Environmental DNA Calibration Study

Interim Technical Review Report

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U.S. FISH & WILDLIFE SERVICE

This report contains technical results from the Environmental DNA Calibration Study (ECALS) through August 2014, and represents an update to the February 2013 interim technical review report. 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 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	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 time 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 stand (~20 bases) of nucleaic 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 Bighead and Silver Carps 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	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 multi-year study to improve the understanding and interpretation of the detection of Asian carp DNA in environmental samples (eDNA) used in early detection monitoring. 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 received 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). Initial ECALS work on potential eDNA vectors included studies of storm sewer transport, fish-based fertilizers, fisheries gear, bird transport and deposition of eDNA, fish carcasses and transport on barges, and sediment eDNA. In 2013 ECALS further investigated vessel hulls, fishing nets, and sediment eDNA. The vessel hull and fishing net trials in 2013 confirmed the presence of very large quantities of eDNA potentially transported by these vectors. Sediment studies confirmed that eDNA sorption on sediments can take place, and low-level long-term eDNA releases are possible from undisturbed and re-suspended sediment (based on 21-day study). It was concluded that sediment eDNA contributions to water samples are likely minimal unless turbidity is high or particulate matter is captured on the filter (current practice is to analyze filtered solids rather than the dissolved filtrate fraction).

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.

The haplotype sequences generated in this study allowed us to develop markers that were better tailored for detecting individuals from the Asian carp populations found in North America. Due to the limited se- quence variation between species across the mitochondrial genomes, design of effective species-specific cPCR and qPCR markers was difficult. The markers designed for this study were chosen from mtDNA regions that were the most divergent between species, which should correspond to markers with the high- est likelihood of being species-specific. However, in initial trials, amplification of DNA from at least one non-target species was observed for most markers. Further testing of cPCR and qPCR assays is needed to adequately evaluate the efficacy of the markers developed in this study. Optimization of PCR is needed, followed by assessment of method sensitivity, and finally further field-testing.

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 has evaluated 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. Initial ECALS work compared different DNA extraction kits, evaluated different field sampling protocols (filtration, centrifugation, sieve cloth), and compared sampling from different depths in the CAWS. Based on these efforts, changes to the Quality Assurance Project Plan (QAPP) have been made.

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 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 also investigated sperm as a source of eDNA over time in static water conditions.

Loading Studies - Loading studies have shown that eDNA shedding rates are consistent over different water-flow rates. We found no correlation between water temperature and eDNA shedding rates. We ob- served a correlation between eDNA loading and fish

density. Studies of eDNA from sperm in water showed that eDNA was detectable for at least 17 days. Water samples can be quantified for carp eDNA using qPCR. The sensitivity of the assay will depend on time-consuming but necessary optimization of the analysis (temperature, reagent amounts). The eDNA signal can be highly variable, likely reflecting clumping eDNA distribution. Fish shed eDNA at higher rates when fed, likely due to cells sloughed off in the excrement. Non-fed fish still shed detectable amounts of eDNA but at approximately 10-fold lower rates compared to the fed fish (especially those fed algae). Non-fed silver carp juveniles and bighead sub- adults shed lower amounts of eDNA relative to algae-fed fish, but differences were not statistically significant. For the bighead sub-adults, shedding rates trended similarly to the bighead juveniles and sub-adult silvers, but did not show significant pairwise differences in shedding rates.

Degradation Studies - Trials assessing the influence of environmental factors on the degradation rate of eDNA were conducted for temperature, pH, microbial loads, light, and water turbulence. The majority of eDNA in these trials degraded either rapidly or very rapidly over a few days, but in all cases a small por- tion of eDNA persisted beyond 2 to 4 weeks. We have identified temperature, pH, and microbial load as factors that can affect degradation, particularly that higher temperature, higher pH, and higher microbial loads are associated with more rapid DNA degradation. In every case, DNA abundances capable of producing positive detections with qPCR or conventional PCR assays persisted beyond the length of trials (14, 15, 28 days, or 91 days).

Probabilistic Model - To integrate what has been learned through ECALS and other Asian Carp Regional Coordinating Committee studies, a conceptual model has been 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. Parameterization of the model is presently underway, building upon the conceptual model previously developed. Information from other ECALS tasks and other sources are being used to parameterize the model.

1 Introduction

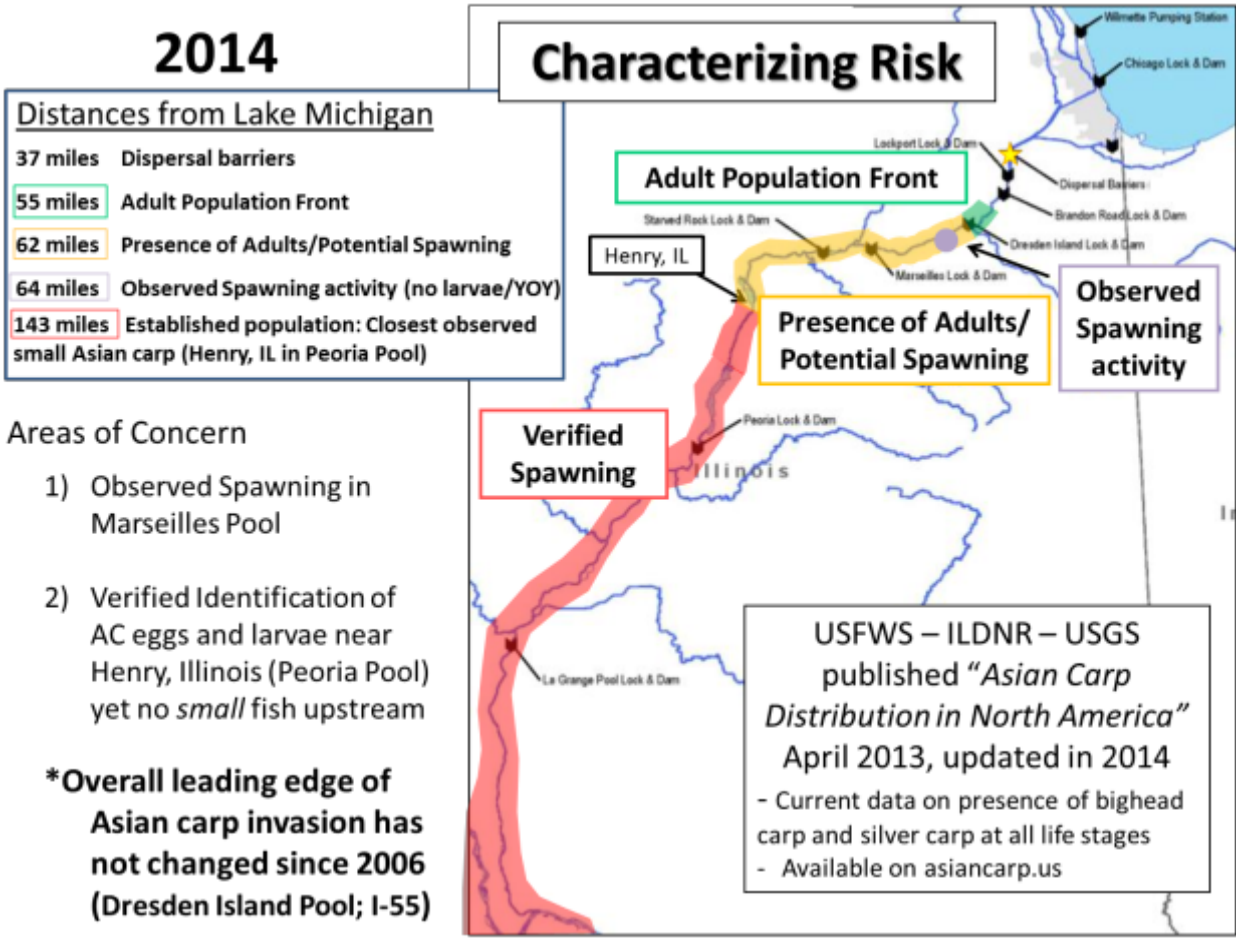
1.1 Project Background

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 1.1.1 to 1.1.3) 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 one adult individual has 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 281.5 in Dresden Island Pool, 15 miles downstream from the barrier and 55 miles from Lake Michigan, and that front has not progressed upstream since 2006. Although spawning activity has been observed in Marseilles pool in 2012, verified capture of eggs and larvae remain downstream in Peoria pool, over 140 miles from Lake Michigan (Figure 1.1.1).

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

Figure 1.1.1. ACRCC depiction of the characterization of risk of Bighead and Silver Carp establishment in the Illinois Waterway.



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

The ACRCC Asian Carp Control Strategy Framework has previously identified three specific Action Items relevant to the use of eDNA, including Action Item Research on the Impacts of Potential Asian Carp Vectors Being a Source of Fish or eDNA Movement in the CAWS, eDNA Calibration and Increased Efficiency, and eDNA Genetic Marker Development. The 2013 version of the Framework, continued work on the eDNA Calibration studies focusing on the development of a probabilistic model.

Figure 1.1.2. Map of the Illinois Waterway, with lock and dam structures labeled.

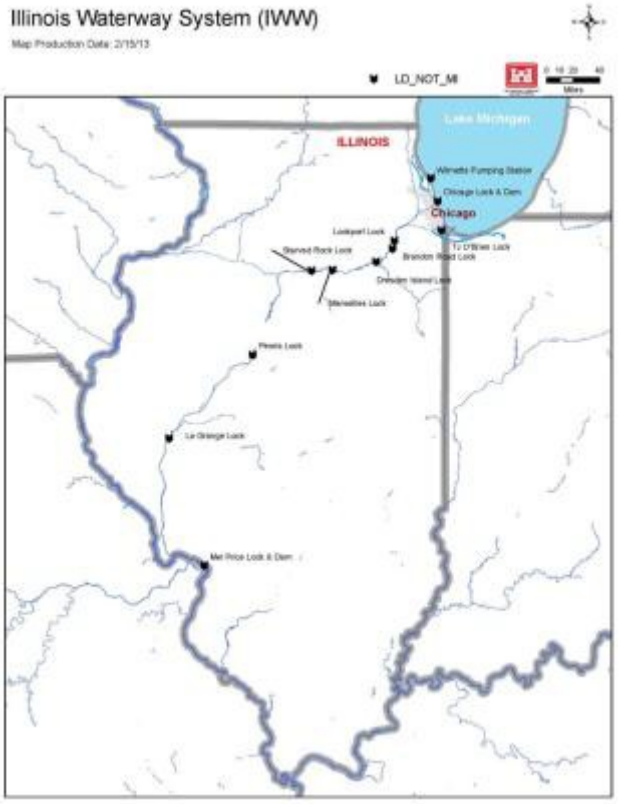
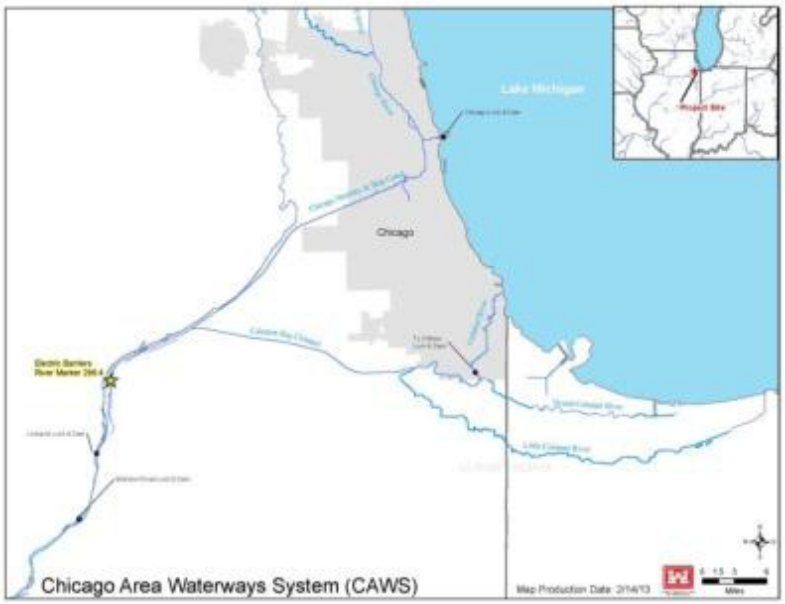


Figure 1.1.3. Map of the Chicago Area Waterway System. The USACE electrical barriers (depicted by the star) are approximately 37 miles from Lake Michigan.



The Environmental DNA Calibration Study (ECALS) was developed by a Federal interagency team (US Army Corps of Engineers [USACE], US Fish and Wildlife Service [USFWS], US Geological Survey [USGS]) 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. 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) 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. 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: 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 third and final 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 (UND) (e.g. Jerde et al. 2011). Their methods were based on conventional PCR (cPCR) analysis in which the presence 0r 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", referring to the *Quality Assurance Project Plan (QAPP): eDNA Monitoring of Bighead and Silver Carps*, which provides standard quality control/quality assurance procedures for the collection, processing, and data reporting for eDNA sampling. The document outlines detailed procedures for Asian carp eDNA sample collection, sample processing (including filtering, centrifuging, DNA extraction, PCR, biomarker analysis, DNA sequencing), data reporting, and quality control/quality assurance protocols to ensure that data are as technically defensible, consistent, and usable as possible. The QAPP ensures continuity among all agencies involved in eDNA sampling activities by setting the same protocols for the collection and processing of eDNA samples. The QAPP was developed by the USACE-USFWS team based on initial protocols from UND, and has been peer-reviewed; the processes and methods have been audited by the U.S. Environmental Protection Agency, verified in at least three independent federal labs as well as academic labs, and the methods have been evaluated by an Independent External Peer Review (IEPR). Released fall of 2011, the eDNA IEPR, conducted by objective panelists with technical expertise in genetics and population ecology, confirmed eDNA sampling and testing methodology is sound for detecting silver and bighead carp DNA but cannot indicate the source of Asian carp DNA (information on the size, gender, number and age of individuals present and can- not distinguish hybrids from pure silver or bighead carp). Based on the levels of peer review, collaboration, and federal oversight going into the development of these procedures, the federal interagency team recommends that all users of eDNA for bighead and silver carp monitoring follow the QAPP.

During the course of the ECALS project, new approaches have been developed with respect to eDNA collection, processing, and analysis (Table 1.3.1). Two new approaches 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, new silver carp-specific markers were developed by ECALS researcher Dr. Jon Amberg (UMESC) specifically for use with real-time quanti- tative PCR (qPCR).

