanche Bays) (3 weeks).
Baker's Lake	119206*	10/7	655	597	77	1329	Movements between 10-60 km within the Baker's Lake/CAWS area; post-breeding: movement about 25-30 km south to Naperville, IL and Fox River (8 weeks); fall/winter: movement >20 km south to Plainfield, IL (around Lake Renwick); another 20 km south of Joliet, IL, along Des Plaines River.
Baker's Lake	119207*	8/4	653	38	0	691	Most movements ≤25 km near Baker's Lake; divided time between Baker's Lake, Deep Quarry Lake, and the Fox River near Eglin, IL.
Baker's Lake	119208	6/29	344	0	0	344	Movements ≤25 km; most time spent near Fish Lake about 20 km north of Baker's Lake.
Baker's Lake	119212*	7/29	673	0	0	673	Movements of 25-60 km north of Baker's Lake at Waukegan, IL; along coast of lake Michigan and inland at Fox Lake.
Baker's Lake	119214*	12/4	325	321	303	949	Movements ≤ 25 km; remained in and around the Baker's Lake area for duration of study and all seasons.
Baker's Lake	119215	6/15	226	0	0	226	Large movements ≥100 km north to Milwaukee, WI, along coast of Lake Michigan.
Subtotal			**6,746**	**3,102**	**1,852**	**11,701**	
Total			**13,081**	**6,745**	**4,249**	**24,076**	

*Birds with sufficient transmitter duration (end of July) plus number of locations to permit future analyses on movement frequency, average distance, and habitat use during the breeding season.

Figure 2.11. Movements of 7 birds tagged at the TNC Emiquon Preserve, near Havana, IL (colored dots show movements of tags 119198, 119199, 119209, 119213, 119216, 119217 and 119220).

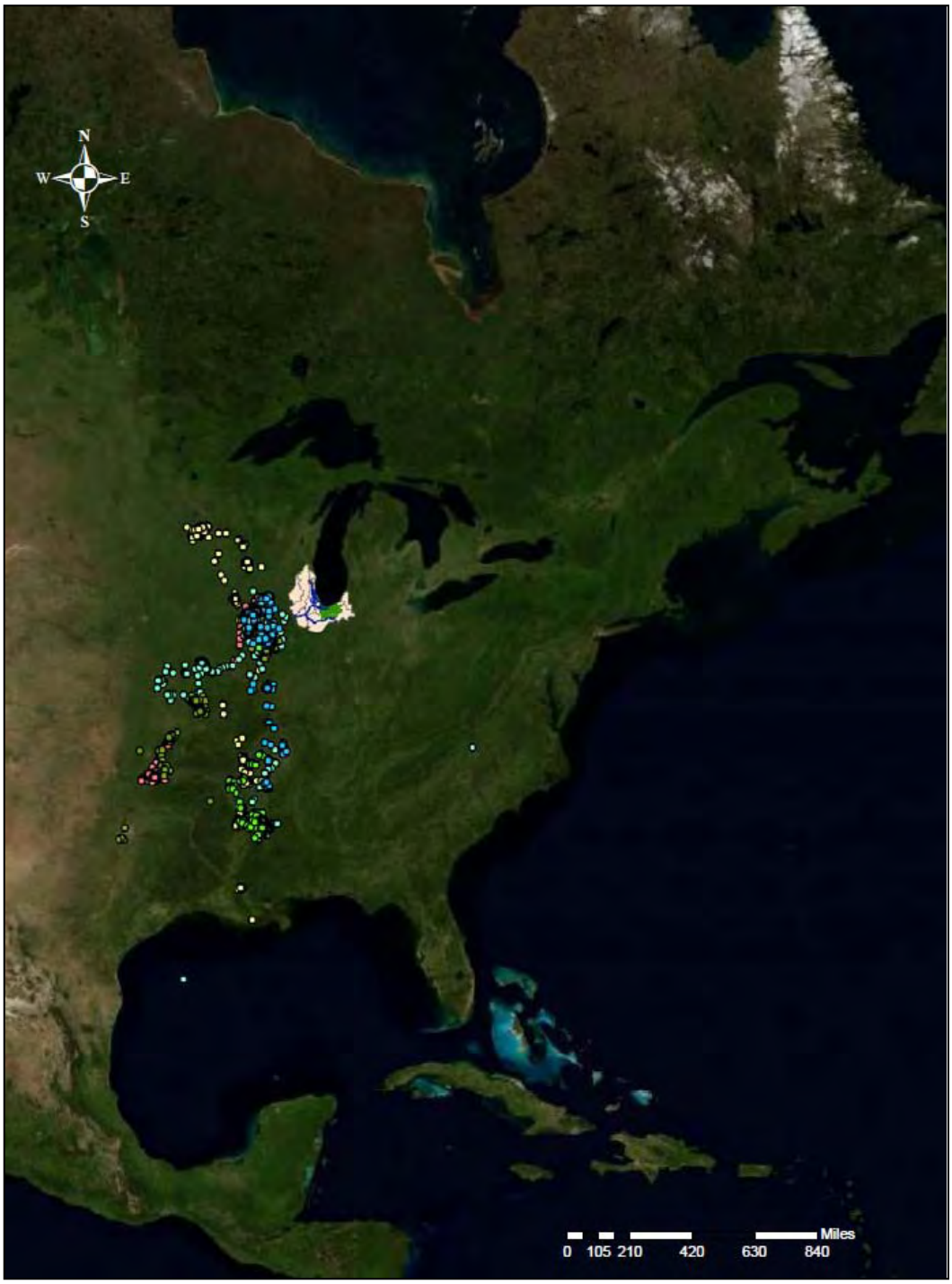
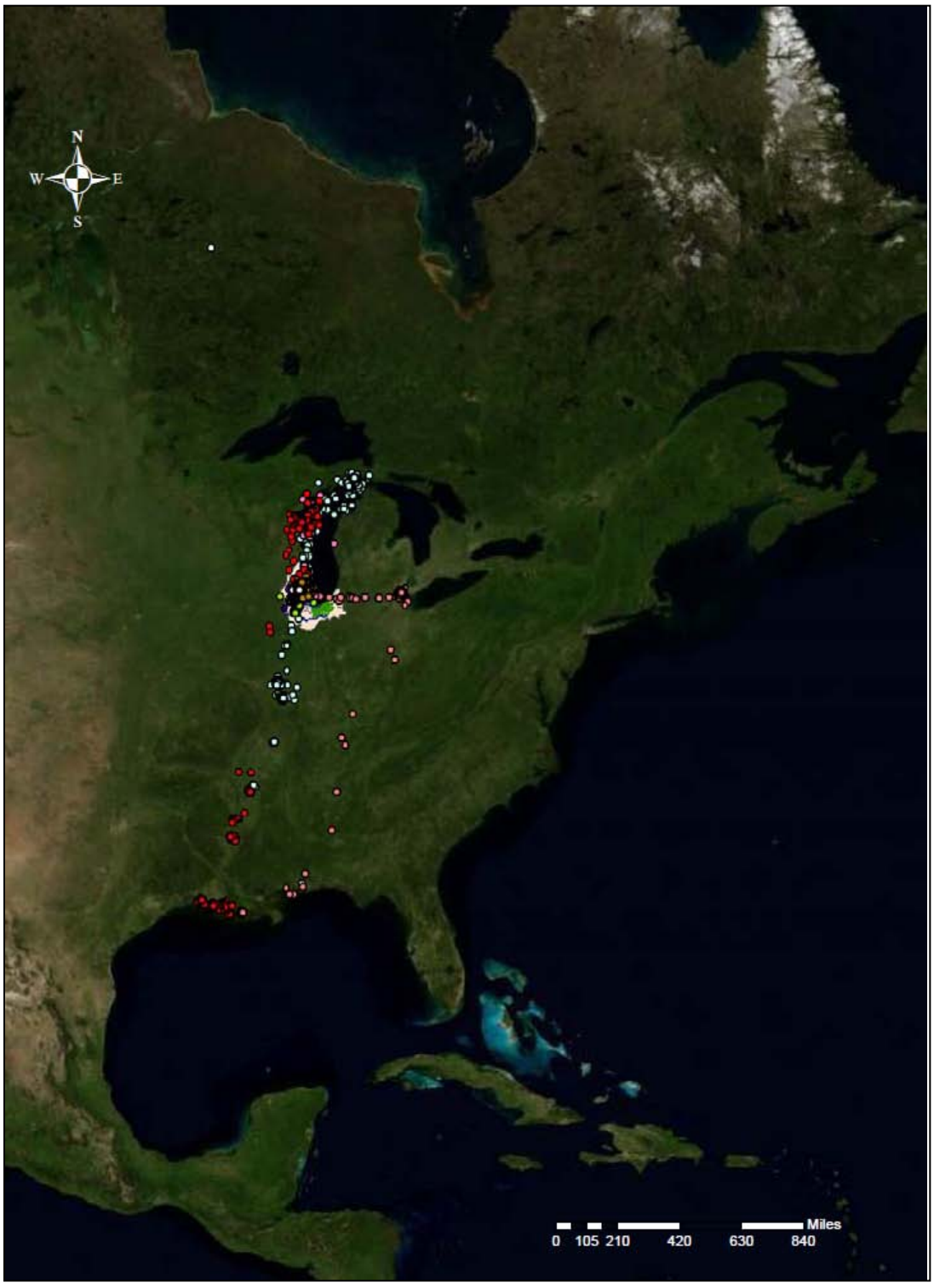


Figure 2.12. Movements of 8 birds tagged at Baker's lake, Barrington, IL within the CAWS (colored dots show movements of tags 119194, 119195, 119196, 119202, 119206, 119207, 119212 and 119214).



Discussion

The smoking gun from this study is shown in the results of the Asian carp eDNA results of the throat and cloacal swabs. These results show that birds from both the TNC Emiquon Preserve and Baker's Lake rookery sites were consuming Asian carp. This result is not surprising for the birds at the TNC site, since the rookery was located in a portion of the Illinois River with a well established spawning carp population. However, it was surprising that 7 out of 15 birds (47%) at Baker's Lake also showed positive results for Asian carp DNA. The satellite tagged cormorants did not show regular flights of birds from Baker's Lake flying to areas with carp and then returning. However, a significant number of these birds must have been feeding on Asian carp at some point during the spring breeding season. Moreover, the location data from the satellite tags showed that the capability and the proclivity of double-crested cormorants to regularly move 50 to 100 km or more at any given time, indicating that they may be significant vectors of Asian carp DNA throughout the CAWS.

Additional research in the Asian Carp eDNA Vectors Unit *(activity 2.3.1: Passage of eDNA through Piscivorous Birds*) has shown that carp DNA can be detected from cormorant feces up to 7 or 8 days after consumption. Moreover, once deposited on a surface (like the artificial nesting structure at Baker's Lake), the carp eDNA can remain viable and detectable through the DNA testing procedure for up to 30 days. This suggests that once cormorants have consumed Asian carp, they can deposit the feces on structures in and around the CAWS, and these structures themselves can act as carp DNA vectors into various water bodies (ponds/lakes, etc.) by serving as sources of carp eDNA during any rain event that washes the fecal material into any standing body of water.

2.4 Asian Carp Carcasses on Barges

2.4.1 Fish Carcasses as Sources of eDNA

Since biologists had reported the presence of dead Asian carp on decks of barges above the U.S. Army Corps of Engineers Electric Dispersal Barrier in the CAWS and slime from those decaying carp trailing down the sides of barges to the water line, concerns have existed regarding the capacity of fomites like barges to transport Asian carp DNA (in the form of carcasses or slime) from areas where Asian carp are present to areas where they are not present or abundant. The goal of this study was to assess whether the Asian carp DNA could be transferred to waters where Asian carp are not present by movement of Asian carp carcasses or

residual slime on fomites such as barges or boats. The specific objectives of this study were: 1) Determine how long detectable amounts of DNA remain on the surface of a dead Asian carp; 2) Determine the persistence of Asian carp DNA in slime deposited by contact between the body of an Asian carp and a simulated barge surface; and 3) Determine how long a carcass immersed in water sheds detectable amounts of DNA.

Methods

Juvenile silver carp were obtained from stocks held at the USGS Upper Midwest Environmental Sciences Center (UMESC), La Crosse, WI. Silver carp were euthanized by overdose in MS-222 (FINQUEL, Argent Chemical Laboratories, Redmond, WA, USA) then stored frozen at -20/-80°C. All samples were stored at -80°C after collection. The DNA in a sample was extracted using the DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions; silver carp DNA was amplified by cPCR using the procedures described in section 2.3.1.

DNA persistence on carcass surface – The carcasses of four silver carp (79.50 ± 4.35 g) were placed on cooking sheets (one carp per sheet). The sheets were intended to simulate the surface of a barge; foam blocks were attached to each sheet and each sheet was floated in an assigned tank of water. All work was completed in outdoor mesocosms at UMESC; mesocosms were enclosed in a wire cage to exclude scavengers and plastic covers were placed over the sheets to prevent rainfall from altering DNA persistence. Carcasses were otherwise exposed to ambient environmental conditions (e.g. temperature, humidity, light). Effects of plastic covers on UV will be determined. Each carcass was sampled in triplicate by gently rubbing a sterile cotton swab on the carcass surface. Each carcass was sampled on Day 1 and then every other day for 18 days.

Shedding of DNA from carcasses – Eight 1.00 L chambers were evenly assigned to contain 1 or 10 carcasses of silver carp. The average carcass mass in chambers containing 1 carcass was 97.73 ± 6.72 g whereas chambers containing 10 carcasses had an average carcass mass of 902.58 ± 42.71 g. Each chamber was supplied with well water (12-13°C) at a rate of approximately 0.30 L min^{-1}. Each chamber was maintained in an outdoor mesocosm at UMESC under conditions described above. Triplicate samples (25 mL) of water were collected from the effluent of each chamber on Day 1 and then every other day for 18 days. The water samples were centrifuged at 5,000 x g for 30 minutes immediately

following collection. Water was decanted and the remaining pellet in the original tube was stored at -80°C.

Persistence of silver carp DNA in silver carp slime – Forty silver carp carcasses were placed on steel cooking sheets (10 per sheet). The carcasses remained on the metal sheets for 1 h then the carcasses were removed and discarded. Silver carp carcass contact with the metal sheets left a residual slime mass which averaged 4.15 ± 1.26 g per sheet. The sheets were placed in the mesocosms using the procedures described for the carcasses placed on metal sheets. Triplicate samples of the slime on each sheet were collected by touching a sterile cotton swab to the slime on the sheet. Each sheet was sampled on Day 1 and then every other day for 18 days.

We assumed that if silver carp DNA was detected in any one sample for a given sample period then all of the samples collected within that period were considered suspect positive for silver carp DNA. We chose this classification scheme to provide a conservative estimate of the number of days that DNA would persist in an amplifiable form under the conditions we studied. Thus, sample days were reported as silver carp DNA positive even if silver carp DNA was detected in only one sample whereas sample days were reported as negative for silver carp DNA only if all samples collected on that sample day were found to not contain silver carp DNA. Samples were processed in reverse order of collection except that all Day 1 samples were processed to confirm the presence of silver carp DNA. Samples collected between Day 1 and the first positive silver carp DNA detection in a later sample were considered to be positive (i.e., if silver carp DNA was present in samples collected on Day 18 then all previous samples were considered positive).