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

Table 1.3.1. ECALS approaches used for DNA analyses.*

Sample Media	Sample Processing	Source of Genetic Marker	Analysis
water; fish tissue, feces, scales, gam- etes, 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 1.3.1.

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 been used to follow up on those vectors deemed most likely by experts to transport Asian carp eDNA. These trials will determine whether detectable eDNA (i.e., 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) 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.

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. 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 ge- netic 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 sys- tem?

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 south of the Brandon Road pool have a small Asian carp population which may be a source of eDNA to the CAWS via flood-ing/overflow, pumping, or cracks in the bedrock. Overbank flooding from the Des Plaines River to the Chicago Sanitary and Ship Canal (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 con- sidered 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, combined sewer overflow (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 the condition of the sediment (settled DNA stirred up from turbulence may introduce a signal that is not representative of recent Asian carp presence)? What is the 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?

<u>Question 2: What factors might influence the persistence of eDNA in the water column?</u>

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 Graphical Model

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 was completed in August 2014. Efforts pertaining to progress on the conceptual model through December 2013 have been documented in the 2013 ECALS milestone report. Although there may be some repetition with respect to ECALS background information here, the entire conceptual model milestone report is presented in its entirety, with minimal editing, for completeness in this report.

Introduction

Asian carp were imported into the United States in the 1970s to control phytoplankton and macrophytes in fish ponds and wastewater treatment lagoons (Kolar et al. 2007). Over the past thirty years, these fish have expanded their range within the Mississippi River Basin. Two planktivorous species of Asian carp are of particular concern. Bighead carp (*Hypophthalmicthys noblis)* and silver carp (*H. molitrix*) are highly efficient filter feeders that have caused significant ecological damage in the Mississippi River Basin by undermining food webs and outcompeting native fish populations in the habitats where they become established (Chick and Pegg 2001, Kolar et al. 2007). Were these fish were to become established in Lake Michigan, they could harm native fish populations.

Efforts to prevent Asian carp from colonizing Lake Michigan have focused on the Chicago Area Waterway System (CAWS) because it is the principal hydrologic connection between the Mississippi River Basin and Lake Michigan. The Illinois River, a tributary of the Mississippi River, is connected to Lake Michigan via the CSSC, which was constructed in the late 1890's to transport sewage from Chicago away from Lake Michigan, the source of the city's drinking water (Changnon et al. 1996, MWRD 2008). The leading edge of the Asian carp invasion is presently considered to be at river mile 278 of the Illinois River, at the Dresden Island pool, about 55 miles downstream from Lake Michigan. However, on rare occasions, individual adult fish have been captured and removed from the pool below Lockport Lock and Dam.

Since 2002, the US Army Corps of Engineers (USACE) has operated an electric fish barrier at Romeoville, Illinois, about 35 miles downstream from Lake Michigan. The fish barrier is designed to prevent the Asian carp invasion front from reaching Lake Michigan via the CAWS. Fish that challenge the barrier are stunned by a non-lethal electrical charge. Although the fish barrier greatly reduces the probability that the Asian carp invasion front will advance toward Lake Michigan via the CAWS, several scenarios under which fish might penetrate or circumvent the barrier may exist (Rasmussen et al. 2011) and studies of the barrier's effectiveness are ongoing (ACRCC 2012a). There are also other pathways by which the fish might reach waters upstream of the barrier (ACRCC 2012a). For example, adult Asian carp are occasionally found in land locked lakes and ponds in the Chicago area. These appear to have been released as fry or fingerlings when lakes and ponds were stocked (ILDNR 2011, USGS 2013).

Over the past several years, a conventional fisheries surveillance program has been implemented in the CAWS to detect the possible presence of bighead and silver carp. This program deploys electrofishing boats and nets at fixed and randomly selected sites to determine the numbers and types of species present. Between 2010 and 2012,

monitoring crews logged over 9,600 hours sampling at fixed and randomly selected sites throughout the CAWS upstream of the barrier (MRWG 2013a). No bighead or silver carp have been captured as part of this conventional sampling program. However, there is one reported occurrence of Asian carp in the CAWS. On June 22, 2010, commercial fishermen working in Lake Calumet captured a bighead carp weighing 8.9 kg. The ACRCC's Monitoring and Rapid Response Work Group (MRRWG, which was renamed the Monitoring and Response Workgroup [MRWG] in 2013) poisoned Lake Calumet with the piscicide rotenone on June 23, 2010, but no additional bighead or silver carp were caught (MRRWG 2012).

Between 2009 and 2012, USACE and partner agencies have been collecting water samples from the CAWS and testing for the presence of deoxyribonucleic acid (DNA) specific to bighead and silver carp. Aquatic organisms release DNA into the environment through bodily excretions such as feces, urine, sperm, eggs, and rotting carcasses. This environmental DNA (eDNA) is assumed to degrade quickly. Until recently, it has been assumed that the presence of bighead and silver carp DNA in water samples indicate that a fish has recently been present near the location where the sample was collected. Water samples were collected over the course of 68 sampling
events. Of the samples collected within and upstream of the electric fish barrier, bighead carp DNA was detected in 43 of 5,522 water samples tested for bighead carp and silver carp DNA was detected in 236 of 5,503 water samples tested for silver carp (MRWG 2013c).

Positive detections of bighead and silver carp eDNA at a monitoring location during two or more consecutive eDNA monitoring events may, at the discretion of fisheries managers, trigger rapid response actions to remove the fish using fishing gear or poison. Between 2010 and 2012, eleven rapid response actions were undertaken employing an estimated 11,330 man hours. No bighead or silver carp have ever been captured during these rapid response actions or during the course of any other MRRWG sampling activity undertaken above the electric barrier. The use of eDNA evidence as a trigger for rapid response actions was discontinued in 2013 because of the lack of success in capturing the target species and uncertainty about how to interpret eDNA monitoring results (MRWG 2013b).

It is possible that bighead and silver carp are present in the CAWS in very low numbers, and therefore difficult to capture or detect using conventional surveillance methods (Jerde et al. 2011). While a very low number of individuals might explain the detection of eDNA and the inability to capture or detect the fish, recent studies have also suggested that eDNA evidence should be interpreted carefully (ECALS 2013, Wilcox et al. 2013). The detection of eDNA belonging to a particular species in a water body

should not, by itself, be taken as proof that a live member of that species is present in that water body because too little is known about factors influencing the distribution of eDNA. For example, it has been shown that eDNA can be transported to the CAWS and released by fish-eating birds, boats, barges, fishing gear and storm sewers (ECALS 2013, MRWG 2013b). Any one or a combination of these sources could provide an alternate explanation for the presence of bighead and silver carp eDNA in the CAWS upstream of the electric fish barrier. Similarly, because eDNA can be difficult to detect at low concentrations, the failure to detect eDNA in a system should not be interpreted as proof that the fish are absent.

Potential actions taken in response to evidence produced by eDNA monitoring in the CAWS have very high costs. If it is concluded that the fish are present, this could lead to costly rapid response actions to remove the fish, the construction of additional barriers, and the closure of navigation routes. However, if it turns out that, in fact, the fish are absent from the CAWS, then these costs could have been avoided. Similarly, an error in concluding that the fish are absent from the CAWS might lead to inaction. However, if it turns out, in fact, that the fish are present and the fish eventually do become established in Lake Michigan, the environmental costs may be high. Total expected costs can be minimized by evaluating the strength of conclusions based on available evidence before taking action. Therefore, the objective of this study is to develop a method that will enable fisheries managers to articulate the strength of conclusions about the source(s) of eDNA detected in the CAWS and the presence of bighead and silver carp above the electric fish barrier.

The following summarizes Environmental DNA Calibration Study (ECALS) efforts to understand and interpret eDNA monitoring results. A conceptual model of eDNA occurrence and persistence is developed to identify what factors may be influencing the spatial and temporal pattern of eDNA detections in the CAWS. A second conceptual model is also presented to describe factors influencing the detectability of eDNA using polymerase chain reaction (PCR). The conceptual models are a preliminary step in developing a probabilistic model to facilitate inference from eDNA monitoring results.

The probabilistic modeling objective is to enable natural resource managers to make statements about the relative importance of the potential sources and vectors of eDNA found in monitoring samples and the probability that live fish may be present in the water body where monitoring samples were collected. This conceptual model report is preliminary. The ideas expressed here will evolve as the various ongoing components of the ECALS study are concluded and the structure of the models will change as the realities of parameterization set in. This conceptual model report contains absolutely no conclusions. No conclusions are made regarding the relative importance of potential eDNA sources and vectors and no conclusions are made regarding whether or not live Asian carp are present in the CAWS or in Lake Michigan.

This conceptual model report begins with a brief literature review, the scope of which is to identify other studies that have applied the eDNA methodology as a complement to conventional surveillance methods and highlight emergent issues with the application of that technique. The conceptual model is introduced and explained. Preliminary plans for parameterization and implementation of the probabilistic model are described. The content of this conceptual model interim report is preliminary and subject to revision as the ECALS continues an iterative process of developing the insights and tools needed to interpret eDNA monitoring results in the CAWS more effectively.

Literature Review

Darling and Blum (2007) outlined several ways that DNA-based methods might be used to monitor invasive species distributions. DNA-based methods are most commonly used to confirm a previously identified specimen or to identify specimens that cannot be otherwise classified because of, for example, a lack of trained personnel or a lack of unique morphological characteristics at a given life stage. In these applications, DNA is extracted directly from a known specimen that provides a point of reference to the original source. Other DNA-based methods use DNA that has been extracted from an environmental sample, such as a sample of soil or water, and there is no specimen, scat, or other evidence that might corroborate the source. These methods include screening for the presence of a target species, quantifying propagule pressure (the number and viability of reproductive units of an invasive species arriving in an area (Stohlgren and Schnase

2006)), and conducting biodiversity surveys (i.e., enumerating all of the species contributing eDNA to an environmental sample). The lack of certainty about where the DNA found in an environmental sample originated and how it arrived at the monitoring location is one of the main challenges of using eDNA based methods.

Several studies have tested the ability of eDNA methods to determine species presence in aquatic environments. Ficetola et al. (2008) screened water samples collected from ponds in France to detect the American bullfrog (*Rana catesbeiana = Lithobates catesbeianus*), an invasive species. Goldberg et al. (2011) collected water samples from five streams on the Payette National Forest to test for DNA belonging to the Rocky Mountain tailed frog (*Ascaphus montanus*) and the Idaho giant salamander (*Dicamptodon aterrimus*). Foote et al. (2012) investigated the feasibility of using eDNA methods to detect harbor porpoise (*Phocena phocena*) in the Baltic Sea. Jerde et al. (2011) report the results of monitoring for bighead and silver carp DNA in the CAWS during the period 2009-2010. Jerde et al. (2013) document the occurrence of bighead and silver carp DNA in tributaries of the Lake Saint Clair, Lake Erie, and Lake Michigan basins. Thomsen et al. (2012a) conducted a biodiversity survey of fish in the Sound of Elsinore, Denmark, using eDNA methods. DeJean et al. (2012) compared the sensitivity of eDNA methods to the sensitivity of conventional surveillance methods in a survey of American bullfrogs in freshwater ponds of the Natural Regional Park of Perigord-Limousin, France. Olson et al. (2012) used eDNA methods to test for the presence of a salamander, the Eastern Hellbender (*Cryptobranchus a. alleganiensis*), in three streams located in Missouri and Indiana. Wilcox et al. (2013) characterized the specificity and sensitivity of an eDNA assay to detect bull trout (*Salmo confluentus*) in Montana streams.

Most eDNA studies, including those listed above, employ conventional polymerase chain reaction (cPCR) to test for the presence of eDNA. The cPCR assay detects the presence of eDNA, but provides no indication of the quantity of DNA in a sample. Quantitative PCR (qPCR) is an alternate assay that provides an estimate of the number of eDNA copies present in an environmental sample. Takahara et al. (2013) used qPCR to evaluate the distribution of bluegill (*Lepomis macrochirus*), an invasive species, in freshwater ponds of Hiroshima Prefecture, Japan. Thomsen et al. (2012b) used qPCR in conjunction with cPCR to test freshwater samples obtained from 98 European lakes, ponds, and streams for the presence of DNA associated with six rare species
representing different taxonomic groups, including amphibians, fish, mammals, insects, and crustaceans. The authors report positive correlations between eDNA concentrations (molecules / µl) and estimates of animal density based on surveys completed using conventional auditory and visual surveillance methods. Using qPCR, Takahara et al. (2012) was able to establish a positive correlation between common carp (*Cyprinus carpio*) biomass and eDNA concentrations in aquaria and controlled ponds.

The primary advantage of qPCR relative to cPCR is that the former provides an estimate of the concentration of an eDNA marker in the environmental sample, which makes it possible to analyze the distribution of target species eDNA at a study site. For example, Takahara et al. (2012) used qPCR to analyze the concentration of carp eDNA during the winter season at 21 sites in Iba-naiko Lagoon, Japan. Concentrations were positively correlated with water temperature, which the authors suggest reflects the carp's preference for warmer water. However, there may be tradeoffs in using qPCR rather than cPCR. Both assays can amplify DNA from non-target species that are phylogenetically similar to the target species (Wilcox et al. 2013) and in some cases it will be more difficult to use DNA sequencing to verify target (vs. non-target) detection for qPCR results than it is for cPCR results.

TaqMan qPCR, the type of qPCR that will likely be used for eDNA assays, works through interaction between the target DNA and three oligonucleotides: two primers that amplify the DNA and an internal probe with a molecular tag that fluoresces when DNA strands are copied. Because TaqMan qPCR requires a close match between the target DNA (or marker) and three oligonucleotides, as opposed to just the two primers used in cPCR, TaqMan assays can provide an added degree of target DNA specificity relative to what may be possible with cPCR. For example, in cases where eDNA samples contain DNA from closely related species, the two primers may bind to and amplify the DNA of multiple species, but the probe may be specific to the DNA of the target species and provide quantitation for only that target. However, when the qPCR product for such samples is sequenced, amplicons (i.e. amplified DNA) from all the related species will be present and will likely confound the sequencing process, preventing validation. Amplicon cloning and sequencing, or next-generation sequencing, could be used to overcome this obstacle, but these are costly and time-consuming processes. With these challenges in mind, exhaustive measures to confirm qPCR marker specificity, along with occasional validation of qPCR results by sequencing, should always be implemented, particularly when significant management, legal, or other consequences will depend on the results of eDNA surveys.