Silver carp DNA was detected in all samples taken on Day 1 (i.e., all samples collected from the surface of silver carp carcasses, from the water flowing over silver carp carcasses, and from silver carp slime on metal sheets). Silver carp DNA was detected in all samples collected on Day 18 from the surface of carcasses and from water flowing over silver carp carcasses. Silver carp DNA was not detected in samples of silver carp slime taken on Day 18. Samples of silver carp slime taken prior to Day 18 are presently being assayed as of the writing of this report and will be provided in the next interim report.

Discussion

The detection of silver carp DNA in these samples confirms that carcasses of Asian carp are a potential source of DNA in environmental samples. If carcasses of Asian carp were transported into areas where Asian carp are not present, the results of our investigation suggest that DNA released from the carcasses could be detected if collected as part of an environmental sample. Based on the results from this trial, removal of Asian carp from barges or boats should be accomplished well before transit into areas where Asian carp are not present. Carcasses detected after transit into areas where Asian carp are not present should be removed immediately and secured in containers such that the carcass cannot contact the water. The portion of the fomite where the carcass was in contact should be sanitized with a solution to denature any residual DNA present where the carcass was in contact with the fomite. However, solutions to denature residual DNA must be evaluated to identify the most economical and environmentally friendly solution prior to use by barge operators.

The detection of silver carp DNA in samples of silver carp slime taken from the metal sheets on Day 1 suggests that fomites (e.g. barges, boats, etc.) could move Asian carp DNA from areas where Asian carp are present to where they are absent. However, the lack of silver carp DNA detection in the samples of slime taken on Day 18 suggests that sequences of silver carp DNA present in slime adhering to fomites does degrade to non-amplifiable sequences. The pending analyses of samples of silver carp slime taken between Day 1 and Day 18 will provide estimates of how long Asian carp DNA would remain viable in slime adhering to potential fomites such as barges or boats. Understanding how rapidly Asian carp DNA degrades in slime could be used to estimate the distance that DNA could be transported on a fomite.

2.4.2 Fish Carcass Transport on Barges

A Guideline for Vessel Operators (Appendix 4) was developed in May 2012 for vessels that enter the CAWS that may be carrying dead silver or bighead carp carcasses, and then depositing them on the upstream side of the barrier by removing the carcasses. The guideline document outlined the protocol for documenting these occurrences, verifying the species, and ensuring removal before the vessel crosses. The four Lock and Dam locations included are: Dresden Island, Brandon Road, Lockport, and TJ O'Brien. Signs were installed at Brandon Road and Dresden Island Lock and Dams to remind vessel operators to clear and remove fish from vessels before proceeding upstream (Figure 2.14). This type of

practice will also inform the ECALS team on the frequency of these events. The protocol is as follows:

If a vessel approaching the lock indicates there is a fish that has jumped onto the vessel, the barge pilot should notify lock operations staff. Once it is safe to do so, the vessel/operator should bag the fish using a standard garbage bag for collection by lock staff. If possible, it is encouraged for deckhands to take a photo of the fish BEFORE it is bagged. Lock staff will take a photo of the fish (or fishes) as best as possible after bagging and record the following information:

- Date and Time
- Location
- Direction of Tow that carried the fish (upstream/downstream)
- Type of vessel and commodity
- Approximate draft of vessel
- Approximate length of fish (optional)
- Pool in which the operator believes the fish entered onto vessel (optional)

During the 2012 shipping season there were three reported incidents. On 10 April two silver carp were found on deck of tow at Lockport Lock and Dam; on 12 April one silver carp was found on a barge at the mouth of the Calumet River; and on 8 June one silver carp was found on two separate barges locking upstream at Brandon Road Lock and Dam.

Figure 2.13 Silver carp on upstream bound barge in the Illinois Waterway

Figure 2.14 Photo of signage installed at Brandon Road Lock and Dam

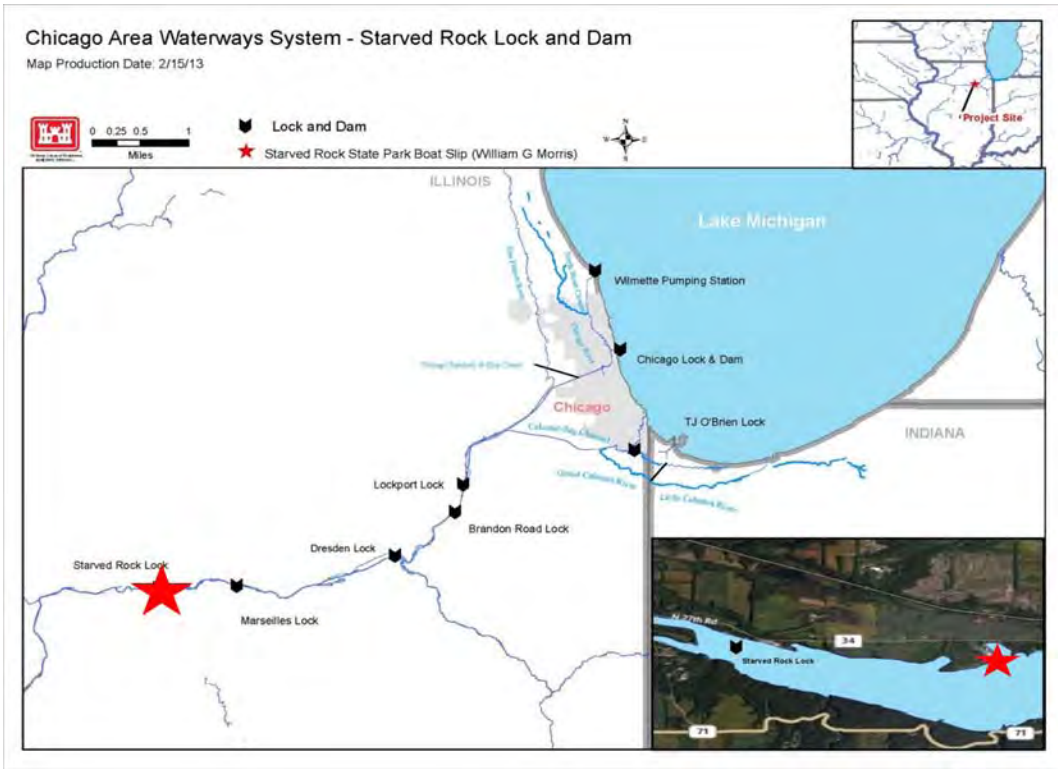
2.5 Sediment eDNA

Collection of sediments in the CAWS took place in October 2012 for the purpose of sediment sorption testing (next section). Additional samples were taken at a stream bank location and from dredged sediment.

Methods

In October 2012, 13 samples were taken from a roughly 1 km extent of the south bank of the Illinois River, starting at the Starved Rock State Park boat slip and moving south -- the locale is about 65 miles south (downstream) of the electric barriers (Figure 2.15). A single 50-ml surface plug of bank sediment (no overlaying water) was taken at each location (which were fairly evenly spaced). Samples were processed at ERDC (DNA extracted with MoBio PowerSoil® DNA Isolation Kit) and assayed using the QAPP markers.

Figure 2.15. Map of the Starved Rock State Park on the Illinois Waterway.



Sediments were also collected from materials associated with the State of Illinois' Mud to Parks Program which transports dredged material from Peoria Lake to publicly owned sites that need topsoil. One location receiving dredged material is the old US Steel site near Calumet Harbor. In October 2012 twenty-eight sediment samples were taken from dredged sediments while being off-loaded at the old US Steel site near Calumet Harbor. The sediments were transported to ERDC in 100ml vials for subsequent DNA analysis (processed in same manner as river bank samples).

Five of the 13 bank sediment samples were positive for silver carp. We did not detect bighead carp in any bank sediment sample. For the Mud to Parks Program sediments, 11 samples tested positive for silver carp, and one sample tested positive for bighead carp.

2.5.1 Sediment Sorption Testing

Activities are scheduled to begin in early 2013.

3 Development of Additional Asian Carp eDNA Markers

The current eDNA markers are both short segments of the mitochondrial DNA control region (or "D-loop") and primarily provide information on presence/absence of that DNA in a sample. Our aim is to develop a suite of markers that provide 1) improved detection probabilities by increasing the number of markers simultaneously assayed, 2) more efficient processing by reducing background non-target PCR amplification and that can be used in real-time PCR (no gel electrophoresis, and reduction or elimination of sequencing), 3) allelic variability (or "polymorphism") to a degree that will allow at least broad estimation or corroboration of Asian carp abundance, and 4) that may provide some indication of the nature or time since deposition of an eDNA sample.

To identify the range of genetic variation that might occur in the CAWS and associated waters, we planned to assay Asian carp of both species from a minimum of 9 North American populations and one Chinese population (targeting 50 fish from each species from each population). Multiple microsatellite DNA loci (targeting 25) developed for use in earlier, published studies will be used to characterize population genetic structure between and within these populations.

We will sequence entire mitochondrial genomes from up to 30 individuals from both silver and bighead carp in order to produce an unprecedented dataset of Asian carp haplotypes. This mtDNA sequence data will provide the material for new marker development, and each new marker will be tested on the acquired samples. DNA sequence variation (single nucleotide polymorphisms (*SNPs*)) between haplotypes will be characterized and used to identify potential markers for population abundance estimation/corroboration (i.e. correlation between estimated population size and numbers of genetic variants (or alleles)). Haplotypes will also be used to characterize population genetic patterns across the North American range of the two species.

We have acquired multiple samples from 29 non-target fish species from the CAWS to allow us to directly test new markers for Asian carp specificity. These assays compliment specificity data obtained from searches of GenBank for non-

target species with DNA sequences that closely match primer sequences used in Asian carp assays[1].

3.1 Sequencing the Asian Carp Mitochondrial Genome

3.1.1 Acquiring Fish Samples for Population Genetic Analyses

We focused on obtaining samples of bighead and silver carp from geographically distant populations, with the assumption that such an approach would maximize the breadth of haplotype diversity that would be observed. By sampling multiple populations for the microsatellite-based analyses, we expect to arrive at a better understanding of the expected genetic diversity within established populations and to provide some insight into general dispersal (or invasion) patterns.

Methods

Genetic material from fish was obtained from several sample types, including fin clips, whole fish, and freeze-dried fish tissues (see Appendix 2 for Asian carp samples, Appendix 3 for non-target fish species).

Results

We obtained approximately 50 samples from each of 18 populations of silver carp and 20 populations of bighead carp. For both species, five pairs of sampling localities overlapped geographically, in which case we will exclude the lower-quality sample from genetic analysis. Three populations of each species were obtained from rivers in Asia. Thus, genetic analysis of silver carp will be carried out using samples from 10 North American and 3 Asian populations, and genetic analysis of bighead carp will be carried out using 12 North American and 3 Asian populations.

Genomic DNA has been extracted from all samples that will be used for haplotyping and population genetic analysis. The freeze-dried samples, which are not a standard way of preserving tissue for DNA analysis, were somewhat problematic. DNA integrity in these samples appears to be adequate for microsatellite DNA genotyping, but samples have experienced some DNA degradation or damage, and could not be used for long-PCR (enrichment step

[1] The National Center for Biotechnology Information's GenBank is an open database with DNA sequences from many species and many loci. We will search this database to identify any species with DNA sequences that might result in PCR amplification in the presence of primers developed for new markers.

employed in mitochondrial genome sequencing, or "haplotyping"). Also, when the freeze-dried samples were worked with, contents were observed to readily disperse as a fine-powder and had to be handled under a fume hood to avoid lab contamination.

3.1.2 Exploring Asian Carp Mitochondrial Genomes for New Markers

Typical DNA marker development is based on one or a few known DNA sequences per species resulting in a potentially significant risk that sequence variability within and among taxa will be underrepresented. Additionally, because of poor sequence coverage in existing databases and Sanger sequencing limitations, marker development is often limited to consideration of just a small number of gene regions. Our aim was to use next-generation sequencing to capture a much greater extent of the within- and between-taxa mtDNA variation than is typically employed in marker development efforts. Our basic assumption was that this comprehensive sequence database, which would provide robust coverage of DNA sequence differentiation between individuals of the same and different species from across the entire mtDNA molecule, would result in more efficient and accurate marker design.

Methods

Carp tissue samples were procured by ERDC personnel and through Jim Lamer (Western Illinois University). DNA was extracted using Qiagen DNeasy Blood and Tissue kits. Long-range PCR using Qiagen Long-Range PCR kits, with reaction recipes and cycling conditions following the manufacturer's protocol, was used to enrich for mitochondrial DNA using 1 or 3 long-PCR reactions that targeted the entire mtDNA of each sample (Miya and Nishida 2000). After library preparation with Nextera DNA sample prep kits, the resulting mtDNA-enriched solutions were sequenced using paired-end sequencing with a next-generation DNA sequencer (Illumina MiSeq), and assembled to obtain whole mtDNA sequences (haplotypes or haplotypes), with high coverage (the number of times a particular nucleotide is represented in the sequence data), for each sample.

Results

Increasing diversity in the sequences used for marker development allowed for more accurate genetic markers. For example, the large numbers of sequenced

genomes used for marker development allowed us to avoid placing primers in intraspecies polymorphic regions of the genome, which would cause unequal amplification efficiencies for different individuals of our target species. Furthermore, the reference sequences of bighead carp and silver carp in GenBank were Asian in origin; the DNA sequences from North America allowed us to develop markers that were more representative of the individuals that will be found in our North American eDNA samples.

To date, complete mtDNA sequences have been obtained for 33 bighead carp and 25 silver carp from 8 locations (Marseilles Reach, LaGrange Reach, Starved Rock State Park, Pool 26, Ohio River/Laketon KY, Arkansas River, Steele Bayou/Big Sunflower/Vicksburg MS area, Red River/Atchafalaya; see Appendix 1 for location details). Average sequence coverage across each whole sequence was 1367X (range 1.5-11971X coverage). Based on preliminary data, mitochondrial sequence divergence between bighead and silver carp species in North America is approximately 4.5% (~750 base pairs), which is consistent with findings from the native Asian range (Li et al. 2009). Genetic variation across populations in North America, revealed by the data thus far, is approximately 0.17% and 0.23% for silver and bighead carp, respectively. Efficient marker development requires sequence differences to be clustered over relatively short regions of the genome for species-specific primer design. Given the limited genetic variation between species spread over the entire 16620 base pair mitochondrial sequence, suitable sites for species-specific primer design were somewhat limited. As a result, a series of general Asian carp primers, which would amplify both bighead and silver carp, were also designed (see Sec. 3.2).

3.2 Testing the Asian Carp Genome

3.2.1 Testing New Markers within Species

When eDNA is highly degraded, as it may often be, detection can be a probabilistic function of what DNA segments happen to be intact when the water is sampled. When an adequate number of copies of a segment (or marker) that is targeted by a PCR assay are intact in a sample a species DNA can be detected. Increasing the number of markers that can used to assay a sample will increase the chance that one or more marker segments will be found in adequate abundance, which increased the odds of detection. Mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) gene regions identified as potential eDNA markers for silver carp, bighead carp, or for the combined species must be tested for 1)

consistent or accurate detection within target taxa and 2) discrimination between target taxa (e.g. silver vs. bighead carp). Desired new markers include:

- Simple presence/absence markers, including markers of different lengths and nuclear DNA markers for which relative abundance might provide some insight into time since deposition
- Quantitative/Real-time PCR markers that facilitate determination of amounts of detected DNA
- Markers with DNA sequence variation (single-nucleotide polymorphisms; SNP data) that can provide some indication of numbers of individual Asian carp responsible for eDNA sample.

Ideally, qPCR data and SNP data will be used as corroborative lines of evidence for estimates of basic population size. Next-generation sequencing or single molecule sequencing may be able to simultaneously provide quantitative and SNP data.

Methods

We sequenced whole mitochondrial genomes from bighead and silver carp (see 3.1.2 above) and aligned them with those obtained from GenBank for fish species that may occur in areas where eDNA samples are collected, including black carp, grass carp, common carp, channel catfish, goldfish, yellow perch, bluegill, largemouth bass, smallmouth buffalo, freshwater drum, and gizzard shad. We visually scanned sequences to identify gene regions that were conserved within species but diverged between species. We used Primer 3 (http://frodo.wi.mit.edu/primer3/) to design primers in the selected gene regions. Markers were tested for amplification of target and non-target species using the QAPP method.

Results

For presence/absence markers, we identified 12 markers to be tested for selective amplification of silver carp, 11 for bighead carp, and 17 to be tested for selective amplification of both Asian carp species to the exclusion of all other species. Testing is presently in progress; preliminary results are presented in Table 3.1. Because these markers encompass most of variable mtDNA sites for which selective primer development is possible, we do not anticipate development of additional markers. Testing of markers using qPCR methods is also in progress; preliminary results are presented in Table 3.2.

Table 3.1. Standard presence/absence markers designed for detection of Asian carp species and result/progress of amplification trials

Species	Primer Names	Progress	Retain	Species	Primer Names	Progress	Retain
SC	SC-1-F and R	Good specificity but weak product-still testing		AC	AC-1-F and R1	Cross reacted with non-target species	NO
SC	SC-1-F and BH-1-R	New-to be tested		AC	AC-1-F and R2	Cross reacted with non-target species	NO
SC	SC-2-F and R	Cross reacted with non-target species	NO	AC	AC-2-F and R	Did not work	NO
SC	SC-3-F and R1	Cross reacted with non-target species	NO	AC	AC-3-F and R1	Cross reacted with non-target species	NO
SC	SC-3-F and R2	Cross reacted with non-target species	NO	AC	AC-3-F and R2	Cross reacted with non-target species	NO
SC	SC-4-F and R	Cross reacted with non-target species	NO	AC	AC-3-F and R3	New-to be tested	
SC	SC-5-F and R	Good specificity, still testing		AC	AC-4-F1 and R	Did not work	NO
SC	SC-6-F and R	Good specificity, still testing		AC	AC-4-F2 and R	Fair specificity-still testing	
SC	SC-7-F and R1	Good specificity, still testing		AC	AC-5-F and R	Cross reacted with non-target species	NO
SC	SC-7-F and R2	New-to be tested		AC	AC-6-F and R	Good specificity, still testing	
SC	SC-8-F and R	Fair specificity, still testing		AC	AC-7-F and R	Weak product, still testing	
SC	SC-9-F and R	Cross reacted with non-target species	NO	AC	AC-8-F and R	Weak product, still testing	
BH	BH-1-F and R1	Did not work	NO	AC	AC-9-F and R	Weak product, still testing	
BH	BH-1-F and R2	New-to be tested		AC	AC-10-F and R	Cross reacted with non-target species	NO
BH	BH-2-F and R	Cross reacted with non-target species	NO	AC	AC-11-F and R	Cross reacted with non-target species	NO
BH	BH-3-F and R1	Cross reacted with non-target species	NO	AC	AC-12-F and R	Did not work	NO
BH	BH-3-F and R2	Fair specificity-still testing		AC	AC-13-F and R	New-to be tested	
BH	BH-4-F and R	Cross reacted with non-target species	NO				
BH	BH-5-F and R1	Cross reacted with non-target species	NO				
BH	BH-5-F and R2	New-to be tested					
BH	BH-6-F and R	Good specificity, detected eDNA	YES				
BH	BH-7-F and R	Cross reacted with non-target species	NO				
BH	BH-8-F and R	Good specificity, still testing					

Table 3.2. TaqMan markers designed for detection of Asian carp species and result/progress of amplification trials.

Species	Marker Names	Result	Retain
SC	SC-TM-1	Good specificity, still testing	
SC	SC-TM-2	Cross reacted with non-target species	NO
SC	SC-TM-3	Cross reacted with non-target species	NO
SC	SC-TM-4	Cross reacted with non-target species	NO
SC	SC-TM-5	New-to be tested	
BH	BH-TM-1	Cross reacted with non-target species	NO
BH	BH-TM-2	Cross reacted with non-target species	NO
BH	BH-TM-3	Good specificity, still testing	
BH	BH-TM-4	Cross reacted with non-target species	NO
AC	AC-TM-1	New-to be tested	
AC	AC-TM-2	New-to be tested	
AC	3	e tested	

Presently, additional markers are being designed using this new sequence information to increase species specificity. Using the published sequences, silver carp and bighead carp DNA sequences were aligned to identify both unique and conserved regions. Using Primer 3.0 v.0.4.0 (http://frodo.wi.mit.edu), new markers were designed to silver carp, bighead carp or both. Sequence specificity was determined by BLAST analysis. Only those markers that amplified targeted species were further considered for marker development. Markers for conventional PCR and quantitative PCR have been designed to target the mitochondrial genome, nuclear genome and ribonucleic acid (RNA). Additionally, a series of markers that amplify different length segments of the same portion of the Asian carp genome has been designed to assess degradation of DNA. This “stair step” marker, or similar markers designed from the Asian carp mtDNA genomes, will be used to evaluate the condition of the DNA in from potential vectors, loading and degradation studies.

Table 3.3. Additional marker development including the "stair step" marker sequences.

Target	Forward	Reverse	Probe	Length
mtDNA	GGTGGCGCAGAATGAACTA	TGGAATTTTACAGCCATGTCC	TTACTTGCATTTGGCTTGGAATCTCAC	67
mtDNA	ATTTGGCTTGGAATCTCACG	CATCATTTAACCAGATGCCAGA	CATGGCTGTAAAATTCCACCCTCCA	78
mtDNA	GGTGGCGCAGAATGAACTA	TCACATCATTTAACCAGATGCC	CCATGTCCGTGAGATTCCAAGCC	108
mtDNA	TATTGTTACTGCCCATGCCT	GAAGGAAAGATGGGGGTAGG	ACTGACTCGTACCCCTAATGATTGGGGCACCT	164
mtDNA	CATAGAATACTACGAAGCACCAT	ATGTTCGGATGTAAAGTGGTATT	TTCGTTGCCACAGGATTCCACGGACTACACGT	160
mtDNA	CACTGGCAGGATGACTAGAA	AGAATTCTGGCTTTGGGAGT	TCGGAGGTTAAATTCCTCCCTAGCGCCCAGAA	165
mtDNA	TTAGAAGCAACAGCAATTAAACC	CATTAGCGATTTTCATTAGTGGG	ACCGAGACCAGTGACTTGAAGAACCACCGTTGT	184
nDNA	CTCCCAGCGTCCCTAAACAA	AACAGGTTTACACTCTGGCA	ATGTTGAGCAATGAGGATGCTGCCGCCCATGT	120
nDNA	GAGCAAGGCTTTTATTGCCC	ATGTGATGGGCAGCTGATAC	ACCAAGTTTCTGGGCCACGAGCATAAACGGCT	117
nDNA	CTTGTCATCTGGTAGAGGAGT	TGAGTGGAACTTTTGTCAAGGT	ACTGGTCTGAAGAGAGGACCTCCATTTCAGGCA	110
rRNA	CAGCTCTACTGTGCCAGA	TTTTCACCACCTCTGGTTTC	----------	130
rRNA	CAGCTCTACTGTGCCAGA	ACTCTCCAAGGTAATGACCAA	----------	254
"Stair-step" Marker				
Step 1	TCCTCCATCAATGAACCCCT	AGCTAGTAGTCAGTGGGAGC	----------	97
Step 2		CATGATTATTGCTGCTGCGG	----------	223
Step 3		ATAATTAGGGCGAATGGGGC	----------	438
Step 4		TTATTCAGCCCATGTGAGCG	----------	581
Step 5		GGCGGTAATTGGAAGGTTCT	----------	847
Step 6		AGTCCTCTCCCTATCACAGC	----------	1387

3.3 Detection of Multiple Alleles

Activities are scheduled to begin in 2013 upon completion of genome testing.

4 Asian Carp eDNA Increased Efficiency and Calibration

Presently, the time from field sampling to analytical results for eDNA can take as long as two weeks, and shorter turn-around times could significantly benefit rapid response actions in the CAWS. Even before a sample can be processed, very intensive fieldwork, followed by laborious sample filtering that can take several hours, are required. ECALS is evaluating ways to reduce time and effort for this process.

ECALS "calibration" tasks consist of studies to determine the relationship between Asian carp size, number, and behavior on eDNA loading (or shedding) by Asian carp. Additional studies will evaluate the effect of environmental factors on degradation of eDNA. Study results will be incorporated into a hydrodynamic model of the CAWS.

4.1 Increasing Efficiency and Throughput of eDNA Processing

There are multiple methods by which DNA can be extracted from eDNA samples. Also, there are multiple qPCR platforms (instruments, reaction mixes, dyes) that may be utilized in future monitoring efforts. Identification of the most cost and time-efficient extraction approach and most robust cross-platform qPCR approach will benefit future monitoring efforts.

Trial 1. Faster Sample Pulping

Methods

Normally, the vortex step (QAPP) in DNA extraction requires around five to ten minutes to ensure thorough shearing of sample filters for DNA lysis. A commercially available instrument, the Mini-Beadbeater-96 (Biospec Products, Inc., Bartlesville, OK), was tested to see if an adequate level of shearing could be accomplished in a much shorter time period.

Ten 1:200 dilutions of Asian carp tissue slurry were filtered through 934-AH filters (QAPP standard). DNA from each filter was extracted using the MoBio PowerWater® DNA Isolation Kit (QAPP), with 5 samples vortexed for ten

minutes at the maximum setting (QAPP) and 5 samples vortexed in Mini-Beadbeater-96 (Biospec Products, Inc., Bartlesville, OK) for 1 min. 20 sec. DNA elutions from both treatments were assayed using standard PCR and with qPCR.

There was no significant difference in apparent DNA yield or quality relative, as measured by PCR success in dilution trials with extracted DNA, to extraction method treatment. We concluded that the faster, bead-beater approach could replace the existing QAPP approach.

Trial 2. Faster DNA Isolation and Purification

Methods

We have been using the MoBio PowerWater, as described in the QAPP, to isolate and purify DNA from water samples. The procedure required about 3.5 hours for every set of 20 samples. Epicentre® Biotechnologies' BuccalAmp™ DNA QuickExtract™ Solution and associated protocols had the potential to reduce the time for this step to about 1 hour. Preliminary trials have indicated that the DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA, USA) may result in a greater yield of amplifiable DNA than the MoBio PowerWater™ DNA Isolation Kit.

Tests with the DNeasy Blood & Tissue Kit are ongoing. The BuccalAmp™ extraction provided efficient and quality DNA yields from tissue, but failed to provide consistently amplifiable DNA from environmental water samples spiked with known Asian carp genomic DNA concentrations. It was apparent that PCR inhibitors persisted in the BuccalAmp™ extractions.

4.2 Optimizing Field Sampling Methods

4.2.1 Test Different Approaches for eDNA Sampling

There are at least 4 different approaches currently being utilized for eDNA sampling (Ficetola 2008, Jerde et al. 2011, Lance and Carr 2012, Goldberg personal communication). We tested 3 different approaches:

- 2-L water sample grab followed by collection of genetic material and other sample constituents on a 1.5 micron, 5.5 cm diameter glass fiber filter; ERDC refers to this approach as the QAPP-type (Quality Assurance Project

Plan) method, as developed by the University of Notre Dame (Jerde et al. 2011)
- 15- or 50-ml water sample grab, followed by centrifugation of sample and pelletization of sample constituents; adapted from Ficetola (2008); ERDC refers to this approach as UMESC-type (USGS Upper Midwest Sciences Center) method
- Pouring approximately 10-L of sampled water, *in situ*, through a 40 micron sieve cloth; ERDC refers to as ERDC-type method (Lance and Carr 2012).

The fourth method, utilized by University of Idaho staff, requires a specially-made field–deployable handheld vacuum and water filtration apparatus. The three tested approaches do not require specially-designed apparatuses, with the exception of a simple PVC pipe & coupler arrangement for the ERDC-type method.

Methods

Three sampling trips were undertaken. In the first (November 2011), 15 locations in the Dresden Island Pool of the Illinois River, were sampled. At each location, both QAPP-type and ERDC-type samples were taken. On the second trip (June 2012), 40 locations were sampled in the Marseilles Pool of the Illinois River. At each location, all three sample types were procured (including 5-10 UMESC-type samples per location) from the top of the water column and the sampling time required to take ERDC samples was recorded. On the third trip (October 2012), 12 locations in the Dresden Island Pool of the Illinois River, (roughly same locations as first trip) were sampled. At each location, all three sample types were procured (including 5 UMESC-type samples per location) from the top of the water column.

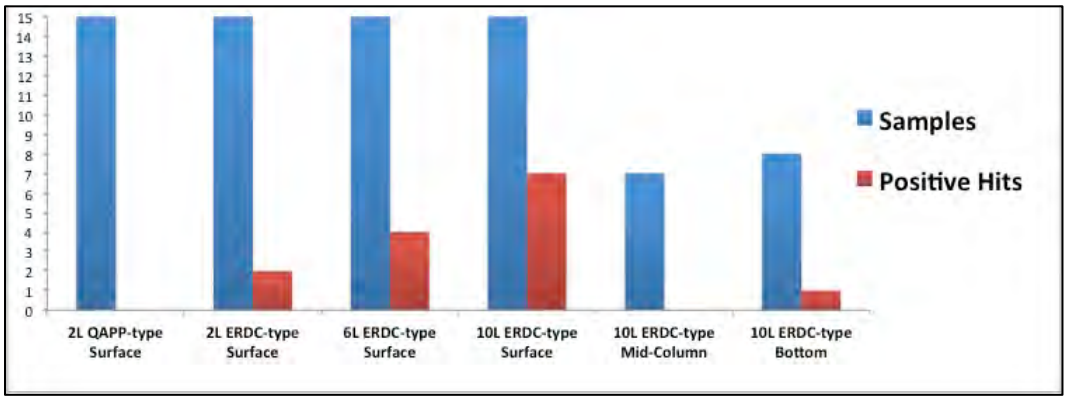
QAPP-type samples were processed and assayed according to QAPP guidelines. ERDC-type samples were processed in the same manner, with the exception of not requiring collection onto a filter paper prior to shipping (essentially filtered *in situ* in field). UMESC-type samples were centrifuged for 30 min. at 4,000 RPM and the pellet was preserved in 200µL of 0.01M Phosphate Buffered Saline solution. DNA was extracted from pelleted materials in UMESC-type samples using the Qiagen DNeasy Blood & Tissue Kit. Samples from the Marseilles Pool were surprisingly lacking in positive hits for any of the sampling approaches and it was surmised that high levels of PCR-inhibiting compounds may have been present in the samples and persisted through DNA extraction and purification. We executed an additional DNA purification step on all samples from this trip

using protocols for the ZR-96 DNA Clean & Concentrator (Zymo Research Corporation, Irvine, CA) kit and re-assayed the samples. In all cases, all samples were assayed with 8 replicate PCR reactions, followed by DNA sequencing of at least one putative positive result (when present) per sample.