The practice of verifying cPCR and qPCR results by sequencing the DNA that has been amplified during PCR is based on the assumption that marker nucleotide sequences are unique to the target species. However, sequence similarities between target and non-target species are often evaluated using a small sample of individuals, which may or may not come from the geographic region of interest. When the results of eDNA assays may lead to outcomes that have significant management, legal, or other consequences, careful and exhaustive measures would be taken to characterize both within- and between-species DNA sequence diversity for the selected marker region. ECALS is addressing these issues for Asian carp eDNA markers through careful development and

screening of multiple individual Asian carp and several individual samples from Asian-carp related species that occur in the CAWS and Lake Michigan.

The concentration of eDNA present in an aquatic system will be a function of the target species population size, shedding rates, and eDNA degradation rates (Dejean et al. 2011, Wilcox et al. 2013). Larger populations and higher shedding rates will tend to increase concentrations and higher degradation rates will tend to reduce concentrations. The shedding rate, the rate at which DNA is released from an organism, will vary between species and individuals, and as individual metabolic rates vary with life stage and season (Goldberg et al. 2011). In closed systems, such as ponds, eDNA concentrations can be expected to accumulate over time to a level that reflects local hydrodynamics (flows and dilution volumes) as well as a balance between shedding and degradation rates. In open systems, such as streams, rivers, and oceans, higher flows or currents will increase the dilution volume and tend to reduce eDNA concentrations (Goldberg et al. 2011, Foote et al. 2012, Wilcox et al. 2013). Flows and currents entering a study site may also import eDNA that has been released elsewhere either by the target species or by a non-target vector, leading to higher concentrations than might otherwise be observed at the study site (Thomsen et al. 2012b, Wilcox et al. 2013). ECALS is conducting studies to quantify eDNA shedding rates from bighead and silver carp (Klymus et al. 2013).

Most investigators tend to agree that eDNA degrades rapidly in the environment, and this has provided one of the primary justifications for inferring target species presence at the study site where eDNA is detected (Ficetola et al. 2008, Jerde et al. 2011). Degradation of eDNA occurs by hydrolysis and may be influenced by environmental conditions, such as temperature, pH, microbial activity, and light or ultra-violet radiation. Matsui et al. (2001) reported that extracellular DNA fragments up to 400 base pairs (bp) in length can persist for up to one week in lake water at 18 deg. C (Ficetola et al. 2008). Thomsen et al. (2012a) found that even small eDNA fragments, up to 100 bp in length, degrade beyond detectability within days. Dejean et al. (2011) quantified extracellular DNA degradation rates using American bullfrog tadpoles and Siberian sturgeon (*Acipenser baerii*) sub-adults (20 cm); these authors found that DNA could be detected in more than five percent of samples for up to 25 and 17 days, respectively. However, DNA fragments may persist in the environment for very long periods of time. Adsorption to mineral and humic substances protects the DNA from extracellular microbes that would otherwise degrade unbound DNA in solution (Levy-Booth et al. 2007). Very cold conditions can also retard degradation. Willerslev et al. (2004) report that eDNA may persist for several hundreds of thousands of years in very cold environments (Dejean et al. 2011, Thomsen et al. 2012). ECALS is investigating how temperature, turbidity, light, pH, and other environmental factors such as adsorption to sediment particles might influence the degradation of bighead and silver carp eDNA.

There are numerous questions about what types of inferences eDNA monitoring can support and how eDNA monitoring studies can be used to inform natural resource management decisions. Two basic inferences are possible. The cPCR assay can be used to infer the presence or absence of a genetic marker in an environmental sample, and an eDNA monitoring program can be used to infer the presence or absence of a target species in the habitat where the environmental sample was collected. Darling and Mahon (2011) considered potential causes of false positive and false negative conclusions based on the results of eDNA assays using PCR. False positive conclusions are those that infer either the marker or the target species is present when in fact it is not. False negative conclusions are those that infer either the marker or the target species is absent when in fact it is present.

Conceptual Model of eDNA Occurrence and Persistence

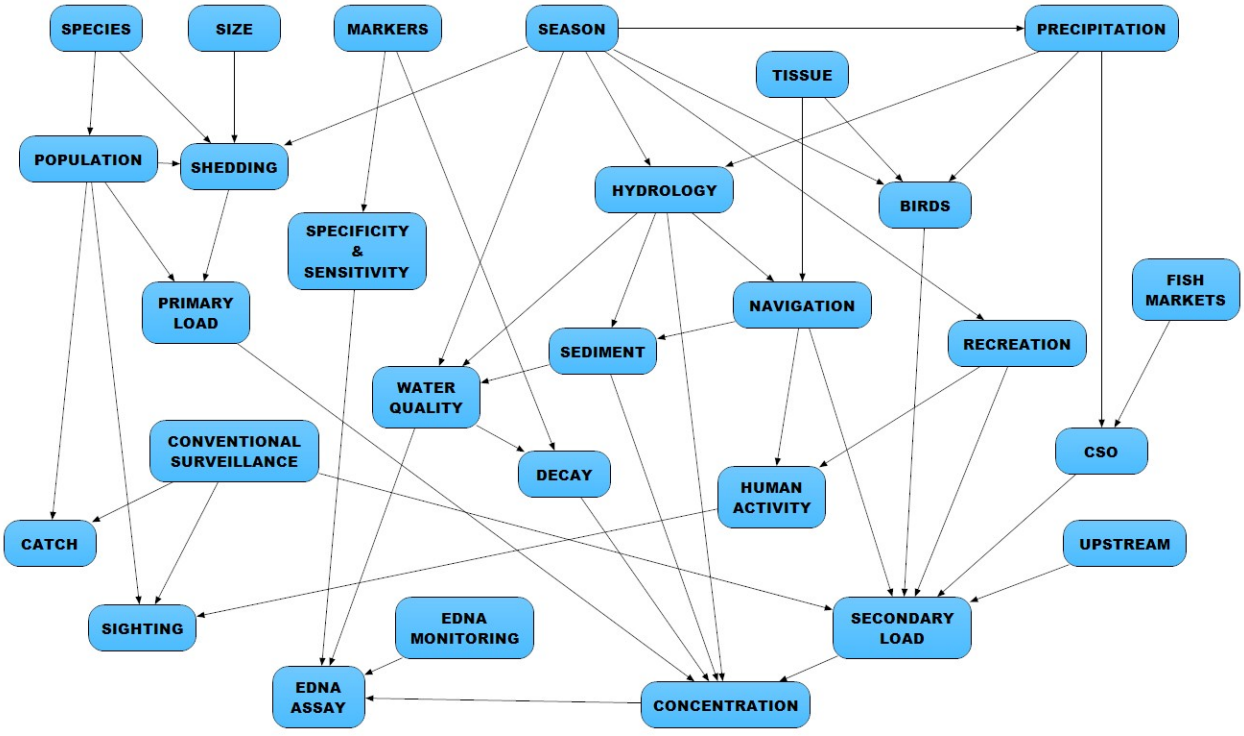
The ECALS conceptual model describes factors or variables that are believed to influence the occurrence, persistence, and detectability of eDNA on the outcome of the eDNA surveillance program. The conceptual model is presented in the form of a graph consisting of nodes representing random variables and directed edges between nodes. Random variables are sources of uncertainty in interpreting eDNA monitoring results. Directed edges, or arrows between nodes, signify the influence of one random variable on another random variable in the direction of the edge. Very complex joint probability distributions can be represented by illustrating the dependence and conditional independence relationships between random variables. Although the graphical models presented here are conceptual, they provide a point of departure for developing a fully quantitative probabilistic model for the interpretation of eDNA monitoring data from a monitored water body. Considerable iterative refinement of the conceptual model presented here will be needed before such a transformation can occur.

The conceptual model summarizes knowledge or beliefs about the sources and vectors of eDNA in the CAWS, and the factors influencing eDNA occurrence, persistence, and detectability in a monitored water body. A very general conceptual model of eDNA occurrence, persistence, and detectability is presented in Figure 2.1.1 to introduce the concepts. In this version of the conceptual model, the formalities of graphical models are relaxed so that concepts can be introduced briefly in general terms. This should make it easier to follow discussions of a more detailed conceptual model in the next section of this report. The issues raised in the presentation of this preliminary conceptual model will be revisited later in a more detailed conceptual model.

The conceptual model in Figure 2.1.1 terminates in three nodes in the lower left hand corner. These nodes represent modes of Asian carp detection in the CAWS: CATCH, SIGHTING, and EDNA ASSAY (For clarity in the presentation, the titles of nodes will be capitalized and potential states of random variables represented by these nodes will be italicized throughout this report). One can think of these nodes as binary random variables with potential states True or False. Catch represents the physical capture of an Asian carp in the CAWS and depends primarily upon the amount and type of CONVENTIONAL SURVEILLANCE employed and the POPULATION of the SPECIES (Bighead carp or Silver carp) that might be captured. Higher populations and higher levels of CONVENTIONAL SURVEILLANCE should result in higher probabilities of capturing a live Asian carp. SIGHTING refers to the casual observation of an Asian carp that does not result in capture. This is a function primarily of the level of HUMAN ACTIVITY on the waterway (e.g., the amount of NAVIGATION and RECREATION), man hours invested in CONVENTIONAL SURVEILLANCE, and the POPULATION of

Asian carp in the monitored water body. The third mode of detection is an EDNA ASSAY. The outcome of an eDNA assay is the a function of the CONCENTRATION of eDNA at the location where the eDNA monitoring sample is collected, characteristics of the eDNA monitoring program (EDNA MONITORING) such as frequency, intensity, and distribution of sample collection efforts, and the SPECIFICITY & SENSITIVITY of the genetic marker that the assay is meant to detect. SPECIFICITY describes how well we know that the genetic marker being tested for is unique to the target species. SENSITIVITY describes how easily the marker being tested for is detected using PCR. WATER QUALITY influences the outcome of an eDNA assay because certain water quality constituents inhibit PCR reactions and may lead to false negative assays.

Figure 2.1.1. General conceptual model of eDNA occurrence, persistence, and detectability in the CAWS.



This conceptual model emphasizes factors influencing CONCENTRATION, the concentration of eDNA in the waterway. The graph contains two nodes representing sources of eDNA: PRIMARY LOAD and SECONDARY LOAD. PRIMARY LOAD is the number of copies of the marker that are released directly from a live fish in the monitored water body. PRIMARY LOAD is a function of the fish POPULATION present in the waterway and the eDNA shedding rate (SHEDDING), which will likely be influenced by SPECIES, the age structure or size distribution (SIZE) of the target species population, and the life-cycle stage or SEASON in which monitoring occurs.

The SECONDARY LOAD node represents all other potential sources of eDNA in the waterway. Known potential sources of secondary load include NAVIGATION, RECREATION, BIRDS, COMBINED SEWERS, CONVENTIONAL SURVEILLANCE, and UPSTREAM sources (ECALS 2013). NAVIGATION captures the potential for barges travelling from below the electric fish barrier to points above the electric fish barrier to inadvertently carry eDNA on their hulls, in bilge water, and in carcasses of fish (especially silver carp) that may have landed on deck. BIRDS captures the potential contribution of eDNA to the waterway when fish-eating birds known to prey on Asian carp such as eagles, cormorants, and pelicans defecate in the water or when runoff transports feces from nearby nesting areas. NAVIGATION and BIRDS are both influenced by a variable called COPKG. COPKG is the eDNA load in one kilogram of fish tissue. This variable is important because the potential contribution of eDNA from

NAVIGATION and BIRDS will depend on the quantity of fish carcasses on barges and the quantity of fish in bird diets. RECREATION captures the potential for pleasure boats and fishing boats, such as those participating in Lake Calumet fishing derbies, to transport copies of eDNA markers from carp-infested waters on hulls and in bilge tanks. Asian carp eDNA has also been detected in discharges from combined sewers, which may carry kitchen waste from homes and restaurants where Asian carp are consumed or from fish markets where Asian carp waste may be disposed of in the sewer system (COMBINED SEWERS). CONVENTIONAL SURVEILLANCE is also a potential source of eDNA in the water body because eDNA can be imported to carp-free waters on nets that have previously been used in carp-infested water bodies. Finally, the node UPSTREAM represents the potential for inflows to transport eDNA that has been re-leased into the environment outside the geographic limits of the study area.

Three other factors contribute to the occurrence, persistence, and detectability of eDNA. These include DECAY, HYDROLOGY, and SEDIMENT. While several studies have shown that eDNA decays rapidly in the environment, there is also evidence that eDNA can persist for long time periods when adsorbed to sediment or in cold temperatures. If eDNA decay is a random process that occurs as the bonds between nucleotides become broken, rendering the marker non-detectable by an eDNA assay, decay rates may, in part, be a function of marker length. Genetic markers consisting of a larger number of nucleotide bonds may have a higher probability of becoming cleaved. This explains the relationship between DECAY and MARKERS, which describes the characteristics of the eDNA marker that is being tested in the assay. Decay rates may also be influenced by WATER QUALITY. Not much is known about how water quality may influence decay rates, but factors such as pH, water temperature, microbial content, and turbidity may be important.

The HYDROLOGY node represents all variables related to the movement and flow of water in space and time, including hydrography, flow, velocity, stream geometry, hydrological residence time and age of water. HYDROLOGY has an important and complex influence on CONCENTRATION. For example, hydrologic variables such as stream geometry and flow will influence eDNA concentrations by altering the dilution volume, the travel time through the system, and the age of water in the system. Higher flows will tend to reduce eDNA concentrations by increasing the dilution volume and reducing the time that eDNA particles remain in the system. Slower moving water may be less likely to contain eDNA that has been imported from outside the system because its residence time in the system is longer, providing more opportunities for eDNA to degrade before it is detected. HYDROLOGY influences WATER QUALITY, NAVIGATION, and SEDIMENT. The effects of HYDROLOGY on WATER QUALITY may be similar to those summarized for eDNA concentrations. While sufficient flows must be available to sup- port NAVIGATION, the importance of this relationship for understanding eDNA occur- rence, persistence, and detection in the CAWS is unclear. The importance of the influ- ence of HYDROLOGY on SEDIMENT is more obvious. Higher flows tend to reduce sediment deposition rates and increase shear stress, inducing resuspension of settled sediments and increasing suspended sediment concentrations in the water column.

The SEDIMENT nodes represent all variables related to the suspension, deposition, and resuspension of sediment, the adsorption of eDNA to sediment particles, and the potential accumulation and burial of eDNA in the sediment layer. Higher flows tend to keep sediment particles in suspension, reduce net-deposition, increase scour, and increase suspended sediment concentrations, increasing the opportunity for eDNA particles to become sorbed to sediment. Once sorbed to sediment particles, eDNA may no longer be susceptible to degradation in the same way that it would be if it were in solution. Sorbed eDNA may also have a tendency to settle out of the water column and become buried in the sediment layer. Thus, the sediment layer may become a reservoir for eDNA. The influence of NAVIGATION on SEDIMENT is similar to that of HYDROLOGY. Barge traffic may tend to increase scour, leading to higher concentrations of suspended sediment in the water column. Overall, the net effect of SEDIMENT on CONCENTRATION is ambiguous. It may be that eDNA concentrations are reduced when eDNA settles out of the water column, but may also be that eDNA concentrations are increased during periods of resuspension if eDNA particles in the sediment layer are resuspended into the water column.

SEASON has a potentially large influence over several variables in the conceptual model. The patterns of PRECIPITATION and its influence on HYDROLOGY may vary by SEASON. SEASON may also influence SHEDDING, BIRDS, and WATER QUALITY. For example, Asian carp may shed more eDNA during those seasons when their metabolic

rates are highest or during mating season when sperm and eggs are disseminated in the water column. SEASON influences BIRDS because piscivorous birds may be more active during some seasons of the year than others, or may migrate during fall and winter months. WATER QUALITY and HYDROLOGY are influenced by SEASON because inflows from Lake Michigan into the CAWS are less likely to occur during winter months than at other times of the year; therefore, water in the system may be more likely to originate at sewer and wastewater treatment plant outfalls where total dissolved solids concentrations are believed to be higher than in Lake Michigan.

The conceptual model in Figure 2.1.1 has been introduced to briefly describe the range and complexity of issues that should be considered in interpreting eDNA monitoring results. Several of the nodes represent active areas of research within ECALS, including SHEDDING, MARKERS, SPECIFICITY & SENSITIVITY, HYDROLOGY, BIRDS, CSO, DECAY, and EDNA ASSAY. Others such as CONVENTIONAL SURVEILLANCE and EDNA MONITORING represent ongoing efforts of multiple federal and state agencies involved in ACRCC efforts. ECALS will draw on these and other available sources in developing and parameterizing a probabilistic model for inference from eDNA monitoring data. While much progress has been made in understanding the issues described here, insights are continuously emerging to inform development of the probabilistic model. As new insights emerge, the conceptual model will be updated and refined to reflect emerging knowledge.

A Refined Conceptual Model

In this section, a more refined version of the conceptual model is presented. The conceptual model is presented in three parts. The first part focuses on hydrologic influences within the CAWS, which determines eDNA transport. The second part focuses on the occurrence and persistence of eDNA in the CAWS. The third part focuses on the eDNA assay and the detectability of eDNA. While the concepts and ideas described in the more general version of the conceptual model are preserved, each node of the graphs presented here represents a well-defined variable within the system that can be quantified in some way. However, there may be no direct mapping of the variables described here to the nodes described above. Preliminary plans for quantification of the variables are also described. The conceptual model should be considered a “work in progress” that is subject to revision rather than an end product. As noted above, considerable refinements of the conceptual model may be needed before it can be transformed into a probabilistic model for interpretation of eDNA monitoring results.

1. Hydrologic Influences in the CAWS

The backbone of the conceptual model of eDNA occurrence and persistence in the CAWS is a graph that describes hydrologic influences among reaches, or stream seg- ments, that make up the CAWS (Hydrologic influences illustrated in Figure 2.1.2 repre- sent ECALS current understanding of the system. ECALS is currently modeling hydro- dynamics in the CAWS to validate this understanding). Nodes in Figure 2.1.2 represent the concentration of eDNA (copies/L) in each reach. The edges between nodes indicate the direction of the dominant hydrologic influence between reaches and the flow of eDNA. Development of this hydrologic map of the CAWS is based primarily on infor- mation contained in Metropolitan Water Reclamation District (2008) and on a detailed hydrologic grid developed by ECALS for the purpose of hydrodynamic modeling to sup- port interpretation of eDNA monitoring results. A map of the CAWS is provided in Fig- ure 2.1.3 to assist in locating the reaches.

In Figure 2.1.2, the CAWS main stem is divided into 24 variable length reaches. Each reach of the CAWS main stem between Lake Michigan and Dresden Lock and Dam is represented by a three or four digit code that is defined in Table 2.1.1. A three digit code is used for main stem reaches. These are defined in Table 2.1.1. A four-digit code is used to reference the 37 boat slips and harbors that form backwaters along the canal. Boat slips and harbors are labeled by appending a single letter to the three- digit code begin- ning at the top of the reach and proceeding to the bottom of the reach in order of occur- rence. Tributaries and backwaters to the left of the main stem in Figure 2.1.2 are on the north or west bank of the CAWS, those on the right are on the south or east bank.

Hydrologic influence in Figure 2.1.2 flows from upstream to downstream in direction of the edges, from Lake Michigan (LMI) towards Dresden Lock and Dam, at the base of CR8. There are four major inflows to the CAWS. These include Lake Michigan (LMI), which contributes flow at three locations in the CAWS, the North Branch of the Chicago River (NBC), the Grand Calumet River (GCR) and the South Branch of the Little Calu- met River (LCR). Flows from Lake Michigan enter the system at the Wilmette pump sta- tion, the Chicago Lock and Dam, and at the head of the Calumet River. Other sources of inflow include Bubbly Creek (BCR) and Lake Calumet (LKC), but these contribute flows to the system only after rainfall events. Water reclamation plants also contribute flow to the system. Edges between main stem nodes and backwaters are typically in the direc- tion of main stem to the backwater. This reflects our current hypothesis that net DNA transport is from the main stem into stagnant boat slips and harbors. However, boat slips and harbors may also concentrate surface runoff and sewer discharges from the Chicago Area to the CAWS following rain events that periodically reverse the direction of DNA transport toward the main stem.

Figure 2.1.2. Representation of the main stem of the CAWS. Each node represents a reach and each edge denotes hydrologic influence from upstream to downstream in direction of the edges.

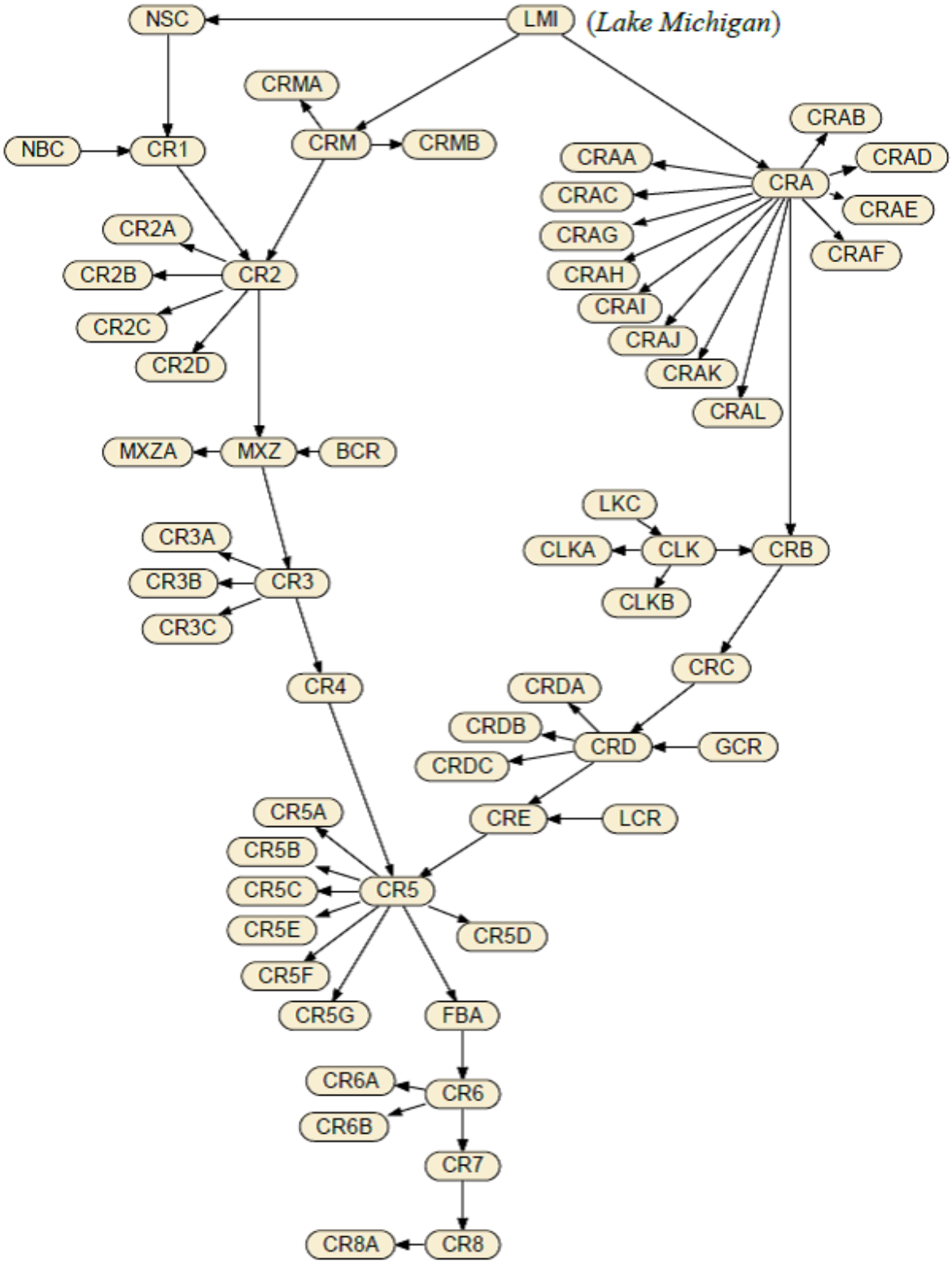
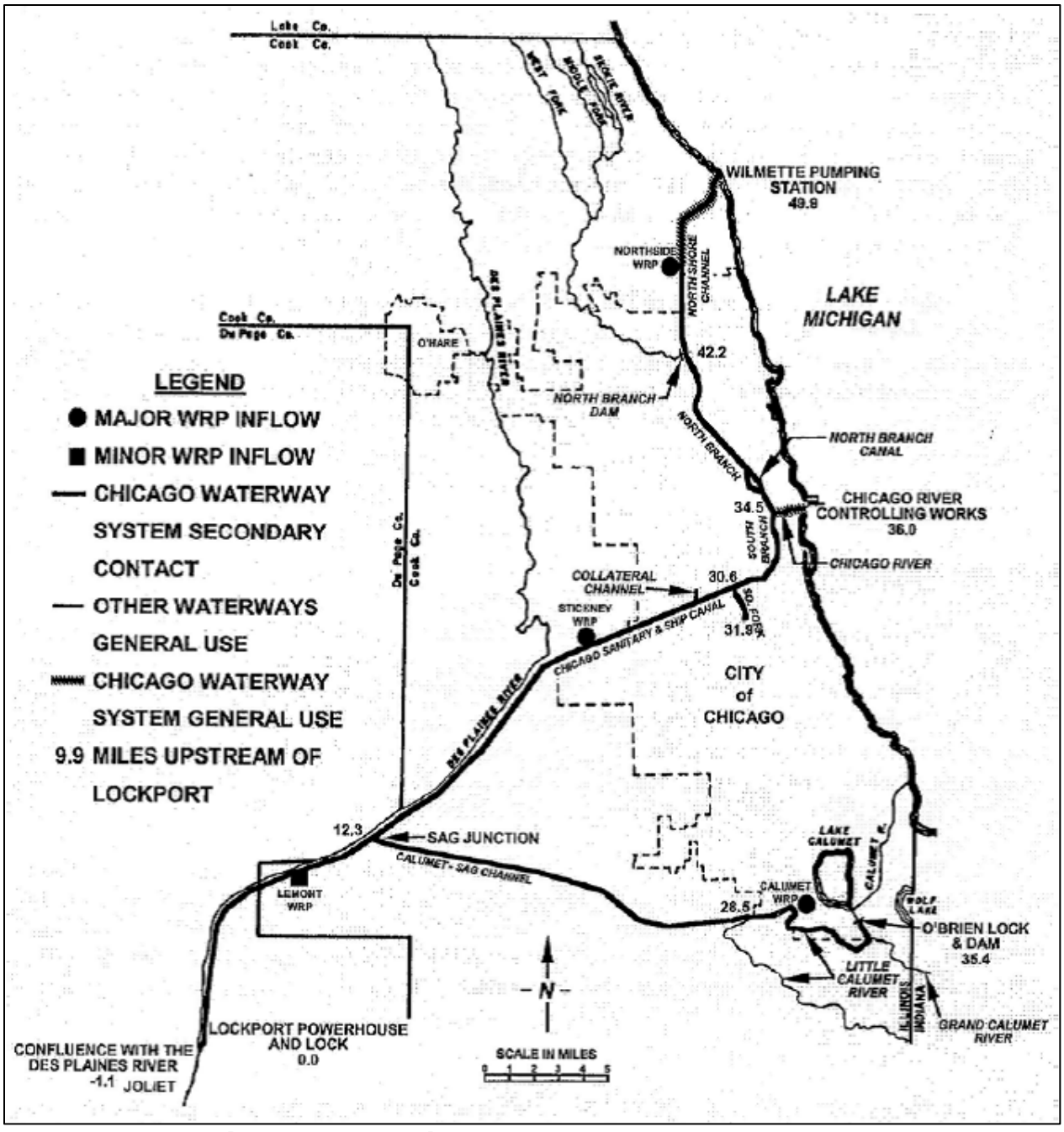


Figure 2.1.3. Map of the Chicago Area Waterway System illustrating major features of the CAWS between Lake Michigan and Lockport Lock and Dam (MRWD 2008).



Source: Metropolitan Water Reclamation District.

Table 2.1.1. Nodes representing the main stem of the CAWS.