Results

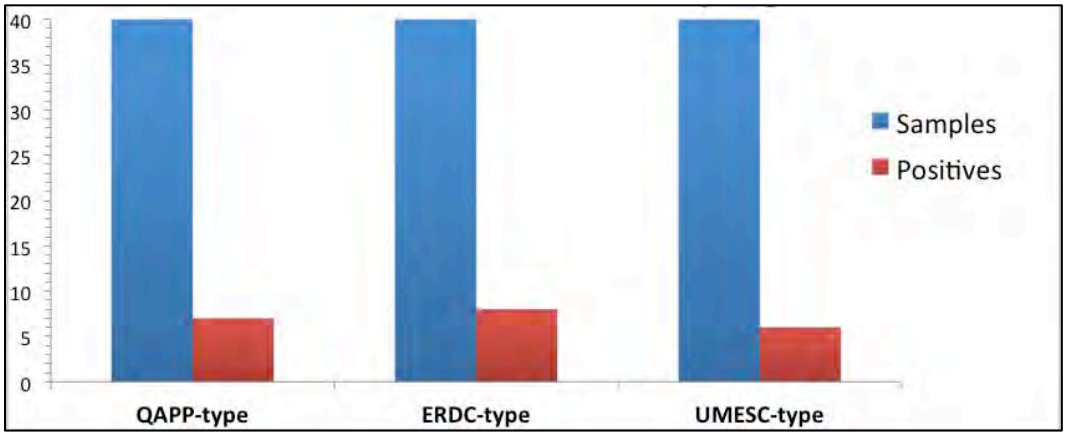
The results of the first alternative sampling trial indicated that ERDC-type sampling performed as well or better than QAPP-type sampling (Figure 4.1). Further, the results indicated sampling from the top of the water column was as effective as sampling from mid-column or from the bottom of the water column.

Figure 4.1. Alternative sampling experimental results, trial 1.



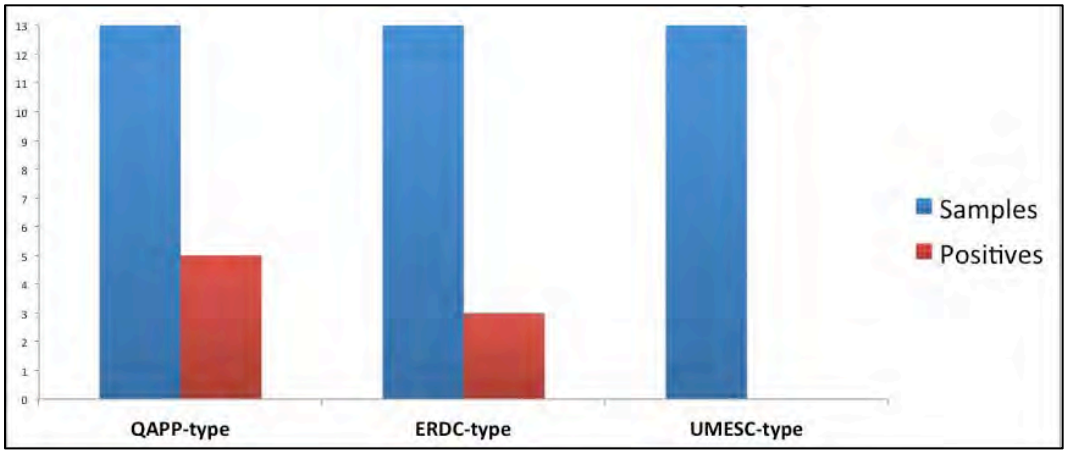
For the second trial, there appeared to be complete inhibition within samples Figure 4.2). After a second DNA purification with ZR-96 kits a significantly higher numbers of positives were observed, with ERDC-type samples seemingly outperforming QAPP- and UMESC-type samples. However, contamination was observed in some field negative controls associated with some ERDC-type samples, and after eliminating these samples from consideration, there appeared to be little difference in performance among the three methods. The additional cleaning with the ZR-96 kits did not occur until after the third sampling trip had been conducted, so the results, including contamination issues, did not inform protocols for the third trip.

Figure 4.2. Alternative sampling experimental results, trial 2.



The third trial exhibited similar contamination issues with ERDC-type samples (Figure 4.3). We surmised that field contamination of ERDC-type samples was likely due to handling the sieve cloths on-board a sampling vessel that had recently been used to capture and handle Asian carp and that conditions on board (e.g. wind) likely lead to too-frequent contact between handlers, sieve cloths (difficult to set onto sampling pipe in wind), and potentially contaminated surfaces (e.g. life jackets, vessel railings, etc.). While the outside of the vessel was decontaminated, it was presumed the live well and safety equipment had most likely not been decontaminated before reuse. Of the apparent contamination-free results from that trip, QAPP-type samples had the highest occurrence of positive hits for Asian carp, with ERDC-type having fewer, and UMESC-type having none. In order to eliminate the high degree of field contamination risk associated with the ERDC-type approach, new protocols for prepping sampling apparatuses for use and for contaminant prevention would have to be devised.

Figure 4.3. Alternative sampling experimental results, trial 3.



4.3 Loading Studies

The purpose of Task 3.2.1 is to investigate the amount of eDNA that is shed by silver and bighead carp under controlled conditions (treatments) using qPCR), and estimate the relationship (if any) between these variables. The different conditions to be tested include: biomass (or number of fish), temperature, and diet type. Studies will be executed with both silver and bighead carp, run independently. Diet and biomass studies will be performed with fish from two age classes (sub-adult and juvenile), the temperature study will only be conducted with sub-adult. We will also investigate sperm as a source of eDNA over time in static water conditions.

Methods

Shedding of eDNA Experiments

Fish will be held in tanks under flow-through conditions at a turn-over rate of roughly once a day (379 L tanks for sub-adult fish and 40 L tanks for juvenile fish). Preliminary studies showed that in the first week, eDNA quantities spiked, but they leveled off by the second week. Therefore, 50 ml water samples will be taken throughout the 3.5 weeks of the study, but only samples from the second week and on will be analyzed with qPCR. Each qPCR run will include a standard curve to infer the quantity of eDNA per sample. Samples from each tank (experimental unit) will be averaged over the 1.5 weeks of sampling to determine

the average eDNA shedding rate of the fish. These averages can then be compared to determine treatment effects on shedding rates.

Shedding/ Degradation of Sperm eDNA Experiments

Milt was collected from silver and bighead carp, sperm counts were determined, and subsamples were placed in static aquaria at different dilutions. Water samples (50 ml) were taken at the time sperm was added to tanks as well as 24 hours, 48 hours, 72 hours, 7 days and 10 days after. These samples were processed and extracted for DNA. They will be analyzed with qPCR as well.

Collection and Extraction of eDNA

For all loading and sperm eDNA degradation studies, 50 ml, below surface water samples are taken. Cellular debris containing eDNA is collected by centrifugation. DNA samples are extracted with a phenol/chloroform protocol.

qPCR of samples

We used the qPCR primers and probe designed by Jon Amberg (see Section 1.3). The 20 µL volume reactions were comprised of 10.0 µL of 2X SsoFast Probes MasterMix (Bio-Rad), 375 nM of each primer, 125 nM of probe, 5 µL DNA and 2 ul molecular grade water. All assays were carried out in triplicate using a BioRad CFX 96 Real-Time PCR Platform. PCR conditions were as follows: 95°C for 2 minutes for initial denaturation of the DNA, then 40 cycles of 95°C for 5 seconds and 58°C for 10 seconds.

Currently the primers used are not specific to either bighead or silver carp, nor are they specific to the genus. Thus the quantities estimated by qPCR in this set of experiments, are only comparable among lab experiments with single species, and not field sampling (as these primers will amplify other species' DNA). Nevertheless, we will be able to observe if shedding rates are affected by treatments.

Expected results

We expect that as biomass increases so will the amount of detectable eDNA. We do not know if eDNA shedding rates will be affected by temperature (a previous study by Takahara (2012) did not find a correlation between temperature and eDNA shed by common carp). We also expect that feeding fish will shed more eDNA than non-feeding fish, and that diet type (rough versus soft) may also affect shedding rates. Results of these studies will provide information necessary to determine the degree to which qPCR can be used to determine abundance or biomass of bighead and silver carps from environmental DNA samples.

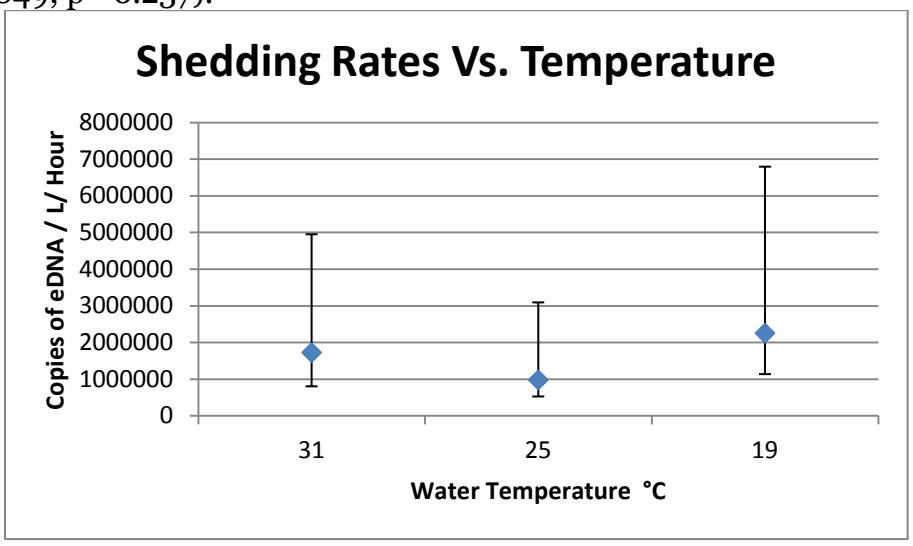
Results

Preliminary studies showed that eDNA shedding rates are consistent over different water flow rates. Thus, under controlled conditions, single juvenile silver carp shed at similar rates once water flow rate is corrected for (Table 4.1). Currently, one experiment assessing how temperature affects shedding rates on silver carp sub-adults has been completed. We found no effect of temperature on shedding rates (One Way ANOVA on log transformed data, $F(2, 6) = 1.849$; $p = 0.237$), a result consistent with results of Takahara et al. (2012) (Figure 4.4). Preliminary studies of eDNA from sperm in water showed that eDNA was detectable for at least 17 days.

Table 4.1. eDNA shedding rates of juvenile silver carp. N=1 for each flow rate.

Shedding Rates	**Low Flow (1 L/hour)**	**Medium Flow (2 L/hour)**	**High Flow (3 L/hour)**
Copies of eDNA/L	6053.771	3303.704	1815.978
Copies of eDNA/L/hour	6053.771	6607.409	5447.935

Figure 4.4. Shedding rates of sub-adult silver carp under three temperature treatments (n=9). Data shown are back-transformed averages and 95% confidence intervals. No significant differences observed (One Way ANOVA; $F(2, 6) = 1.849$; $p = 0.237$).



4.4 Degradation Studies

Activities are scheduled to begin in early 2013.

4.5 Validation Trials

Artificial stream and outdoor mesocosm activities are scheduled to begin in 2013 upon completion of the loading and degradation studies. The design of pond studies will be informed by the results of the laboratory eDNA shedding studies and by the results of eDNA degradation studies occurring at UMESC and ERDC. The goal of the pond studies is test predictions generated using laboratory information in a more complex system.

4.6 Fish Supply

Task 3.3 activities have included acquisition of field Asian carp specimens as well as maintenance of live juvenile Asian carp at the ERDC Aquatic and Wetland Research Center. These activities will supply fish to support other ECALS tasks. Fish were obtained from hatcheries (Osage Fish Hatchery, Osage, Missouri; USGS, Columbia, MO; Bonnet Carre Spillway, Louisiana). In addition, ERDC is prepared to collect sub-adult and adult Asian carp in the Mississippi River and tributaries using nets and electro-shocking as the need arises. ERDC also prepares protocols for the Institute of Animal Care Committee and permits (Lacy Act) for interstate transport. The following paragraphs provide summaries of transport and husbandry techniques to maintain fish in the laboratory.

Transportation Containers – Fish are transported from the hatchery to ERDC in commercially manufactured "live haul" tanks carried in the bed of a ¾ ton pickup truck (Figure 4.5). Tanks are filled with fresh water and fish transferred to tanks during early morning and driven directly to ERDC (estimated transport time of 10-12 hours). Water is re-circulated and aerated continuously during the trip so that water changes will be unnecessary. Dissolved oxygen, water temperature, and condition of fish are checked at 2-hour intervals. Low dissolved oxygen or elevated temperatures will be mitigated with compressed oxygen and ice. Any fish that die in-route are removed from the tank, placed in ice chests, and brought to ERDC for documentation. No fish is discarded during the trip. Voucher specimens of dead fish are preserved in formalin and deposited in a museum collection (e.g., Mississippi Museum of Natural Science, University of Louisiana Museum of Natural History). The remainder are desiccated and buried on-site at ERDC.

Holding Facilities – Fish are maintained in a secure laboratory facility (Figure 4.6) at ERDC with a closed-system of individual re-circulating tanks (Figure 4.7). The laboratory is approximately 400 m^2 in a building that is approximately 1672 m^2. A generator is automatically started during local electrical failures to ensure that

Figure 4.5. Transportation truck with aerated live wells.

Figure 4.6. Laboratory building where silver and bighead carp are housed.

there are no interruptions of power. Doors lock automatically and are opened by punching in a multi-character security code. Water and sewer service is provided by City of Vicksburg (MS). Water entering the building is potable and requires de-chlorination prior to aquaculture use; water leaving the building enters the municipal sewage system and receives tertiary treatment.

Figure 4.7. One of four 1500 gallon recirculating aquaculture tanks.

Holding tanks are made of fiberglass, reinforced plastic insulated to an R-9 factor. Each tank is 8 feet in diameter, filled to a depth of 4-ft for a working water volume of 1,500 gallons. Tanks have a dual stand pipe center drain with 1/16" mesh surrounding the external 6" stand pipe (Figure 4.8). The inner 3" stand pipe is elevated 42" to protect from complete tank drainage. All tanks are re-circulating. There are two tanks per filtration system with a 2.86 times per hour turnover rate. Six 800 µm bag filters act as the primary mechanical filter, 4.4 ft^3 bead filter acts as the fine particulate mechanical filter, a low space bioreactor acts as the biological filtration, and two UV sterilizers outputting 42,667 $\mu Ws/cm^2$ dosage are capable of killing microorganisms such as bacteria, viruses, molds, and algae (Figure 4.9). Water from tanks does not come in contact with any other aquatic system (natural or man-made). Water from tanks is removed from a bottom drain and flows directly into laboratory floor drains. Tank water is never discharged directly into the environment. The laboratory is "double escape-proof" – a single room within a larger secure building. Tanks are also "double escape-proof" – isolated tubs that do not connect with the environment and which are filtered when emptied.

Figure 4.8. Recirculating tank details. From left to right: A close up image of the 1/16" mesh that covers the external stand pipe. The middle photo is an overview photo of the tank. The right image shows the 6" external stand pipe covering the 3" internal stand pipe that drains the tank.

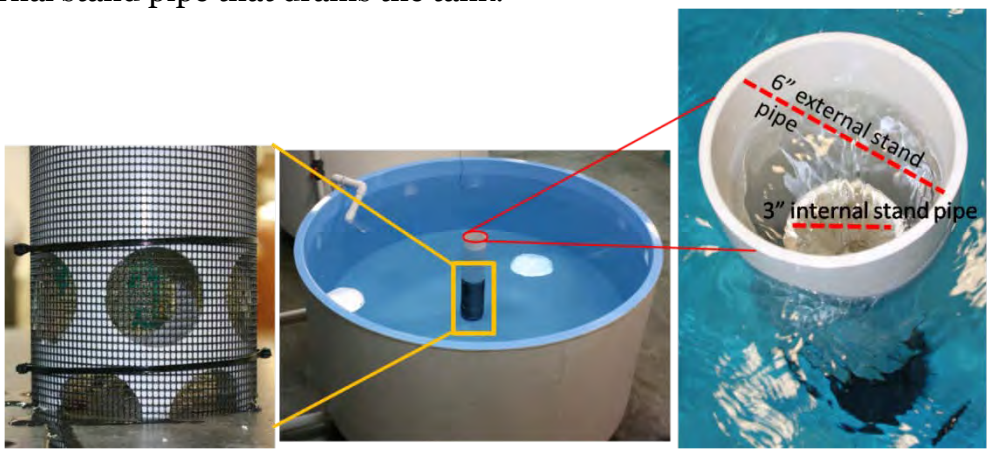
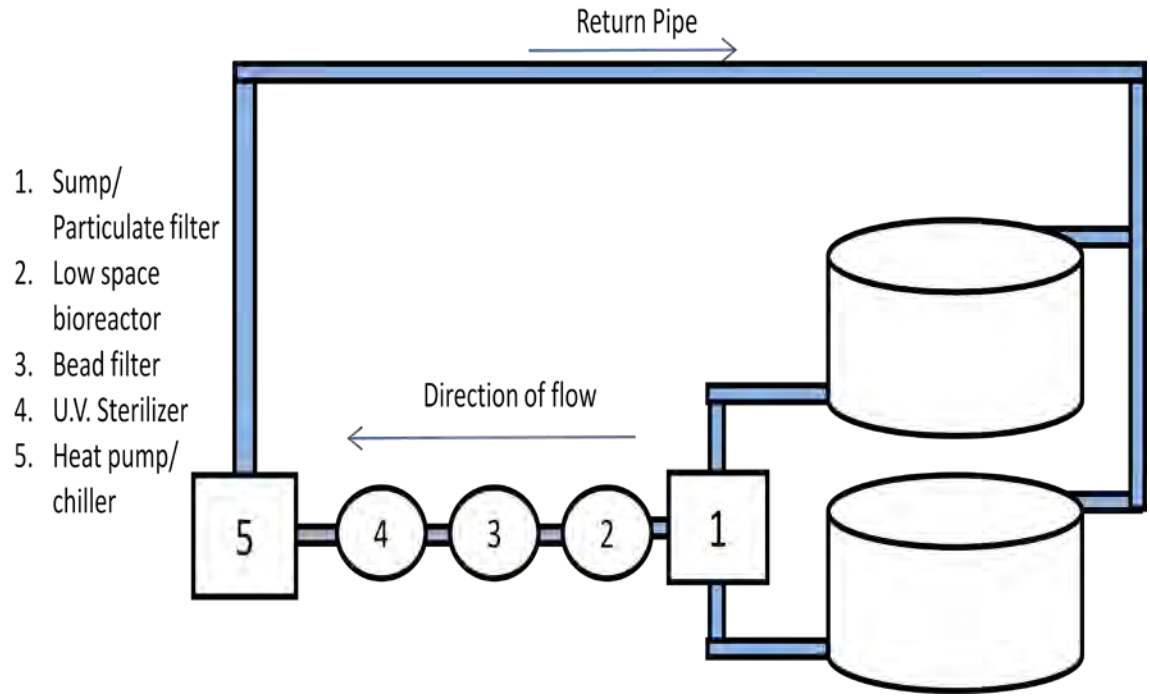


Figure 4.9. Recirculating aquaculture system flow drawing.



Feeding and Care – Water quality is monitored daily during the first week of acclimation and twice weekly afterwards, and recorded in a Daily Care Record. Water temperature, conductivity, dissolved oxygen, and pH are measured using a

Hydrolab multi-parameter water quality probe dedicated to laboratory use (i.e., not used in the field). Turbidity (in NTUs) is measured concurrently with a Hach 2100P turbidimeter. Ammonia, nitrites, and nitrates are measured using aquarium test kits (i.e., indicator solutions provide colorimetric estimates of concentrations). Water quality outside normal ranges or exhibiting abrupt (e.g., within two days) changes and which could be physiologically stressful are immediately reported to the principal investigator and laboratory manager.

Fish are fed at least twice each day, in late morning and late afternoon, by hand. They are fed as much food as they will eat in 10 minutes. Uneaten food is removed after that. Fish feeding on dry foods (flakes, pellets) are fed smaller quantities 2-4 additional times during the day by mechanical battery-powered feeders (Eheim Model 3582000). Carps are fed flakes and pellets. Time of feeding is recorded and entered on experiment data sheets. Frozen and live foods and kept in a laboratory micro-fridge dedicated to that purpose.

4.7 Hydrodynamic Modeling

Activities commenced in fall 2012 upon acquisition of funding, but notable progress has already occurred as of December 2012. The hydrodynamic grid has been completed for the area to be modeled, and protocols have been established to enable passing of information back and forth between the hydrodynamic (i.e. water quality) and eDNA transport (i.e. water quality) parts of the model. Results from other ECALS investigations (e.g. eDNA degradation studies) will be incorporated into the eDNA transport model when available. Three-dimensional simulations of the hydrodynamics of the barrier area are also underway, and the electrical field modeling is in preliminary development at present.

4.8 Probability Modeling

Activities are presently not funded.

Literature Cited

Asian Carp Regional Coordinating Committee. 2012a. *FY 2012 Asian Carp Control Strategy Framework*. February 2012. http://asiancarp.us/documents/2012Framework.pdf.

Asian Carp Regional Coordinating Committee. 2012b. *Monitoring and Rapid Response Plan for Asian Carp in the Upper Illinois River and Chicago Area Waterway System*. ACRCC Monitoring and Rapid Response Workgroup. May 2012. http://asiancarp.us/documents/2012mrrp.pdf.

Battelle. 2010. Short-term analysis service (STAS) on Revised Final Independent External Peer Review Report Environmental DNA (eDNA) Science and Methodology. Prepared for U.S. Army Corps of Engineers Ecosystem Restoration Planning Center of Expertise, Rock Island District. December 7, 2010. http://www.usace.army.mil/Portals/2/docs/civilworks/Project%20Planning/eDNA_review.pdf

Deagle B. E., A. Chiaradia, J. McInnes, and S. N. Jarman. 2010. Pyrosequencing faecal DNA to determine diet of little penguins: is what goes in what comes out? Conservation Genetics 11: 2039-2048.

Doehm J., A. Juen, K. Nagiller, S. Neuhauser, and M. Traugott. 2011. Molecular scatology: how to improve prey DNA detection success in avian faeces? Molecular Ecology Resources 11: 620-628.

Ficetola, G.F., C. Miaud, F. Pompanon, and P. Taberlet. 2008. Species detection using environmental DNA from water sample. Biology Letters 4: 423-425.

Guillaumet, A., B. Dorr, G. Wang, J. D. Taylor, R. B. Chipman, H. Scherr, J. Bowman, K. F. Abraham, T. J. Doyle, and E. Cranker. 2011. Determinants of local and migratory movements of Great Lakes double-crested cormorants. Behavioral Ecology doi: 10.1093. 2011.

Hatch, J. J., and D. V. Weseloh. 1999. Double-crested cormorant (*Phalacrocorax auritus*). Account 441 *in* A. Poole and F. Gill, editors, *The Birds of North America*. The Academy of Natural Sciences, Philadelphia, PA, and the Ornithologist's Union, Washington, DC.

Jerde, C.L., A.R. Mahon, W.L. Chadderton, and D.M. Lodge. 2011. "Sight-unseen" detection of rare aquatic species using environmental DNA. Conservation Letters 4: 150-157.

Koller, D., and N. Friedman. 2009. *Probabilistic Graphical Models*. The MIT Press, Cambridge, MA p. 1,229.

Lance, R. F., and M. R. Carr. 2012. *Detecting eDNA of Invasive Dreissenid Mussels, Report on Capital Investment Project*. ANSRP Technical Notes Collection. ERDC/TN ANSRP-12-2. Vicksburg, MS: U.S. Army Engineer Research and Development Center.

Li, S.F., J.W. Wu, Q.L. Yang, C.H. Wang, Q. Chen, D.C. Chapman, and G. Lu. 2009. A comparison of complete mitochondrial genomes of silver carp Hypophthalmichthys molitrix and bighead carp Hypophthalmichthys nobilis: implications for their taxanomic relationship and phylogeny. Journal of Fish Biology 74:1787-1803.

Miya, M, and M. Nishida. 2000. Use of mitogenomic information in Teleostean molecular phylogenetics: a tree-based exploration under the maximum-parsimony optimality criterion. Molecular Phylogenetics and Evolution 17: 437-455.

Pearl, J., 1988. *Probabilistic Reasoning in Intelligent Systems: Networks of Plausible Inference*. Morgan Kaufmann Publishers, Inc., San Francisco, CA, p. 552.

Pearl, J., 2000. *Causality: Models, Reasoning, and Inference*. Cambridge University Press, New York, NY, p. 464.

Schultz, M.T., T.D. Borrowman, and M.J. Small. 2011. *Bayesian Networks for Modeling Dredging Decisions*, Engineer Research and Development Center Technical Report ERDC-EL/TR-11-14, US Army Corps of Engineers, Vicksburg, MS, p. 74.

Sutherland R.M. 2000. Molecular Analysis of Avian Diets. PhD Thesis, University of Oxford, UK.

Takahara, T., T. Minamoto, H. Yamanaka, H. Doi, and Z. Kawabata. 2012. Estimation of Fish Biomass Using Environmental DNA. PLoS ONE 7(4): e35868. doi:10.1371/journal.pone.0035868.

Thomsen P. F., J. Kielgast, L. L. Iversen, P. R. Møller, M. Rasmussen, and E. Willerslev. 2012. Detection of a diverse marine fish fauna using environmental DNA from seawater samples. PLoS one 7:e41732.

United States Army Corps of Engineers. 2012. *Quality Assurance Project Plan, eDNA Monitoring of Invasive Asian Carp in the Chicago Area Waterway System*. USACE Chicago District, May 2012. http://www.asiancarp.us/documents/USACE-eDNA-QAPP.pdf.

Appendix 1 Distribution of eBird Locations for Various Species year-round (2005-2012) from Havana, IL to the CAWS (Figures A-1 through A10)

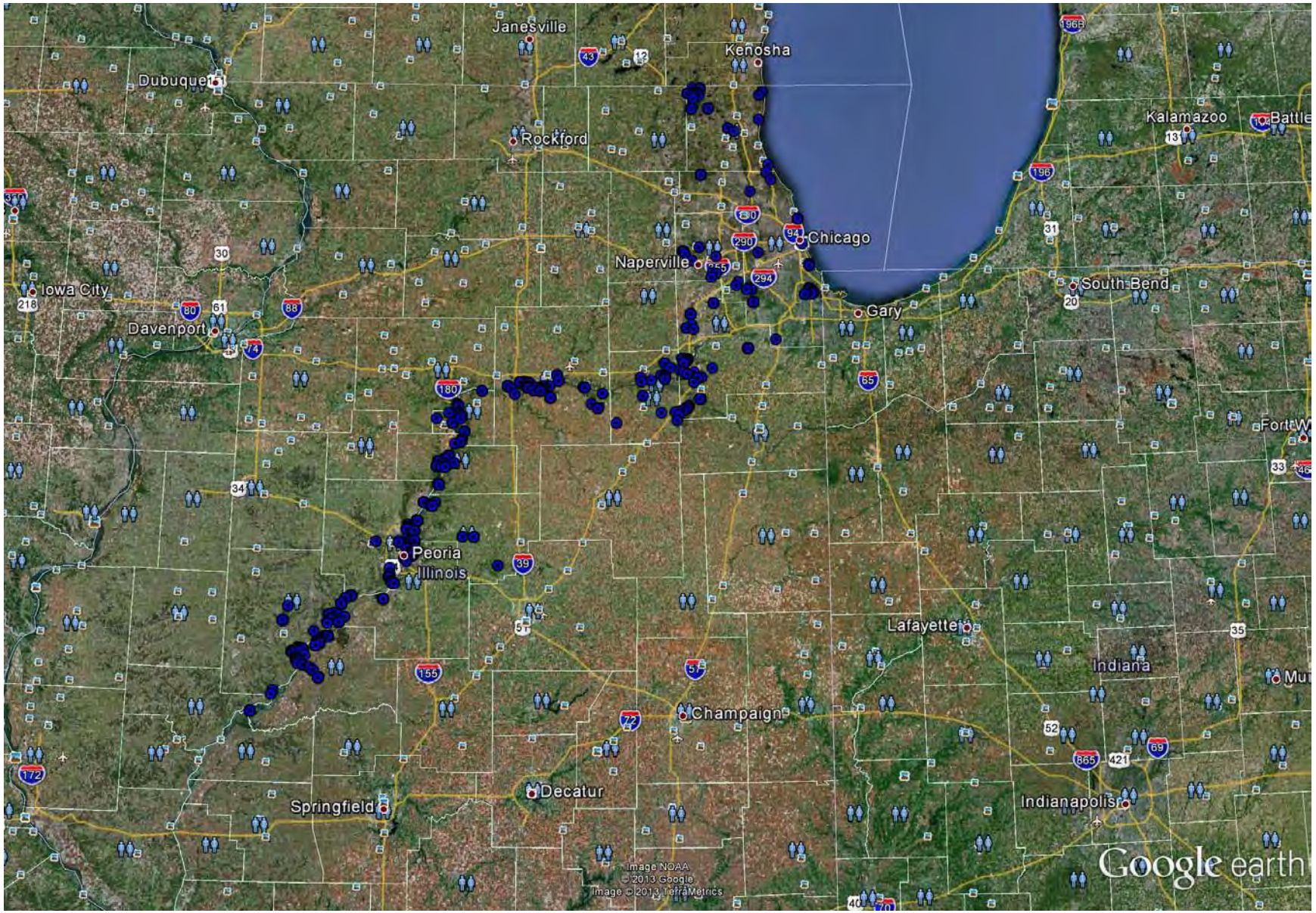


Figure A-1. American white pelican.