SYMBOL	D
LMI	Lake Michigan (LMI)
NSC	N C
NBC	North Branch of the Chicago River (NBC) upstream of its confluence with NSC
CR1	North Branch of the Chicago River (CR1) below the confluence of NBC and NSC to the South Branch of the Chicago River (CR2)
CRM	Chicago Sanitary and Ship Canal (CSSC) from LMI at the Chicago River Controlling Works (CRCW) to its confluence with CR2
CR2	CSSC from the confluence of the North Branch of the Chicago River (CR1) and CRM to the upstream boundary of CR3
BCR	Bubbly Creek, a canal extending south from the main stem of the CSSC at MXZ to its terminus, 1.3 miles upstream
M	A
CR3	CSSC from MXZ to a point just upstream of Stickney Water Reclamation Plant (WRP)
CR4	C
CR5	CSSC from the confluence of CR4 and CRE to the upstream boundary of the electric fish barrier
FBA	C
CR6	CSSC from the downstream boundary of FBA to the Lockport Lock and Dam
CR7	Il

CR8	Illinois River from Brandon Road Lock and Dam to the Dresden Lock and Dam
CRA	C
CLK	The canal linking the Calumet River to Lake Calumet
LKC	Lake Calumet
CRB	A mixing zone at the confluence of CRA and CLK
CRC	Little Calumet River from the base of its confluence with CLK and CRA to its confluence with GCR
CRD	Little Calumet River from its confluence with GCR to the LCR
CRE	Cal-Sag Canal from the South Branch of the Little Calumet River to CR5
GCR	Grand Calumet River
LCR	South Branch of the Little Calumet River

The load of eDNA at each node in the system originates either from an upstream node or from primary and secondary sources within the reach. Primary sources, secondary sources other than upstream loads, and other factors influencing the occurrence and persistence of eDNA in the waterway are not represented in this part of the conceptual model, but are discussed in the following section.

2. Occurrence and Persistence of eDNA in the CAWS

The second part of the conceptual model describes the occurrence and persistence of eDNA in the CAWS, which is some function of how much eDNA is released into the system, when and where it is released, how it is transported, and how quickly it degrades. The conceptual model is presented in Figure 2.1.4 for a single reach. Each node represents a random variable that is a source of uncertainty in estimating either the concentration of eDNA in that reach or the outcomes of conventional surveillance (capture or

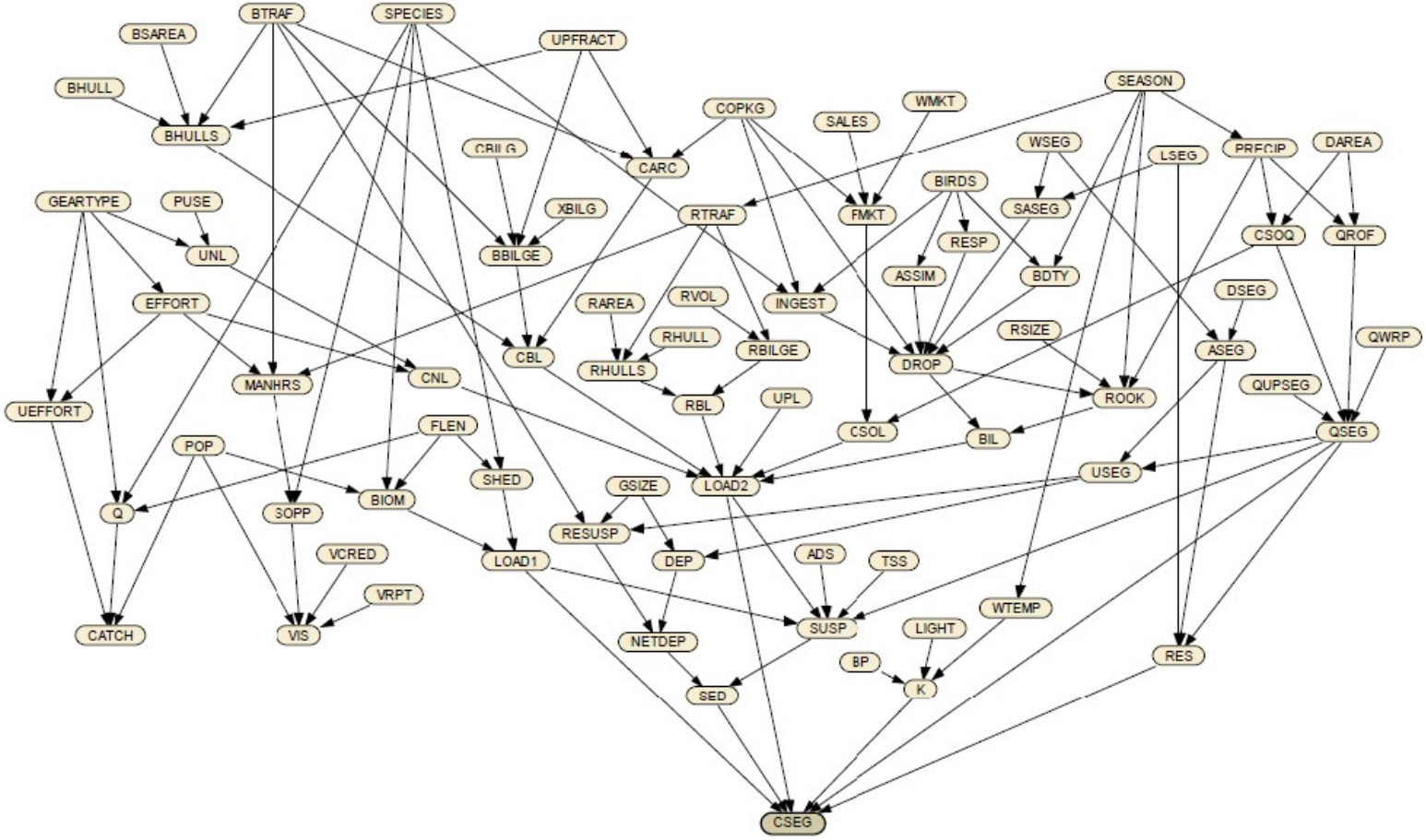
sighting of an Asian carp). Each node in the conceptual model is labeled with a brief title. Random variables are described in the text and defined in Appendix A.

There are three terminal nodes in the network: VIS, CATCH, and CSEG. VIS describes the visual detection of a fish. CATCH describes the capture of an Asian carp in the course of conventional surveillance. Either one of these events could resolve the question about whether or not bighead or silver carp are present in the waterway; although there might still be uncertainty about the source of eDNA detected in monitoring samples. CSEG is the average daily concentration of eDNA in the reach (copies/L). The CSEG node provides a conceptual link between the graph of eDNA occurrence and persistence and the graph representing hydrologic influences in the CAWS (Figure 2.1.2) because the random variables are identically defined in both graphs. The graphs have been developed this way to facilitate inference in each reach.

CSEG is a function of the degradation rate (K, day^{-1}), residence time of water within the reach (RES, days), flow (QSEG, m^3/day), primary eDNA load (LOAD1, copies/day) and secondary eDNA load (LOAD2, copies/day). The primary load is generated by live fish in that reach; therefore, LOAD1 depends upon the biomass of the target species (BIOM, kg) and the unit shedding rate (SHED, copies/kg/day). The unit shedding rate depends upon the species and size distribution of the population. Therefore, SHED is a function of SPECIES and fish length (FLEN, mm), which is a proxy for the age or size structure of the population within the reach. FLEN is related to biomass by a length-weight function for the target species. As described for the general conceptual model (Figure 2.1.1), LOAD2 is the sum of all eDNA loads to the reach other than those from live Asian carp. Secondary sources of eDNA load may include upstream load (UPL, copies/day), bird load (BIL, copies/day), combined sewer loads (CSOL, copies/day), recreational boat load (RBL, copies/day), commercial boat load (CBL, copies/day), and commercial net load (CNL, copies/day).

Sources of secondary load (LOAD2) include UPL, BIL, CSOL, RBL, CBL, and CNL. UPL is the combined load from all upstream reaches of the waterway that may contribute eDNA to the reach of interest. Potential sources of BIL include eDNA in excrement from birds that may be flying over or otherwise in contact with the waterway (DROP, copies/day) and eDNA in excrement from birds that may be carried in surface runoff from rookeries or high density nesting sites near the waterway (ROOK, copies/day). We dis- tinguish these two sources because DROP is a distributed source and ROOK is a

Figure 2.1.4. Conceptual model of eDNA occurrence and persistence in a single reach of the CAWS.



point source. DROP is a function of bird density in the reach (BDTY, birds/day), the amount of target species ingested (INGEST kg/day), the rate of assimilation (ASSIM, day^{-1}) and respiration (RESP, day^{-1}), all of which vary with bird species (BIRDS). INGEST depends on BIRDS (bird species) and SPECIES (fish species) because bighead and silver carp may constitute a lesser or greater fraction of the diet of some birds species than others, either because of differences in the availability of bighead or silver carp for consumption or difference in the preference of different birds species for bighead and silver carp. BDTY is likely to vary by SEASON because birds are more likely to be less active during winter months. ROOK is a function of the number of birds nesting at the rookery (RSIZE, birds/day), SEASON, and precipitation (PRECIP, mm/day). ROOK is influenced by DROP because similar biological processes control the rate of eDNA deposition at rookeries as elsewhere.

CSOL represents the eDNA load from combined sewers that discharge to the reach. These discharges may contain eDNA generated by households or restaurants where Asian carp are con- sumed or fish markets were Asian carp are processed and sold. The volume of discharges from combined sewers tends to increase with the amount of precipitation (PRECIP), the amount of Asian carp sold (SALES, kg/day) and consumed and fraction of fish that are discarded as waste (WMKT, kg/day). Waste may be disposed of either through the wastewater system or directly to storm sewers. For example, ice contaminated with fish slime and scales may be dumped into storm sewers directly (ECALS 2013). Commercial fishing nets used to help control Asian carp populations below the electric fish barrier are another potential secondary source of eDNA in the CAWS. Fishing gear can become a vector of eDNA if it becomes contaminated with eDNA and is later used to carry out planned intensive surveillance, fixed and random site monitoring, or rapid response actions above the electric fish barrier. CNL (copies/day) is the eDNA load from commercial fishing gear (especially fishing nets). The CNL will vary with gear type (GTYPE), level of fishing effort (EFFORT, units), and the unit load of fishing gear (UNL, cop- ies/unit/day). UNL depends on GTYPE and PUSE, which is a variable describing the extent to which fishing gear has been previously used in waters known to be infested with bighead and silver carp.

Recreational and commercial boaters may unintentionally distribute eDNA from carp infested waters to waters that have not yet been infested. BTRAF (boats/day) is the amount of commercial boat and barge traffic in a reach. Only the fraction of commercial boat and barge traffic (UPFRACT) travelling from below the electric fish barrier to locations above the barrier is believed to carry eDNA on hulls (BHULLS, copies/m^2), in bilge water ((BBILGE, copies/L), or in fish carcasses that can sometimes be transported on the barge deck (CARC copies/kg). The contribution from barges is dependent on the concentration of eDNA in waters where the barge became contaminated with eDNA of the target species, the surface area of barge hulls

(BSAREA, m^2), the volume of bilge water taken on in contaminated waters, and the frequency with which boats and barges inadvertently transport target species carcasses across the barrier. Similar processes are involved when recreational boats transport eDNA, but it seems much less likely that recreational boats might carry target species carcasses on deck. The distribution of recreational boats in the CAWS (RTRAF boats/day) may differ substantially from the distribution of commercial boat and barge traffic (BTRAF) with recreational boat activity more likely to occur closer to Lake Calumet and the entrances to Lake Michigan.

Once released into the water column, eDNA will be distributed within the waterway by hydrologic forces that are a function of stream geometry (depth (DSEG, m), width (WSEG, m), length (LSEG, m)), and flow (QSEG, m^3/day). Inflows to CAWS reaches come either from upstream reaches (QUPSEG, m^3/day), wastewater reclamation plants (QWRP m^3/day), CSOQ discharges (CSOQ m^3/day), or runoff (QROF m^3/day). Surface runoff is a function of drainage area (DAREA m^2) and precipitation. The occurrence of eDNA in the reach also depends upon the degradation rate and residence time of water. Degradation may be influenced by the length of the marker (BP, base pairs) and environmental conditions such as water temperature (WTEMP, deg C) and ultra-violet light (LIGHT). ECALS is aware that other factors such as pH and microbial activity may also be important, but these factors are not represented in this graph.

The eDNA in the water column (CSEG) may either be free in solution or adsorbed to suspended sediment particles (SUSP, copies/mg TSS). The fraction adsorbed to sediment depends upon the concentration of suspended sediment (TSS, mg/L), and the adsorption rate (ADS, day^{-1}). SUSP is also influenced by QSEG because flows will affect both the concentration of eDNA and TSS in the water column. If eDNA is adsorbed to suspended sediment, it may tend to settle out of the water column and become stored in sediment where it is unavailable for capture and de- tection by an eDNA monitoring program. The load of eDNA stored in sediment layer (SED) de- pends upon the net sediment deposition rate (NETDEP, day^{-1}), which is the difference between the deposition rate (DEP, day^{-1}) and the resuspension rate (RESUSP, day^{-1}). Resuspension may occur as a result of high water velocities (USEG, m/day) or barge traffic (BTRAF, barges/day) and the amount of resuspension that occurs may depend on the sediment grain size distribution (GSIZE, μm) and other sediment characteristics.

Asian carp might also be detected in the CAWS by casual observation (VIS) or conventional surveillance (CATCH). VIS is a binary node that takes the state *True* if a bighead or silver carp has been sighted or *False* if no bighead or silver carp has been sighted in the reach. VIS depends on whether an individual sighting a bighead or silver carp reports the sighting (VISRPT) and the degree of credibility of the report (VISCRED). The probability of visual detection in-

creases with sighting opportunity (SOPP) and the size of the fish population (POP). Opportunities for casual observation of bighead or silver carp are a function of the level of commercial and recreational activity and conventional surveillance in the reach and fish behavior, which will vary by species. CATCH is also a binary node that takes the value *True* if an Asian carp has been captured and *False* if no Asian carp has been captured in the course of conventional monitoring efforts in the reach. This is a function of the catchability of the species given the population number (POP), the population size distribution (FLEN), and the unit effort expended on conventional monitoring (UEFFORT). UEFFORT is a function of gear type employed in monitoring and the level of fishing effort (EFFORT).