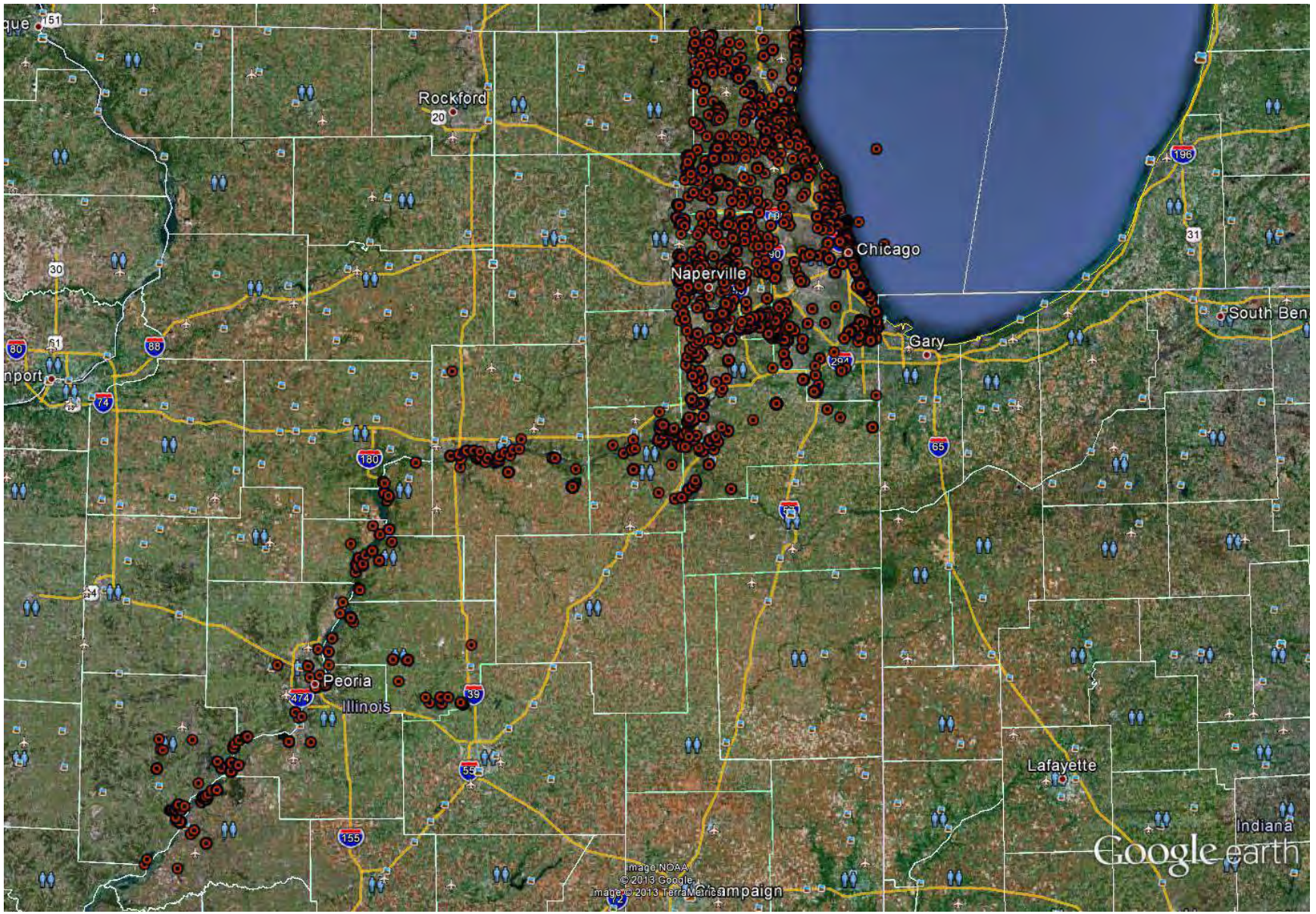


Figure A-2. Double-crested cormorant.

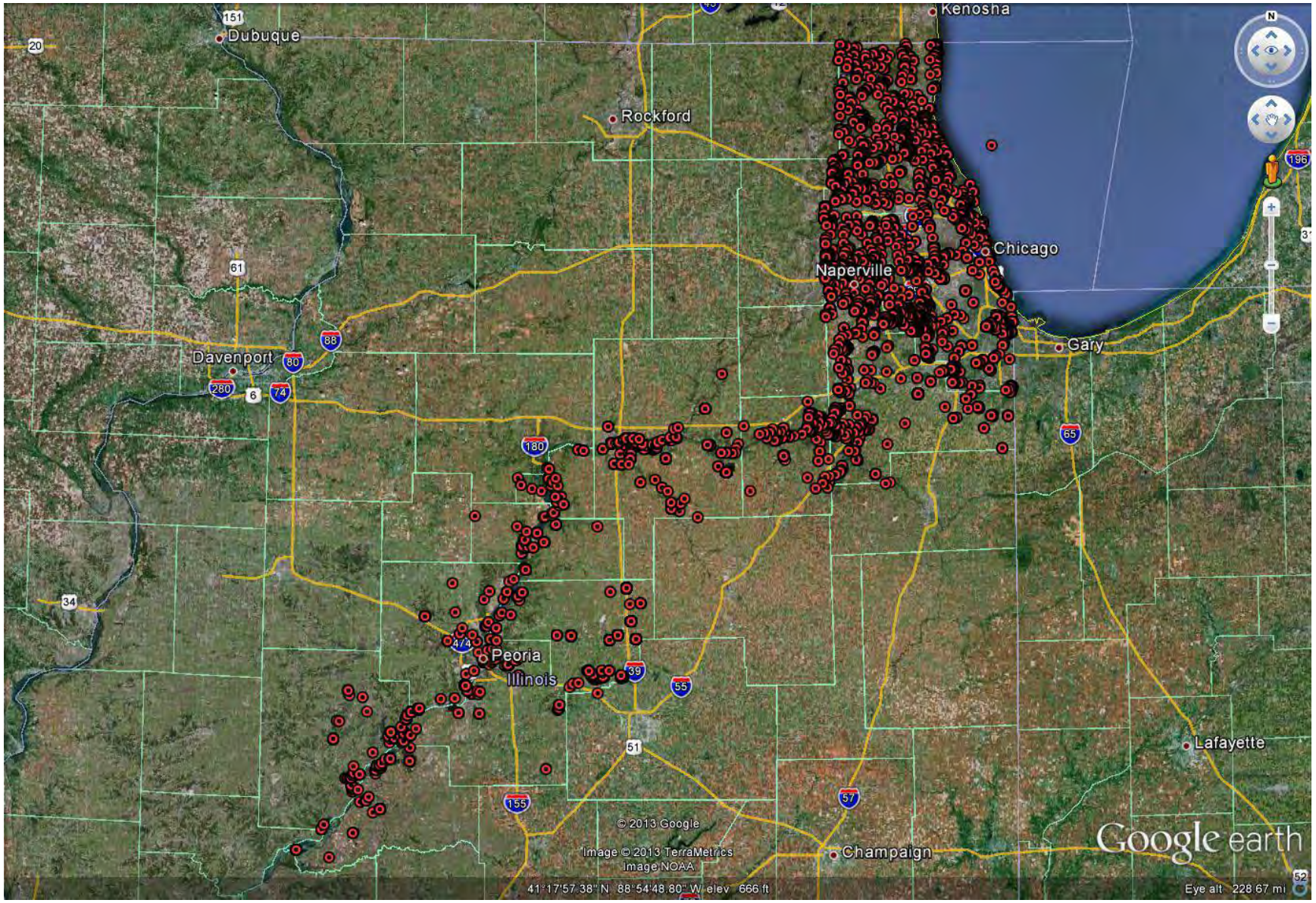


Figure A-3. Great blue heron.

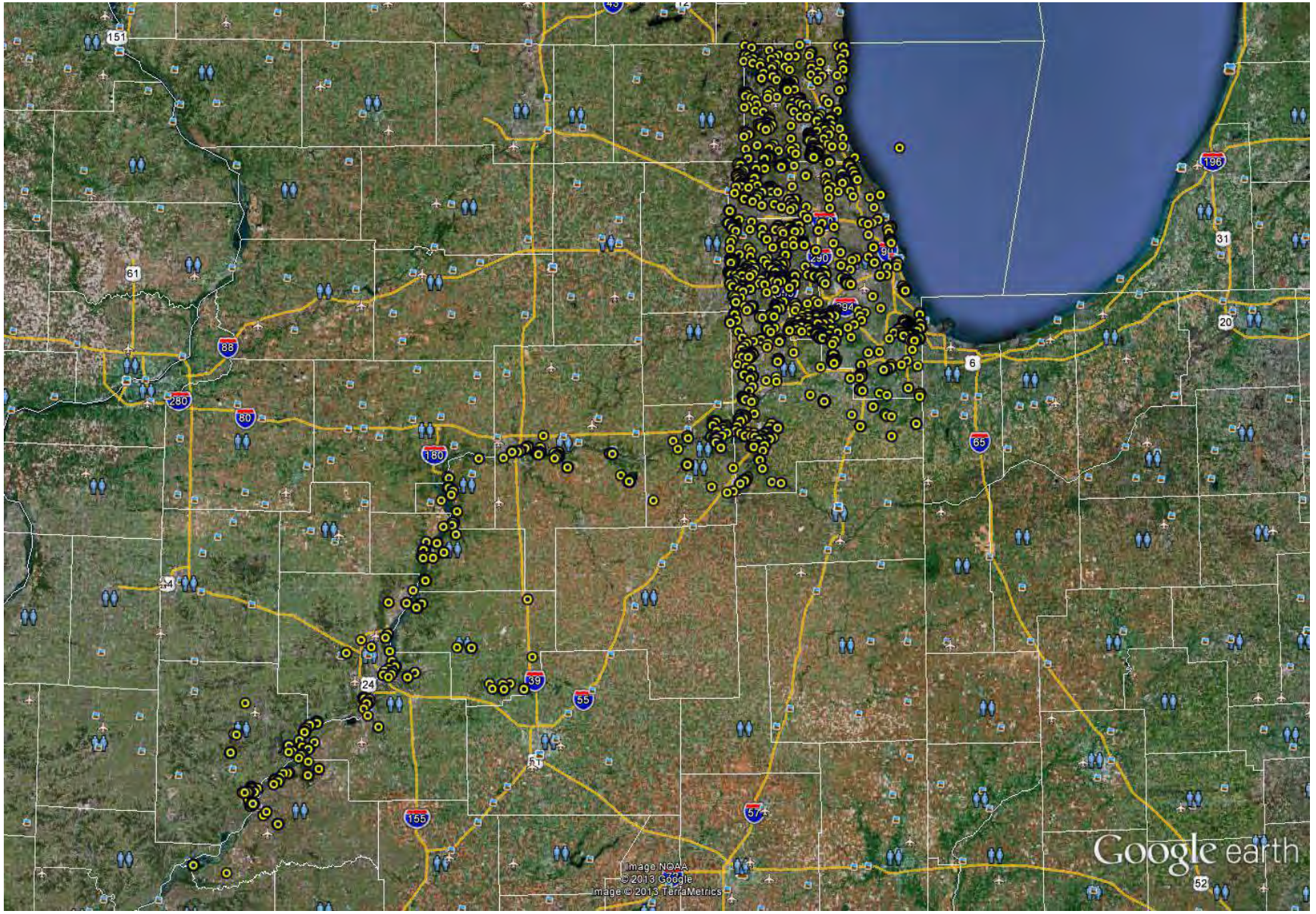


Figure A-4. Great egret.

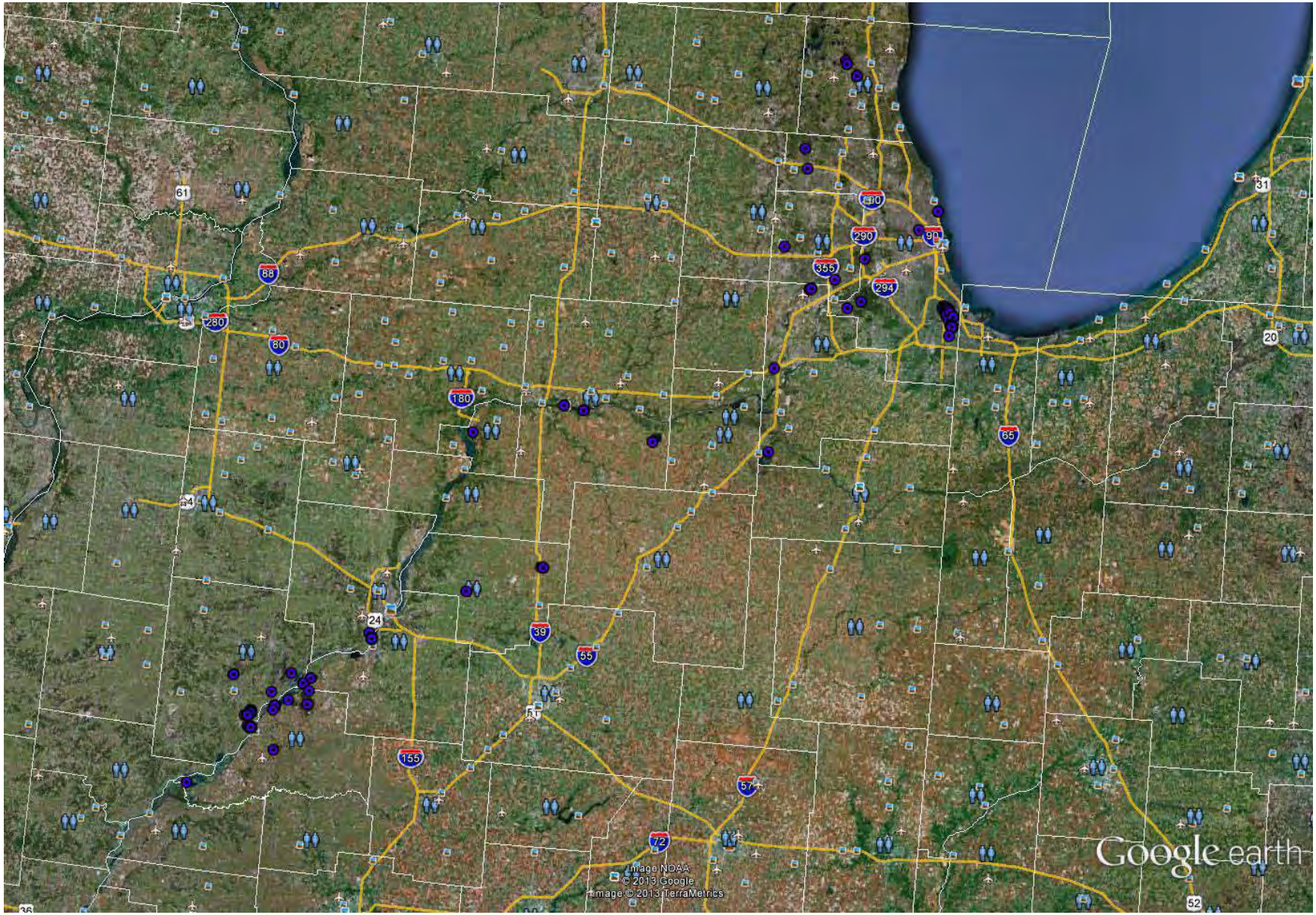


Figure A-5. Snowy egret.

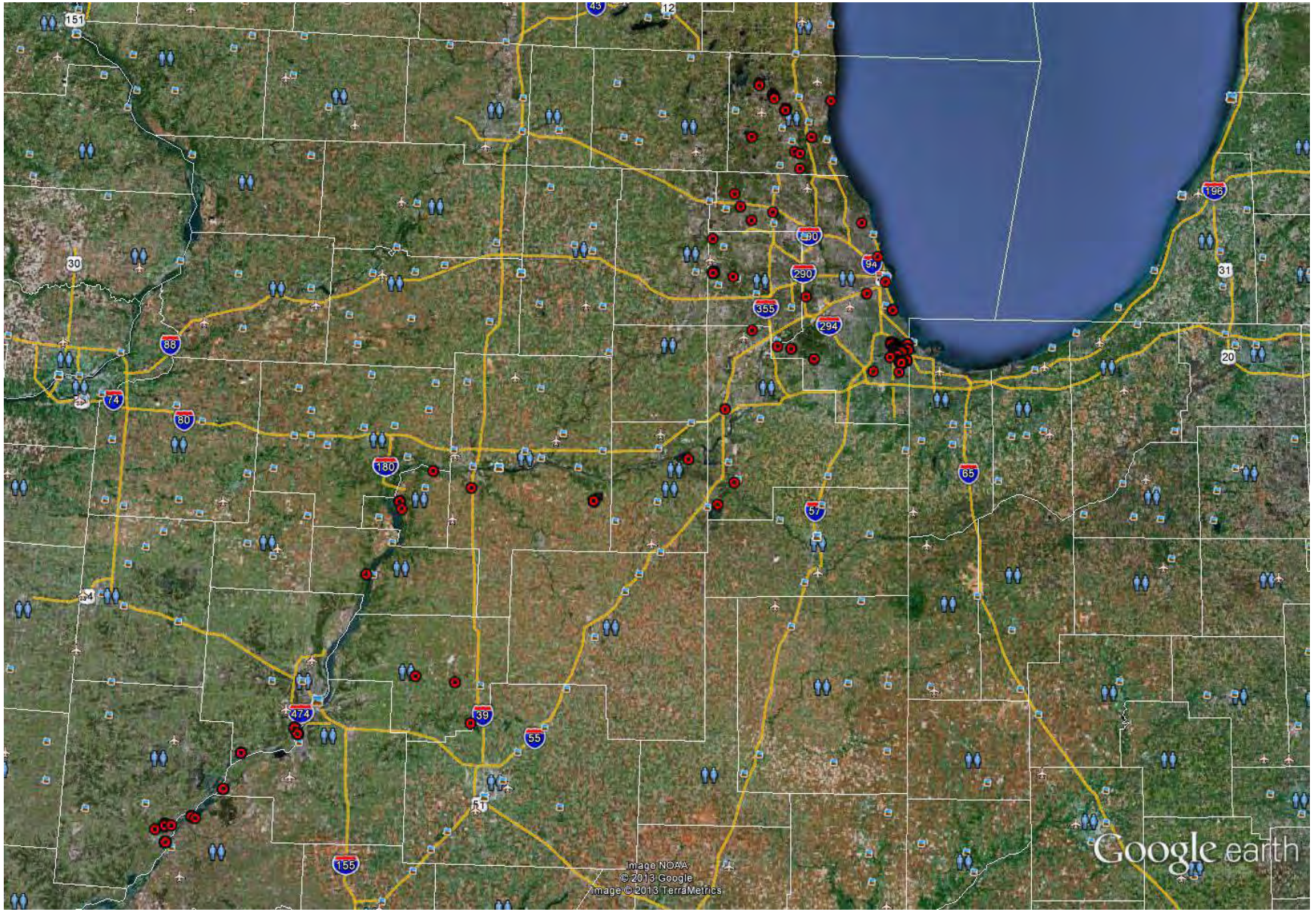


Figure A-6. Little blue heron.

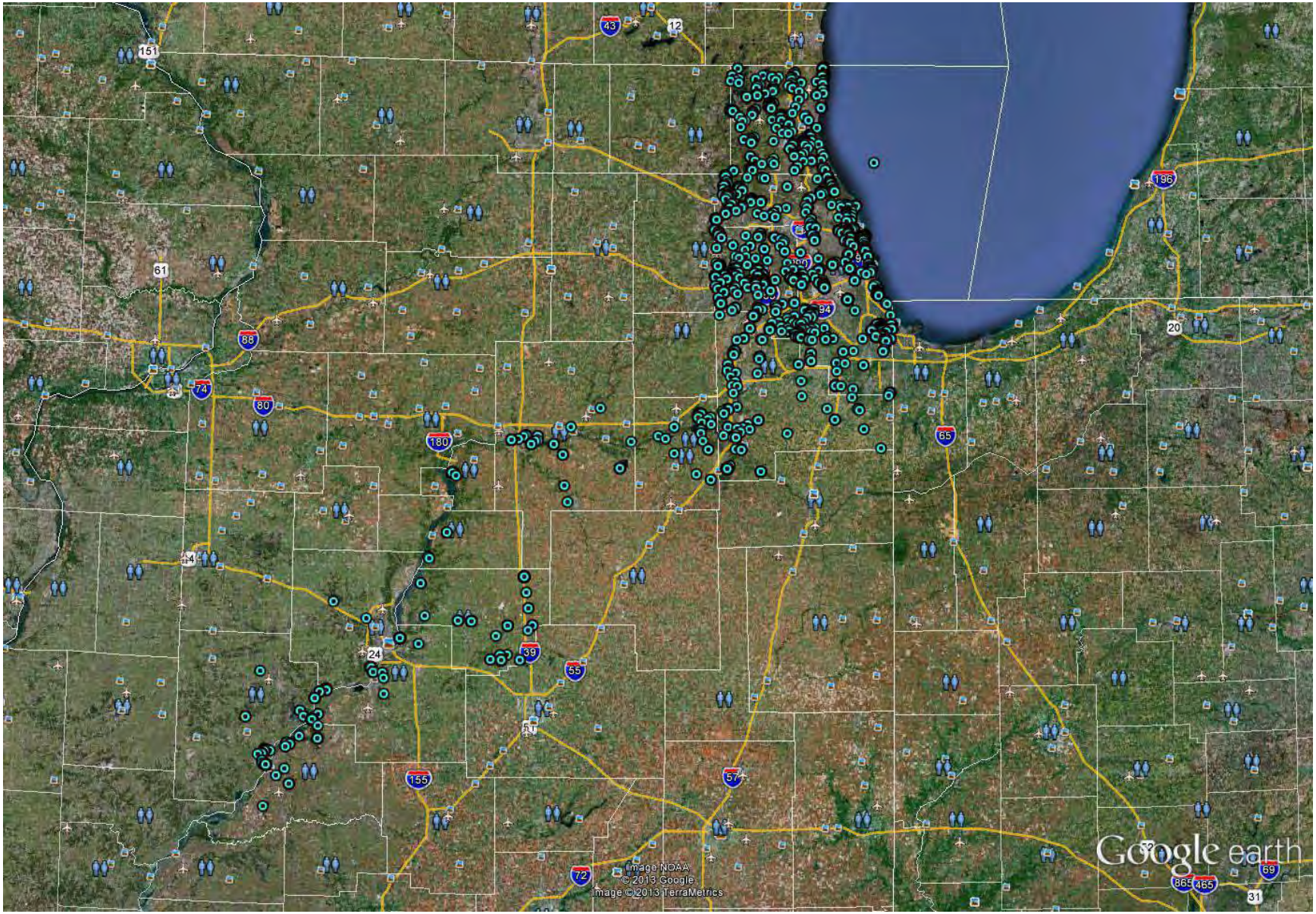


Figure A-7. Green heron.

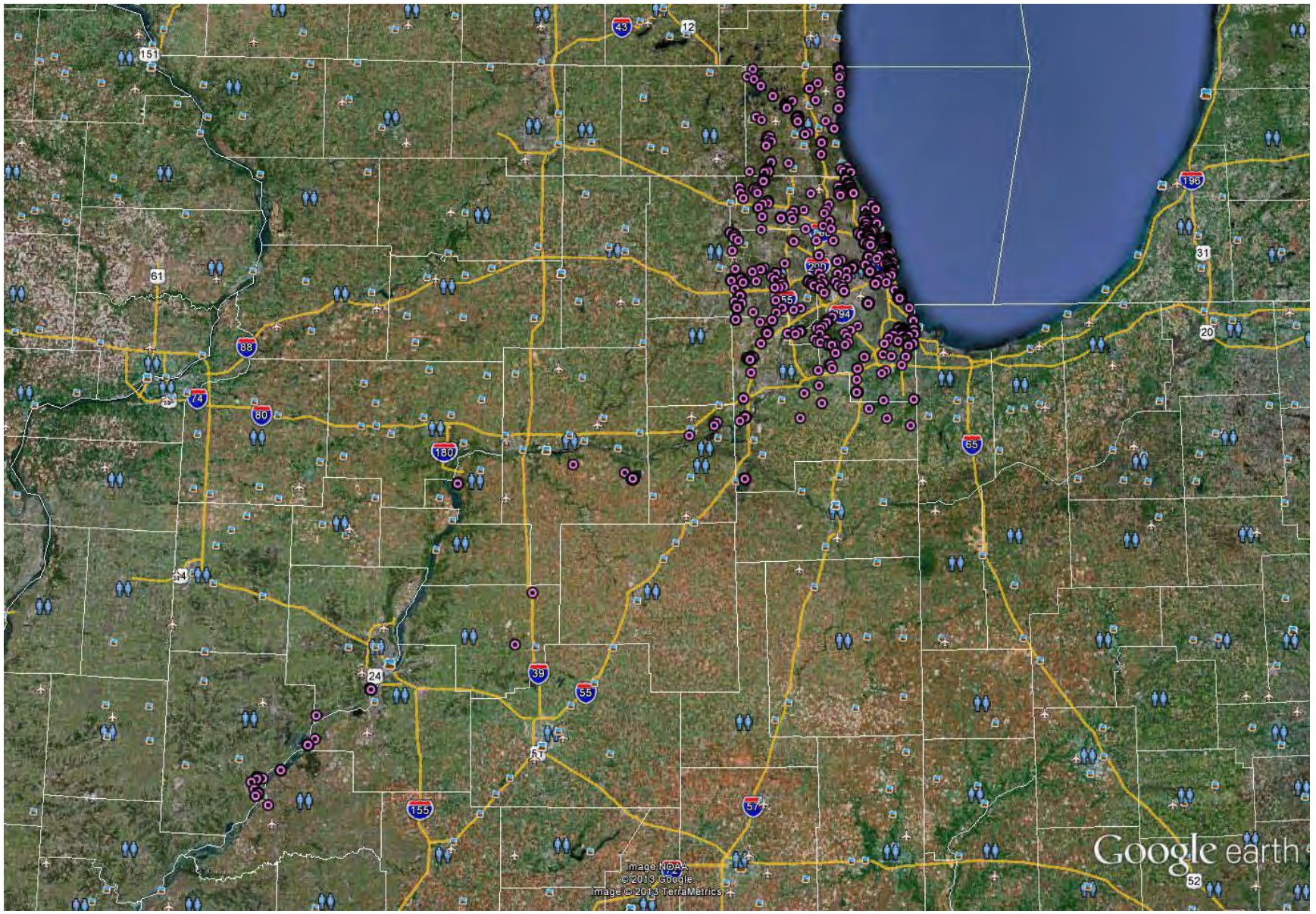


Figure A-8. Black-crowned night-heron.

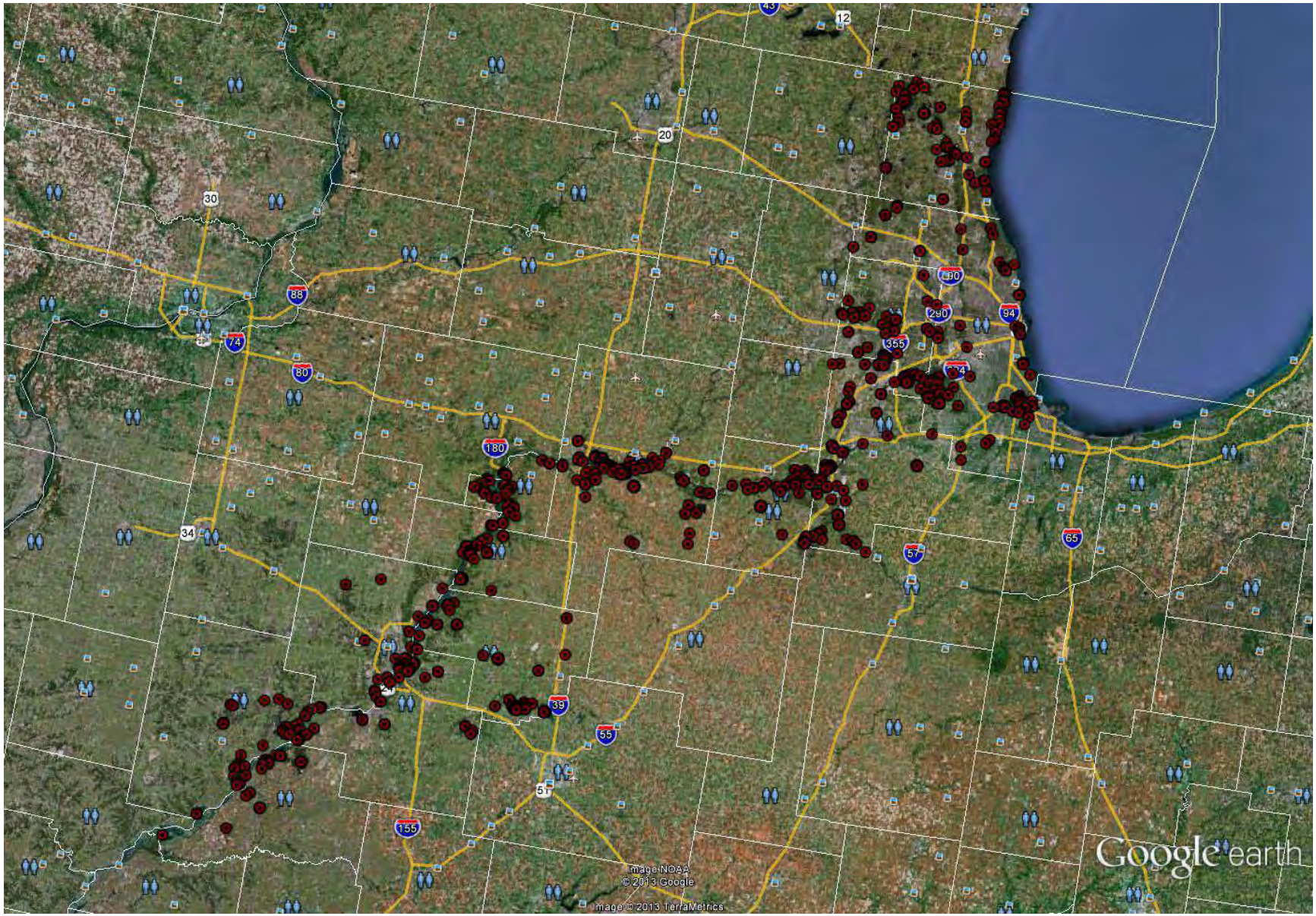


Figure A-9. Bald eagle.