3. Detectability of eDNA in the CAWS

Asian carp eDNA is detected in monitoring samples from the CAWS using PCR, which is capable of detecting very small quantities of a genetic marker in an environmental sample. Sample collection and analysis procedures were originally developed at the UND with funding from USACE (USACE 2012). An independent peer review of the eDNA methodology by Environmental Protection Agency's (EPA's) Great Lakes National Program Office in 2009 assessed the reliability of analytical procedures at the UND. The review expressed confidence in the methodology and procedures. The EPA review did not address interpretation of eDNA monitoring results in regards to the presence or absence, proximity, or abundance of carp in the study area (Blume *et al.* 2010). USACE subsequently contracted with Battelle Memorial Institute for a second independent peer review of the eDNA methodology. The review found that the eDNA methodology was sound in principle and presented several advantages over conventional surveillance methods. However, it also identified some key limitations of the approach. In particular, the review concluded that detection of eDNA does not provide conclusive proof of species presence and does not provide information on the size or age of individuals or the size of a population, if present (BMI 2012).

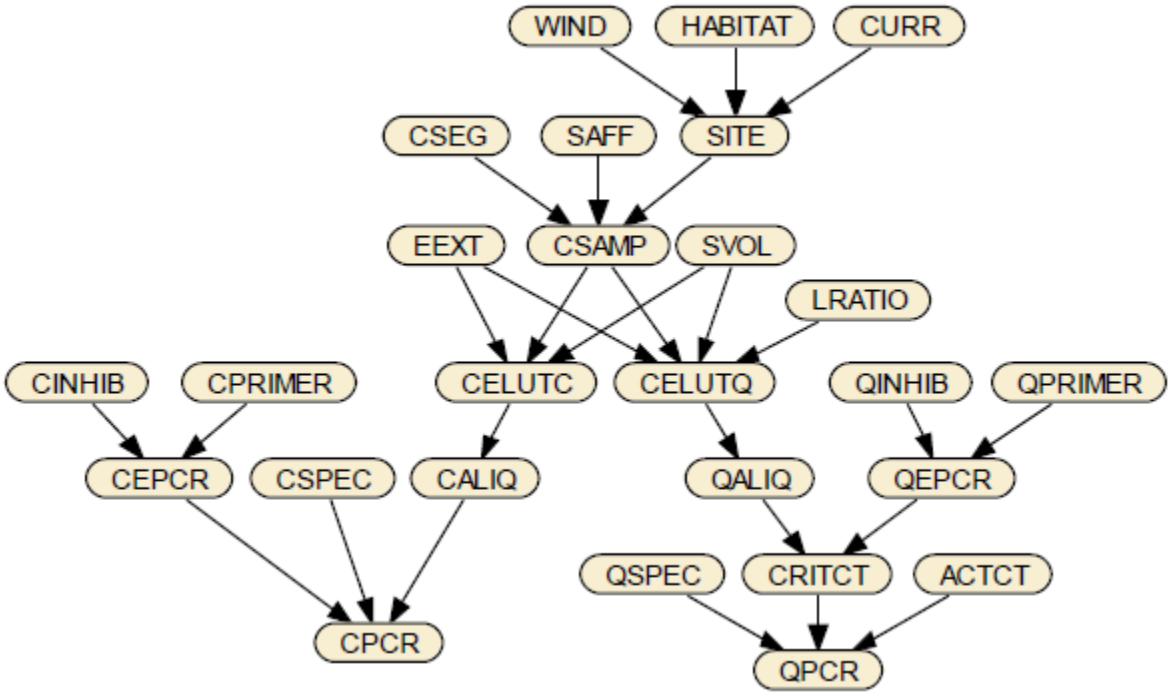
Sample collection and analysis procedures are described in the QAPP and, with a few exceptions, follow those developed by the UND (USACE 2012). Samples of water (usually two liters) are collected from the CAWS and filtered through one or more 1.5 micron glass fiber filters. Filters are then shipped on ice to a laboratory where the eDNA is extracted from the filter paper using a MoBio Power Water DNA Isolation Kit ® and separated from non-DNA extracts by centrifugation. A 100 µl elution containing the sample is then stored at -20 deg. C for PCR. PCR is an iterative process of heating and cooling the sample to denature the eDNA and amplify a genetic marker that is specific to the target species. Theoretically, the concentration of a target species marker will double each time the sample is heated and cooled. Samples can be analyzed using two types of PCR: cPCR and qPCR. The cPCR assay is strictly a test for the pres-

ence or absence of the marker. Samples testing positive for the genetic marker using the cPCR assay are sequenced to confirm that they come from the target species. The qPCR assay (referring, again, to TaqMan-style qPCR) detects the presence of the marker and also provides an estimate of the concentration; however, samples testing positive using qPCR are assumed to be specific to the target species and are typically not sequence confirmed. This assumption, however, will require rigorous testing and occasional verification. Currently, only the cPCR assay is included in the QAPP as an approved technique for Asian carp eDNA monitoring studies in the CAWS, and qPCR is being examined for inclusion in the QAPP. The graph for detectability of eDNA (Figure 2.1.5) terminates in two nodes (cPCR and qPCR) that describe the outcomes of the PCR assays. The cPCR and qPCR assays are performed on separate 1 µl aliquots drawn from a 100 µl elution that is produced using the extraction procedure.

The cPCR node can take one of two possible states, *Negative* if eDNA is not detected or *Positive* if eDNA is detected. In cPCR, DNA fragments other than the target marker can sometimes produce fluorescent bands that may indicate a positive test result. Therefore, the DNA from all positive cPCR assays must be sequenced and sequences are evaluated using BLAST searches in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) (Benson *et al.* 2011) to assess how closely the DNA fragment matches the target species. The outcome of a confirmed positive cPCR test result may remain uncertain if there are questions about target marker specificity. This uncertainty is represented by the variable CSPEC, which is the degree of belief in the specificity of the target marker. The outcome of the cPCR assay depends on the concentration in the aliquot drawn for the assay (CALIQ, copies/µl), cPCR marker sensitivity, and PCR efficiency (EPCR). The cPCR marker sensitivity is the probability of detecting the marker given its concentration. PCR efficiency (EPCR) is difficult to measure in cPCR, but is the ratio of the amount of DNA that is detected and the amount of DNA that should be detected. This is primarily a function of the level of inhibition (CINHIB) and primer quality (CPRIMER). CINHIB is the level of PCR inhibition present in the aliquot. Algae, bile salts and humic acid are just a few of the known substances that, if present, can inhibit PCR reactions (Alaeddini 2012). CPRIMER is the ability of the cPCR primer to locate and bind to the genetic marker. This is important because different primers exhibit differences in their ability to bind to a target.

The QPCR node describes the outcome of the qPCR assay and is the concentration of the target marker in an aliquot withdrawn from the elution. Because qPCR and cPCR utilize different markers, and qPCR markers are generally shorter than cPCR markers, cPCR

Figure 2.1.5. Conceptual model of eDNA detectability using PCR.



and qPCR markers may tend to be present in different concentrations that reflect differences in degradation rates that depend on marker length. If so, the ratio of the two marker concentrations may vary with the age of the DNA and the ratio of the lengths of the markers (LRATIO). The concentration of a qPCR marker is estimated using TaqMan PCR (Holland *et al.* 1991), which is a qPCR method designed to increase specificity of the PCR reaction. A fluorescently labeled probe designed to bind to the target marker is added to the aliquot. As the DNA fragment is copied by enzymes, the probe is cleaved from the template and a fluorescent signal is emitted. The number of copies of the eDNA fragment doubles with every PCR reaction cycle, producing a logarithmic increase in the intensity of the fluorescent signal over time. If no sig- nal is reached after 40 qPCR cycles, the sample is presumed negative. An estimate of the num- ber of eDNA copies in an aliquot is obtained by measuring the amount of fluorescence pro- duced following each PCR cycle and comparing this to the level of fluorescence produced at each cycle by a dilution series of known DNA marker concentrations. The CT value, the number of qPCR cycles required to achieve a critical level of fluorescence, increases as the concentration of eDNA in the aliquot decreases. At very low concentrations of eDNA, qPCR may produce false negatives (failing to detect target species eDNA when it is actually present) because the concentration of DNA in the sample is below the detection limit for the qPCR marker system.

In Figure 2.1.5, the qPCR node depends on the critical CT value (CRITCT, cycles), the actual CT value observed during the assay (ACTCT, cycles), and the specificity of the qPCR marker (QSPEC). The CRITCT node is parameterized by running a set of standards containing known concentrations of the marker. CRITCT is uncertain because, when creating a set of standard fluorescent curves, the number of cycles needed to exceed critical fluorescence at a given concentration will vary. This depends, in part, on the efficiency of the qPCR reaction (EPCR), which is a function of the level of inhibition (QINHIB) and primer quality (QPRIMER). ACTCT is uncertain because at least three aliquots from the elution with unknown copy number are used in each qPCR assay and these may produce fluorescence at different CT values, either because of the random effects associated with the process that produces fluorescence or because of differences in the concentration of a marker among the aliquots. QSPEC is similar to the node CSPEC and represents the degree of belief in the specificity of the qPCR marker.

Each monitoring sample from the CAWS is processed and reduced to a single 100 µl elution of unknown concentration. One possibility would be to assume that the elution is an homogenous solution. However, eDNA markers are discrete particles and aliquots extracted from an elution may contain a variable copy number. For example, at concentrations less than 100 copies/µl, the number of aliquots potentially drawn from an elution exceeds the copy number and some aliquots will contain no copies of the marker. The CALIQ and QALIC nodes describe the uncertain concentration (copies / µl) of cPCR and qPCR markers in an aliquot, respectively. Aliquot concentrations depend on the elution concentration and are influenced by the processes involved with extracting aliquots from the elution.

Marker concentrations in an elution depend on sample volume (SVOL, liters), the concentration of the marker in the water sample drawn from the CAWS (CSAMP, copies/L), and the extraction efficiency (EEXT). Water samples are typically two liters. All else equal, larger water samples should contain a larger number of markers than smaller water samples. The node CSAMP is the unknown concentration of a marker in the monitoring sample. This depends on concentration of the marker in the reach (CSEG, copies/L) and the distribution of eDNA in the water column. After collection, the content of water samples are collected on glass fiber filters and the eDNA is then extracted from the filter. Higher extraction efficiencies will result in higher elution concentrations.

There are significant questions about how eDNA is distributed in the water column, and this may influence the concentration of the sample. For example, one hypothesis is that eDNA has a strong surface affinity (SAFF) and this has been the rationale for collecting water samples from the surface of the water column. There are arguments both for and against this hypothesis. The primary argument for this hypothesis has been that eDNA is associated with fish feces that

have a tendency to float and may therefore be associated with scum that accumulates on the water surface. The primary argument against this hypothesis is that a large fraction of fish feces do not float and fecal matter may disintegrate rapidly, leaving small particles of free DNA in solution to become mixed in the water column. SAFF is a partition coefficient describing the propensity of eDNA to be in the surface layer of the water column. High values of SAFF indicate that eDNA has a strong propensity to be in the surface layer, values of SAFF close to one suggest that eDNA is equally distributed in all layers of the water column, and values of SAFF less than one suggest eDNA is more likely not to be in the surface layer.

The node labeled SITE is another partition coefficient. If eDNA is associated with scum on the water surface, then its distribution on the water surface may be influenced by wind and currents. For example, during windy days, there may be a tendency for surface scum to accumulate along the banks or in backwaters. Similarly, currents may tend to be stronger in the center of the canal than along the edges, making it more likely that surface scum, and possibly eDNA, will be found along the banks rather than toward the center of the canal. The SITE variable is a function of wind speed at the time of sampling (WIND, kmh), currents at the location the sample is taken (CURR, m/s), and a qualitative variable describing habitat characteristics (HABITAT), such as along the bank, in a backwater, or mid-stream.

Preliminary Plans for Parameterization of Network Nodes

The conceptual models presented above represent interdependencies among random variables that influence the outcome of eDNA assays. These graphs can be converted to probabilistic models by parameterizing network nodes. Parameterization proceeds by defining a discrete domain for each random variable that is a mutually exclusive and collectively exhaustive set of potential random variable states (variables that are naturally continuous must be discretized). A random variable whose state does not depend directly upon the state of another random variable (e.g., has no edges directed toward it from another node) is defined by a probability table that gives, for every potential state, the probability of that state. All other variables are defined by conditional probability tables (CPTs) that define the probability of the random variable state for every possible combination of parent node states (Koller and Friedman 2009). Prior uncertainty in the value of each variable may be based on existing data, external model outputs, functions of parent node variables, or engineering judgment. In this study, a strong preference will be given to using data, external models, and functions. Engineering judgment will be used only as a last resort. In general, preference will be given to databases developed during the course of ECALS or through other ACRCC efforts, or through published studies. Additional laboratory or field experiments may be needed to construct the CPTs.

In this section of the report, we provide a more detailed definition for each node and, where information is presently available and summarize the plans for parameterization of each node. Each node of the conceptual model of eDNA occurrence and persistence in the CAWS (Figure 2.1.4) is defined in Appendix A, Table A.1. Each node of the conceptual model of eDNA detectability (Figure 2.1.5) is defined in Appendix A, Table A.2. Preliminary plans for parameterizing each node are described following the definition. Where CPTs are derived from existing models, ECALS will use the outputs of one of two models. A Curvilinear-grid Hydrodynamics 3D (CH3D) model is being developed to simulate hydrodynamics in the CAWS for the period 2009-2012. Twenty main stem reaches between Lake Michigan and Dresden Lock and Dam, as described in Table 2.1.1, are represented in the model. The CH3D model includes the four tributary reaches (LMI, NBC, GCR, LCR) as open-flow boundaries. Outputs of CH3D will support implementation of ECALS' CE-QUAL-ICM model, which will be used to simulate the fate and transport of eDNA markers and coincident water quality constituents. Details regarding calculations or model runs are not provided in this summary.

Conclusion

This interim milestone report has described a conceptual model that will serve as a point of departure for developing the probabilistic model. ECALS vision is to develop a probabilistic model that will enable fisheries managers to interpret eDNA monitoring data more effectively so that appropriate management actions can be taken in response to eDNA monitoring results. The probabilistic model is designed to estimate: 1) the probability that each of the potential sources and vectors of eDNA in the CAWS is, in fact, the actual source of eDNA detected in monitoring samples; and 2) the probability that bighead or silver carp are present in the CAWS given the evidence from eDNA monitoring results and other lines of evidence.

ECALS has identified several design principles to help guide development of the probabilistic model: 1) the model should be available for real-time implementation so that results can be obtained as soon as possible after eDNA monitoring results become available; 2) the cost associated with implementing the model should be minimal; 3) the analytical procedure should be accessible to a trained technician at the Master's level; 4) the analysis should be transparent and credible to support decision making; 5) the model should require little or no updating in the near term; and 6) the model should be transferable to other locations on the Asian carp invasion front with a minimum level of effort. The extent to which each of these goals can be successfully met is unclear because the model is still under development. As ECALS finalizes development plans, these principles will be considered.

2.2 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.

A detailed description of the methods and results can be found in the February 2013 Interim Report. It was 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.

2.3 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

According to New Life, the Super Soil Booster contains about 20% Asian carp per batch. According to Schafer, they use about 30 million pounds of Asian carp in their fertilizers per year, and that the majority of their product contained Asian carp.

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 fertilizer when diluted at 1 part fertilizer: 20 parts water (150 ml total volume) 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, as was likely, either the DNA was degraded during processing or inhibitory substances prevented PCR detection.

2.4 Boat Hulls and Fishing Gear

Fisheries gear (boats, nets) from natural resources agencies, contract fishermen, and 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 USACE Engineer Research and Development Center (ERDC) personnel in October 2012 and November 2013.

2.4.1 October 2012 Trials

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 2.4.1). 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. Swab samples were then shipped to ERDC for cPCR DNA assays using the QAPP markers.

Additionally, ERDC personnel took 8 eDNA samples from 17 gallons of distilled water in which portions of nets used to capture Asian carp were rinsed. Initially, 19 liters (5 gallons) of distilled water was poured into a tub and, prior to rinsing nets, liters of water were taken from

the tub as a negative or “water blank” control as a means to monitor for inadvertent Asian carp DNA contamination of the tubs or water and false positive results. After moderate rinsing (or “swishing about”) of each net in its tub, two liters of water were taken as a sample. Each sample (and its paired negative control) was then processed and assayed using the same procedures as outlined in the QAPP for eDNA samples.

Results

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. Considering the likely very high concentrations of carp DNA on boats and nets, and our efforts to rapidly process samples in order to not significantly delay departure of commercial fishing boats, it is not surprising that DNA may have contaminated sampling personnel clothing after which that clothing came into contact with gloves or the tubs used to rinse nets, or that, perhaps, the tubs were not adequately sterilized between uses (some were used for more than one net) – either of which scenario may have resulted in contamination of negative (“clean water”) controls taken from tubs prior to rinsing nets. However, as observed on gels and measured using a qPCR marker developed by UMESC, the levels of DNA detected in controls were very minor compared to large amounts of DNA associated with net samples (Figures 2.4.2 and 2.4.3). A second effort to measure DNA content of nets in 2013 included careful planning to avoid the same errors that resulted in cross-contamination in this earlier trial and no evidence of contamination was observed (methods and results in next subsection).

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.

Figure 2.4.1. USACE field researchers testing commercial fishing boat hulls for the presence of Asian carp DNA.

Figure 2.4.2. Test results for bighead carp DNA on commercial fishing nets.

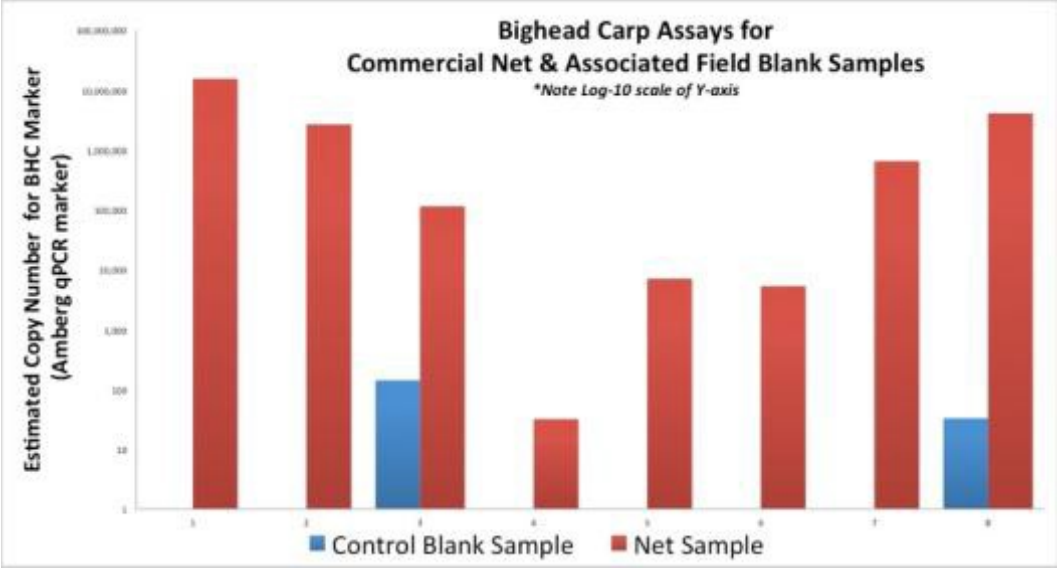
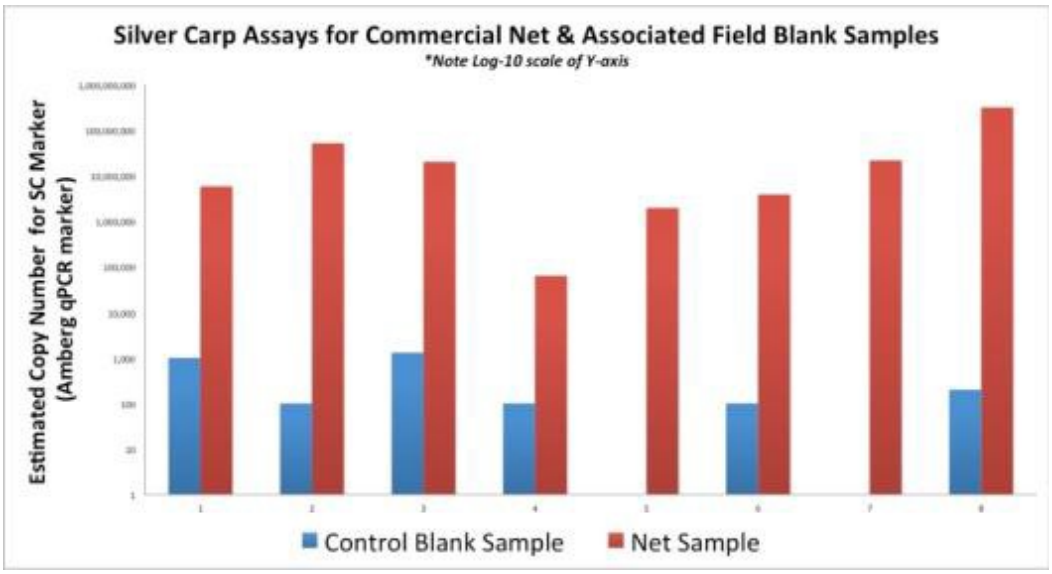


Figure 2.4.3. Test results for silver carp DNA on commercial fishing nets.



2.4.2 Summer/Fall 2013 Trials

Additional examination of the potential for boat hulls and commercial fishing nets to transport Asian carp eDNA took place in late 2013. The primary purpose was to further demonstrate that boat hulls and nets have the capacity to transport large quantities of Asian carp eDNA. Because of the inherently significant variances that might exist in DNA loadings among nets (e.g. use and other factors), as well as the likely variances among boat hulls (e.g. location and frequency of use), as well as other potential factors, the results of these trials are unlikely to represent the entire range of possible values.

Methods

Hull Sampling

On 21 November 2013 we swabbed the outer hulls of commercial fishing boats working Barrier Defense carp netting in the Marseilles Pool of the Illinois River. Our intent was to characterize the amount of eDNA on hulls at three different times: pre-fishing, post-fishing, and after a pressure bleach wash. In the morning, prior to boats entering into the Illinois River at the William G. Stratton State Park ramp in Morris, IL, each of five commercial boats was sampled for hull DNA. On each boat, a 25 cm x 25 cm section just above the keel of the outer starboard bow hull was selected and completely wiped with five 55 mm-diameter glass fiber filter papers. Each filter paper was dampened prior to wiping by spraying the paper with commercial distilled water. Each combined set of five filter papers was placed in a sterile 15 ml screw-top polypropylene tube and stored on ice. Prior to taking each sample, five filter papers were dampened and placed in a sterile 15 ml screw-top polypropylene tube and stored on ice to serve as a negative control for that set of samples. In the afternoon, soon after the boats were pulled out of the river, samples and negative controls were taken in the same manner, except the filter papers did not need moistening prior to swabbing the hull. For these sets of samples, the outer *port* bow hull, just above the keel, was sampled. Finally, after boats had unloaded fish and cleaned their outer hulls using high-pressure steam spray with bleach (cleaning station set up at about 175 m from fish 0ff-loading site), we likewise sampled the outer port *mid* hull, just above the keel and further aft than the spots where earlier samples were taken (since most DNA was removed during the first sample). As with earlier samples, negative controls samples were taken prior to hull sampling. All samples were stored on dry ice and shipped overnight to ERDC, where upon arrival they were stored at -20° C.

Net Sampling

In early summer 2013 ERDC purchased 5 commercial fishing nets, with each gill net being 300 feet long and comprised of 12 foot deep net for 150 feet and 14 feet depth for 150 feet. Each net was provided to a commercial fisherman, who then used the net during every barrier defense event (below the barrier) starting in early July 2013. ERDC collected the nets on 21 November 2013 as boats left the water following fishing for the day in the Marseilles Pool of the Illinois River. The nets had been used that day and the previous 2 days to fish Morris and/or Starved Rock (both have abundant Asian carp populations). We excised a 10 foot section of net from the middle of each net and rinsed (material "swished" around in water) that section for 1 minute in newly purchased 5 gallon buckets holding 15 liters (4 gallons) of commercial distilled water (Figure 2.4.4). Following the 1-minute rinse, five 50-ml grab samples (standard screw-top polypropylene centrifuge tubes) were taken from the water in each bucket. Prior to placing each net section in the rinse water, a negative control sample (50 ml water) was taken from each bucket. The samples (5 samples x 5 nets, plus one negative control for each net for 30 samples total) were kept on dry ice and shipped overnight to ERDC, where upon arrival they were stored at -20° C. The nets were also shipped overnight to ERDC. At ERDC the nets were stored in open tubs within rodent-proof cages in a storage room with uncontrolled interior temperature. At approximately one-month intervals from 21 November 2013, 10-foot interior sections from each net have been excised and similarly processed. As of the date of this report, 5 additional monthly samples have been processed.

Genetic Assays

DNA was isolated and purified from each set of five swabs (single extraction reaction) taken from boat hulls using a modified CTAB protocol. For each net and sampling date, one [1] of five 50-ml rinse water samples was spun down to pellet any material collected in the water. DNA from the pellet was extracted using the same modified CTAB protocol as used for boat hull swabs. The negative control sample from each boat hull or net set was likewise processed. Extracts from boat hull and net samples, as well as associated negative controls were then assayed with the qPCR marker SCTM-5. These qPCR assays included 5 replicate reactions per sample.

[1] The remaining 4 50-ml samples from each net sampling event (net x date) were stored for later use (if needed).

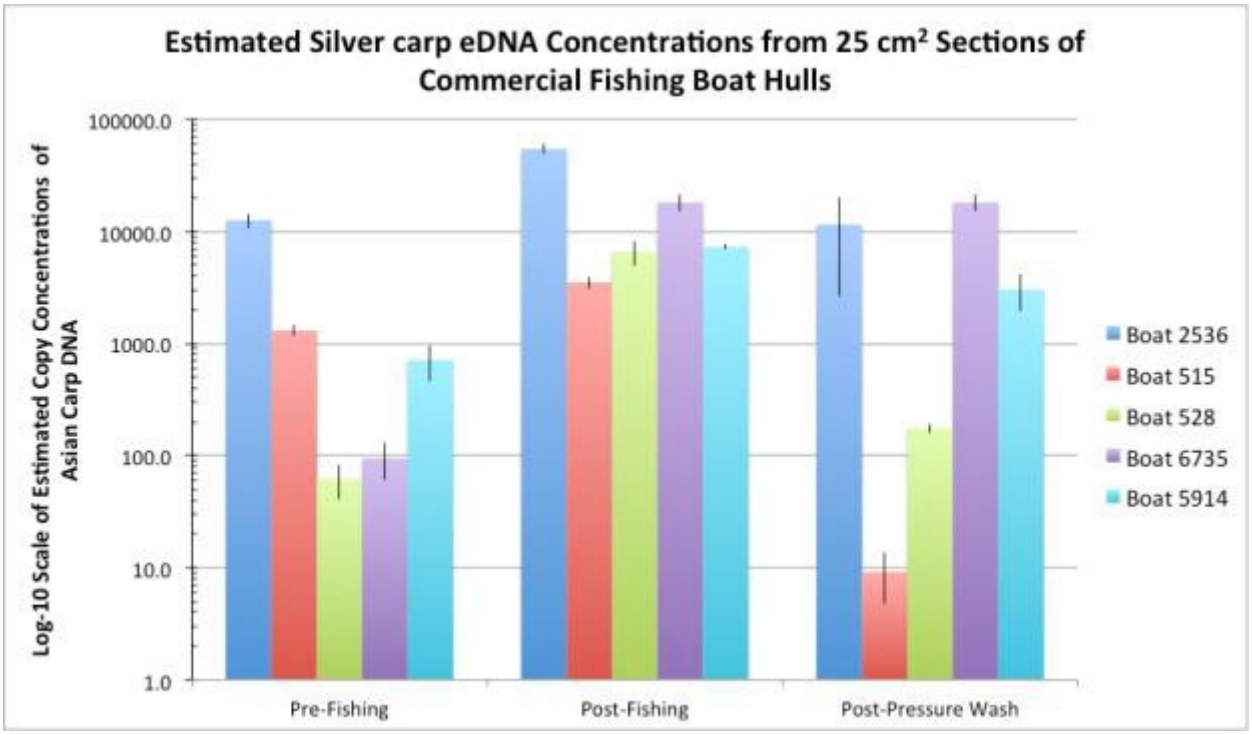
Figure 2.4.4. Net processing. Each net is laid out over a new plastic sheet, a middle portion (10 ft. or ~ 3.05 m) is cut away with sterile scissors, and the excised section is rinsed (with moderate swirling and dunking) in commercial distilled water in a new bucket for 1 minute in order to release DNA into bucket water for sampling.