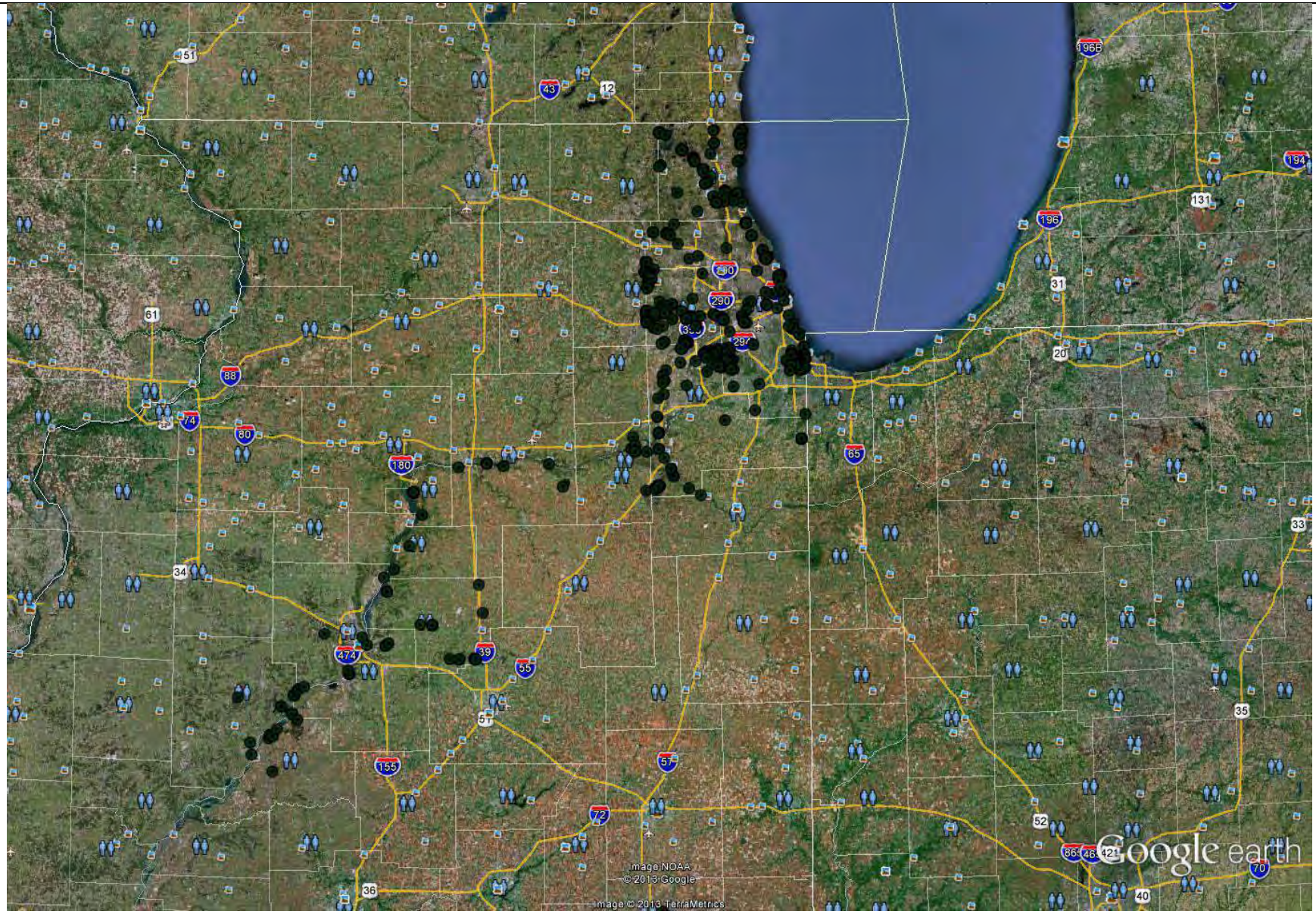


Figure A-10. Osprey.

Appendix 2 Silver and Bighead Carp Collections

Information about samples of silver carp (SC) and bighead carp (BH) collected for genetic analysis, including sampling location, source, number of samples (N), type of samples, and whether they DNA was extracted from the sample to be used for genetic analysis.

Species	Location	Location code	Source	*N*	Type	DNA Extracted?
SC	Site 1: Around Vicksburg: Yazoo, Steele Bayou, Big Sunflower	S1/SB/BS-SC	Kilgore	50	Fin Clip	Yes-50 Quick Extract
SC	Site 2: Red River, Atchafalaya	S2/RR/Atch-SC	Kilgore	50	Fin Clip	Yes-50 Quick Extract
SC	Site 3: Arkansas River	S3/AKR-SC	Kilgore	48	Fin Clip	Yes-48 Quick Extract, 16 also Qiagen
SC	Ohio River: confluence of Ohio and Mississippi Rivers	OHR-SC	Kilgore	48	Fin Clip	Yes-48 Qiagen
SC	LaGrange Reach, Illinois River	ILAG-SC	Lamer		Fin Clip	Yes-48 Qiagen
SC	Marseilles Reach, Illinois River	IMAR-SC	Lamer	47	Fin Clip	Yes-47 Qiagen
SC	Laketon, KY, Lower Mississippi River	MKY-SC	Lamer	--	Fin Clip	No-Same location as OHR
SC	North of Vicksburg, MS, Lower Mississippi River	MMS-SC	Lamer	--	Fin Clip	No, same location as site 1
SC	Near Blair, NE, Missouri River north of Omaha	MOO-SC	Lamer	43	Fin Clip	Yes-43 Qiagen

SC	Throughout pool 20, Upper Mississippi River	PL20-SC	Lamer	48	Fin Clip	Yes-48 Qiagen
SC	Ellis bay, southern end of pool 26, Upper Mississippi River	PL26-SC	Lamer	47	Fin Clip	Yes-47 Qiagen
SC	Big Sioux, Upper Missouri	SVC-S	Chapman	8	Freeze-dried	Yes-8 Qiagen
SC	LaGrange Reach, IL River	SVC-IL	Chapman	--	Freeze-dried	No-Same location as ILAG
SC	Near Columbia, MO	SVC-CI	Chapman	25	Freeze-dried	Yes-25 Qiagen
SC	Illinois River	SVC-IR	Chapman	--	Freeze-dried	No-already have 2 sets of IR samples IMAR, ILAG
SC	Amur River, China	AR-SC	Lu	22	Fin Clip	Yes-22 Qiagen
SC	Yangtze River, China	YR-SC	Lu	18	Fin Clip	Yes-18 Qiagen
SC	Pearl River, China	PR-SC	Lu	22	Fin Clip	Yes-22 Qiagen
BH	Site 1: Around Vicksburg: Yazoo, Steele Bayou, Big Sunflower	S1/SB/BS-BH	Kilgore	50	Fin Clip	Yes-50 Quick Extract, 33 also Qiagen
BH	Site 2: Red River, Atchafalaya	S2/RR/Atch-BH	Kilgore	25	Fin Clip	Yes-25 Qiagen
BH	Site 3: Arkansas River	S3/AKR-BH	Kilgore	5	Fin Clip	Yes-5 Qiagen
BH	Ohio River: confluence of Ohio and Mississippi Rivers	OR-BH	Kilgore	29	Fin Clip	Yes-29 Qiagen

BH	LaGrange Reach, Illinois River	ILAG-BH	Lamer	46	Fin Clip	Yes-46 Qiagen
BH	Marseilles Reach, Illinois River	IMAR-BH	Lamer	49	Fin Clip	Yes-48 Qiagen
BH	Laketon, KY, Lower Mississippi River	MKY-BH	Lamer	--	Fin Clip	NO-Same location as OR
BH	North of Vicksburg, MS, Lower Mississippi River	MMS-BH	Lamer	--	Fin Clip	No-Same location as site 1
BH	Near Blair, NE, Missouri River north of Omaha	MOO-BH	Lamer	48	Fin Clip	Yes-48 Qiagen
BH	Throughout pool 20, Upper Mississippi River	PL20-BH	Lamer	48	Fin Clip	Yes-48 Qiagen
BH	Ellis bay, southern end of pool 26, Upper Mississippi River	PL26-BH	Lamer	32	Fin Clip	Yes-32 Qiagen
BH	Big Sioux, Upper Missouri	BHC -S	Chapman	20	Freeze-dried	Yes-20 Qiagen
BH	LaGrange Reach, IL River	BHC-IL	Chapman	--	Freeze-dried	No-Same location as ILAG
BH	Near Gavin's Point Dam, NE	BHC-N	Chapman	23	Freeze-dried	Yes-23 Qiagen
BH	Near Columbia, MO	BHC -CI	Chapman	30	Freeze-dried	Yes-30 Qiagen
BH	Illinois River	BHC -IR	Chapman	--	Freeze-dried	Already have IR samples IMAR, ILAG (from Lamer)-not used

BH	Franklin Island Conservation area	BHC -YOY	Chapman	--	Freeze-dried	Already have IR samples IMAR, ILAG (from Lamer)-not used
BH	Amur River, China	AR-BH	Li	10	Fin clip	Yes-10 Qiagen
BH	Yangtze River, China	YR-BH	Li	29	Fin clip	Yes-29 Qiagen
BH	Pearl River, China	PR-BH	Li	22	Fin clip	Yes-22 Qiagen

Appendix 3 Non-Target Fish Collections

Most were obtained from Lake Calumet, IL. The ECALS goal for non-target fish is 10 individuals from each of 30 species. Currently, 29 species are represented in our collection.

Common Name	Species name	Total
Brown Bullhead	*Ameiurus nebulosus*	3
Freshwater Drum	*Aplodinotus grunniens*	2
Goldfish	*Carassius auratus*	5
Quillback	*Carpiodes cyprinus*	2
Grass Carp	*Ctenopharyngodon idella*	10
Spotfin Shiner	*Cyprinella spiloptera*	1
Common Carp	*Cyprinus carpio*	4
Mirror Carp	*Cyprinus carpio* sp.	4
Gizzard Shad	*Dorosoma cepedianum*	4
Channel Catfish	*Ictalurus punctatus*	5
Smallmouth Buffalo	*Ictiobus bubalus*	5
Black Buffalo	*Ictiobus niger*	9
Brook Silverside	*Labidesthes sicculus*	2
Green Sunfish	*Lepomis cyanellus*	4
Pumpkinseed Sunfish	*Lepomis gibbosus*	5
Orangespotted Sunfish	*Lepomis humilis*	3
Bluegill	*Lepomis macrochirus*	7
Smallmouth Bass	*Micropterus dolmieu*	2
Largemouth Bass	*Micropterus salmoides*	7
White Perch	*Morone americana*	2
White Bass	*Morone chrysops*	2
Round Goby	*Neogobius melanostomus*	1
Golden Shiner	*Notemigonus crysoleucas*	1
Emerald Shiner	*Notropis atherinoides*	3
Yellow Perch	*Perca flavescens*	3
Bluntnose minnow	*Pimephales notatus*	1
White Crappie	*Pomoxis annularis*	5
Black Crappie	*Pomoxis nigromaculatus*	3
Flathead Catfish	*Pylodictis olivaris*	5

Appendix 4 Carcass Removal Guidelines

Recommendations for
Removal of Asian Carp Carcasses on Vessels

2012

DO NOT MOVE ASIAN CARP CARCASSES UPSTREAM OF EITHER THE DRESDEN ISLAND OR BRANDON ROAD LOCK AND DAMS

Thank you for assisting with Asian Carp Control and Management Efforts!

RECOMMENDATION

As your vessel approaches the **Dresden Island and Brandon Road Lock and Dams:**

- **Check** your decks for fish before entering Dresden Island and/or Brandon Road Lock and Dams
- **Notify** the lock operation personnel that you have a fish on board
- **Remove** the fish and place in a garbage bag

Lock operations staff will dispose of bagged fish and may collect basic information to note probable source or location of fish. These recommendations are not intended to, and should not, result in delays or disruptions in the lockage process, or disruptions to vessel operators. Please exercise caution and ensure all actions and operations are undertaken in a safe manner.

There will be signs at the Dresden Island and Brandon Road Locks reminding you to clear your decks of fish, but we encourage you to employ this protocol at every lock upstream of the Brandon Road Lock and Dam in the Illinois Waterway.

Asian carp have been observed (dead and alive) on decks of boats and barges throughout the Illinois Waterway. Clean, wash, or rinse decks (if it is safe to do so and in compliance with applicable federal and state regulations) to remove fish materials from your vessel downstream of the electric barrier.

Any documentation of fish found in this fashion is appreciated. You can email any information or questions to kevin.irons@illinois.gov or phone 217.557.0719. Leave a brief message with call back information.

1 Illinois Department of Natural Resources www.asiancarp.us

Asian Carp Regional Coordinating Committee

Leading the way in Asian carp control and management.

CONTACT: Questions, comments, or concerns

Kevin Irons, Aquatic Nuisance Species Program Manager
Illinois Department of Natural Resources
One Natural Resources Way, Springfield, Illinois. 62702.
Phone: 217.557.0719 **Email**: kevin.irons@illinois.gov

More information about Asian carp is available on www.asiancarp.us

ILLINOIS DEPARTMENT OF NATURAL RESOURCES

PURPOSE:

This protocol describes the recommendations for disposing of fish, including Asian Carp upon discovery on boats and vessels, moving upstream through the Illinois Waterway System. We encourage you to follow this procedure when a fish carcass resembling an Asian Carp is found on your boat or vessel. The main reason for implementing this recommendation involves our current monitoring strategy for Asian Carp above the barrier, using a genetic surveillance tool called Environmental DNA (eDNA). This tool, used since 2009, detects the genetic material (eDNA) of Bighead and Silver Carp in the water. Asian Carp DNA is released from slime, feces, urine, and blood as well as other fish flesh which in time will degrade in the environment. It is possible that DNA material from a carcass transported by boats and vessels is contributing to the eDNA that we detect in the water above the barrier. To reduce the potential for distorting eDNA results, we request that these practices be followed to the extent possible. Additionally, there may also be a possibility that fish being transported could survive, and introduced to waters above the electric barrier if they are returned to the water.

Commercial vessels have been cooperative in reducing the moving of water through bilge and ballast water management near the electric barrier. We would like to remind recreational traffic that they should also monitor water onboard their vessels. ***Movement of bilge and ballast across the electric barrier is prohibited*** *(title 33 of the Code of Federal Regulations [CFR] part 165.923; information on the rule can also be found in the "Waterway Users" tab on AsianCarp.us). Please plan accordingly to reduce spread of all Aquatic Nuisance Species.*