Results

Hulls

Analysis of boat hull swabs on five commercial fishing vessels in the morning prior to a day of fishing yielded between 10 to 3000 copies of silver carp eDNA per µl of DNA extract. Assuming 100 µl of DNA extract from each sample and the 25 cm^2 sampling area provides a very rough estimate of 40-12,000 copies/cm^2 of Asian carp eDNA on the boat hulls (Figure 2.4.5). Upon return from fishing in the afternoon, boat hull samples yielded between 1,000 to 14,000 copies of silver carp eDNA, and a rough estimation of 4,000-56,000 copies/cm^2 of Asian carp eDNA on the boat hulls. After a high pressure bleach wash of the boat hulls, between 1,000 to 4,500 copies of silver carp eDNA were detected in samples, which would result in the rough calculation of 4,000-18,000 copies/cm^2. Boat 6735, which showed virtually no change in eDNA concentration from the second to the third set of samples was found to have inadvertently skipped the pressure-cleaning.

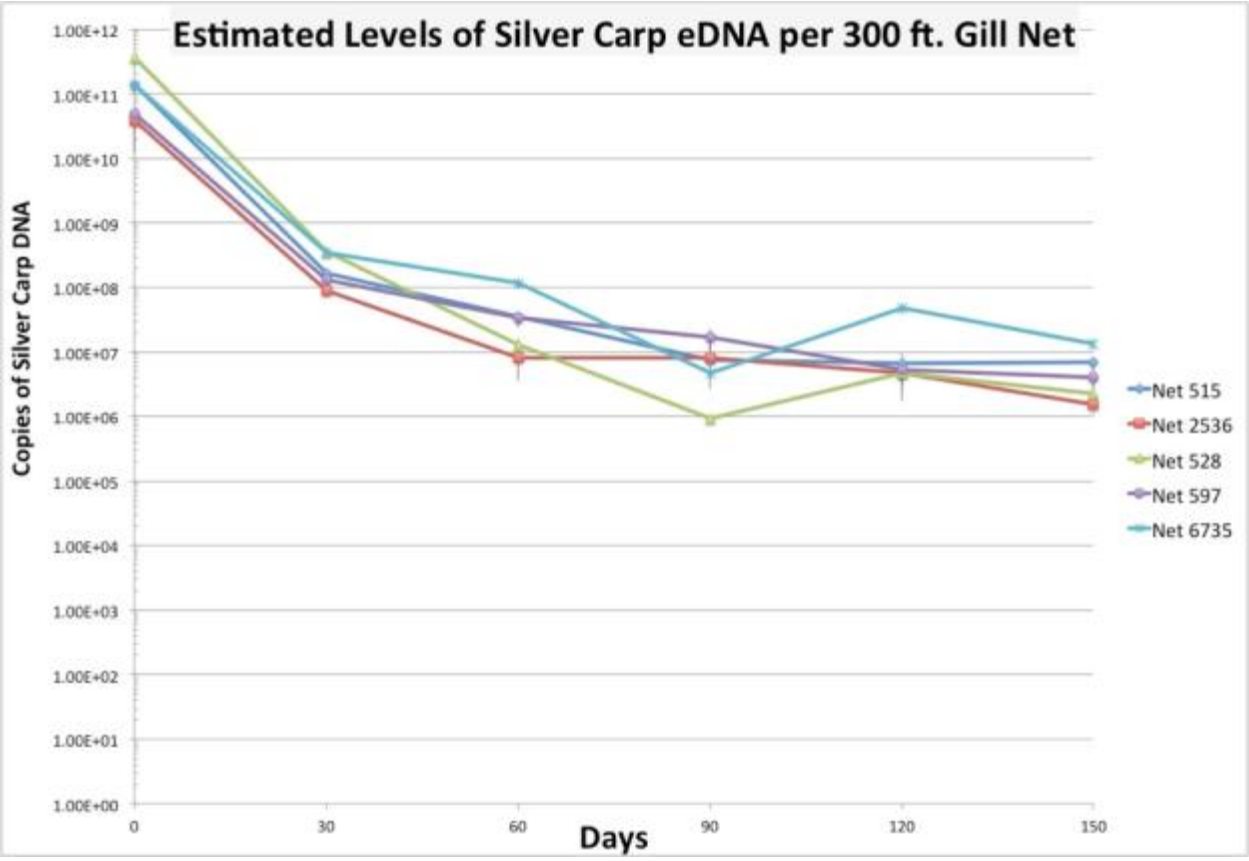
Figure 2.4.5. Estimated concentration of silver carp eDNA (copies/cm^2) on the hulls of five commercial fishing boats prior to entering Illinois River, immediately after exiting Illinois River, and after a high-pressure bleach wash.



Nets

All negative controls were clean. Among the five nets, the qPCR estimates for Day 0 (fresh from use) ranged from 42,350-390,250 copies of silver carp eDNA per µl of DNA extract. Assuming 100 µl of DNA extract from each sample (50 ml), roughly 15,142 ml per sampled volume of water (~ 4 gallons) per 12 m^2 (~130 ft^2; on average, 10 foot section of net ≈ 10 foot x 13 foot), and a total of about 362 m^2 (~3900 ft^2; 300 foot length net with average width of 13 feet) of net, provides a very rough estimate of 2 x 10^9-4x10^{10} copies of silver carp eDNA per 300 ft. net (Figure 2.4.6) immediately after use. Nets have been stored for six months since the Nov. 2103 sampling and sampled once each month in order to demonstrate the DNA levels remaining over time. DNA appears to degrade relatively rapidly, but even at Month 5 we estimate that there are still more than a million intact copies of the silver carp marker associated with each net. If commercial fishermen were moving several nets from waters with large Asian carp populations to above the barrier locales (prior to changes in protocol), they could have moved a lot of DNA.

Figure 2.4.6. Estimated copy number (CN) of silver carp eDNA that is associated with five 300-ft gill nets up to five months after each net was pulled from use.



2.5 Bird Transport and Deposition of eDNA

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 2.5.1 and 2.5.2 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).

2.5.1 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 to 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. A detailed description of the cPCR methods and results can be found in the in Table 2.5.1.

Table 2.5.1. Days silver carp DNA was detected in feces from piscivorous birds following a meal of silver carp. A plus (+) indicates a positive detection of silver carp DNA and 'NA' indicates data results are not available for this sample set.

Bird	Study Day			
	1	4	7	8
Eagle	+	+	+	+
Pelican	+	+	NA	+
Cormorant/Ibis	+	+	NA	+

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.

2.5.2 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). A detailed description of the methods and results can be found in the February 2013 Interim Report.

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.

2.6 Documenting Presence and Satellite Tracking of Piscivorous Birds

Efforts pertaining to the satellite tracking of piscivorous birds have been documented in an ECALS report (Guilfoyle et al. 2014). The following summarizes research conducted on piscivorous birds along the Illinois River into the Chicago Area Water System (CAWS) in 2012. Our first objective was to 1) document the presence and relative abundance of piscivorous birds in the region, and 2) document the seasonal behaviors of double-crested cormorants (*Phalacrocorax auritus*), especially their daily movements and likely foraging behaviors in and around the CAWS. The double-crested cormorant was chosen as the target species for this research because of its large breeding population in the CAWS and its well-known predatory behavior on fish populations.

We used eBird, a national online database of bird observation data, to document the relative abundance and distribution of the 10 most common piscivorous birds in and around the CAWS. From 2005 to 2012, the double-crested cormorant was the most common breeding piscivorous bird in the region, followed closely by the American white pelican (*Pelecanus erythrorthynchos*), and other, less common species including the great egret (*Ardea alba*) and great blue heron (*A. herodias*). The bald eagle (*Haliaeetus leucocephalus*) eagle was the most common winter piscivorous species. Thirty double-crested cormorants were captured and fit- ted with Sirtrack® Argos Satellite Platform Transmitting Terminal (PTT) Harness Transmit- ters (model: K3H 174A KiwiSat 303). Fifteen of these birds were captured at Baker's Lake, Barrington, IL, which is located within the CAWS. The remaining birds were captured at The Nature Conservancy (TNC) Emiquon Preserve near Havana, IL, just 70 km south of Peoria, IL, near the largest northern spawning area for carp along the Illinois River. During the processing of captured birds, cloacal and throat swabs were collected along with feather samples for anal- yses for presence of Asian carp DNA.

Cormorants captured at both colony sites moved in large steps, with the mean distance of just over 60 km per step distance during the breeding season, indicating that this species is capable of moving the distance between the CAWS and carps spawning areas with ease. However, no overlap in daily movements or breeding home ranges was observed between the colonies. Cloacal and/or throat swabs were positive for carp eDNA from birds from both colonies, with 14 of 15 birds being positive from the TNC Preserve and 7 of 15 birds positive from Baker's Lake, indicating conclusively that birds from both colonies were feeding on carp. Movement data from the satellite tags could not determine where the birds from Baker's Lake may have been foraging on carp based on current Asian carp distribution data.

Concurrent research by the USGS showed that captive cormorants and eagles fed a diet of carp had positive carp eDNA in their feces for up to 7 days. Moreover, bird feces placed on metal sheets and exposed to the elements also yielded positive carp eDNA for up to 30 days. These data suggest that large fecal accumulations at cormorant nesting sites throughout the CAWS may potentially contaminate the system with carp eDNA after every rain event. Together, these data reveal that cormorants in particular and piscivorous birds in general, are potentially significant sources of Asian carp eDNA throughout the CAWS. This research could not determine the magnitude of bird-contributed carp eDNA in the system versus other potential sources (e.g., boat hulls, bilge pumps, etc.), but nevertheless, the role of birds as vectors of carp eDNA in the CAWS should not be ignored.

2.7 Asian Carp Carcasses on Barges

2.7.1 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 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. A detailed description of the methods and results can be found in the February 2013 Interim Report.

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 the 2013 Interim Report.

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 ntended 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 and then again on Day 28.

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 and then again on Day 28. 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.

Due to the patchy distribution of DNA in a sample, 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 even if DNA was not detected in aliquots analyzed from those other samples. 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) even if DNA was not detected in aliquots analyzed from those other samples.

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, from water flowing over silver carp carcasses and from silver carp slime taken on Day 18. DNA was detected in the silver carp slime in at least one sample on each day until the end of the study, Day 18.

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.

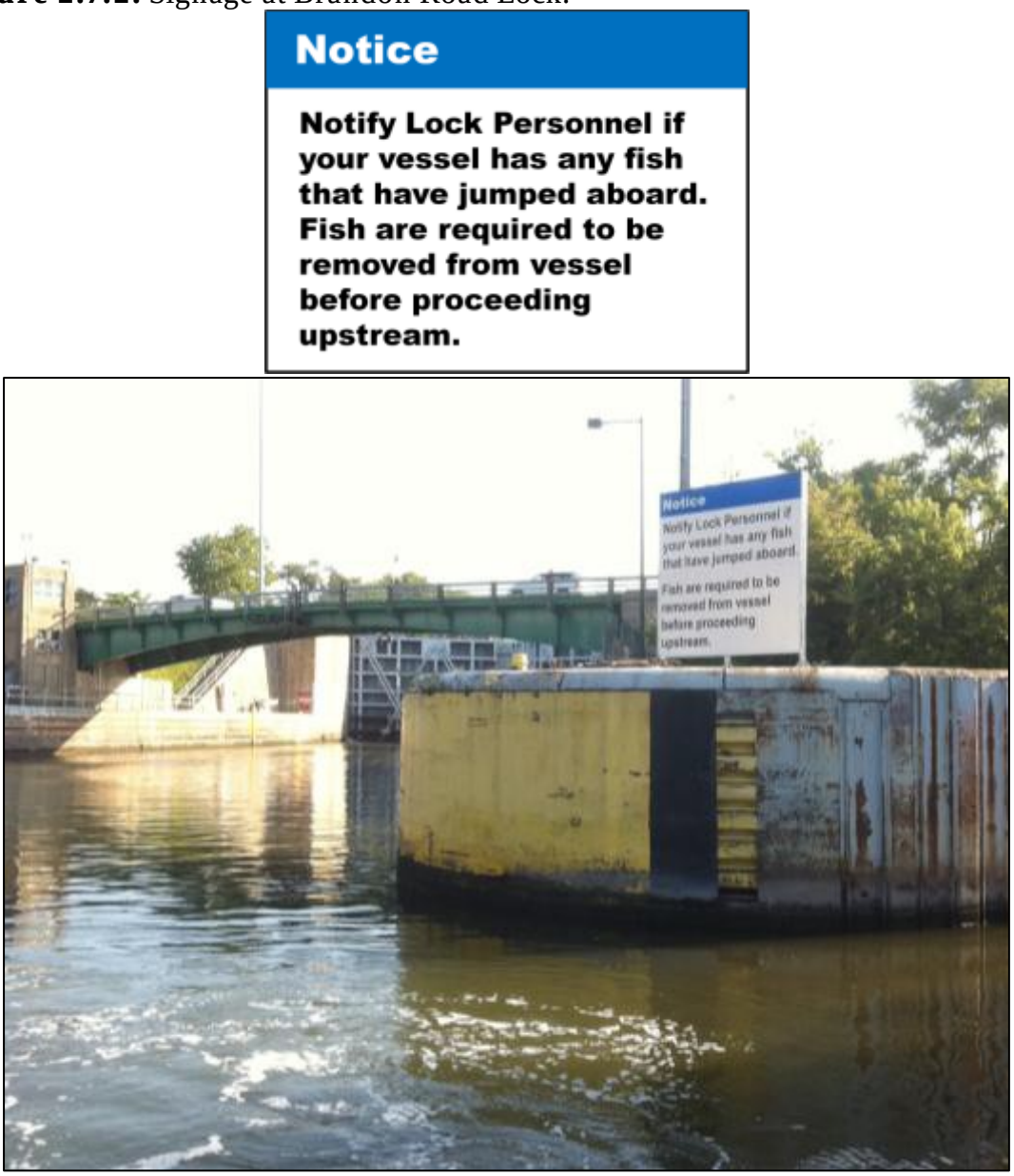
The detection of silver carp DNA in samples of silver carp slime taken from the metal sheets on Day 1 suggests that fomites (e.g. barges, boats, etc.) could move Asian carp DNA from areas where Asian carp are present to where they are absent. Detectible DNA was present in the silver carp slime out to Day 18. This suggests that DNA may persist while adhering to a barge or boat for more than 4 weeks under the right conditions. During this time, these barges and boats can be transferring DNA long distances into areas where Asian carp are not present.

2.7.2 Fish Carcass Transport on Barges

A Guideline for Vessel Operators (refer to February 2013 Interim Report) 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 2.7.2).

Figure 2.7.2. Signage at Brandon Road Lock.



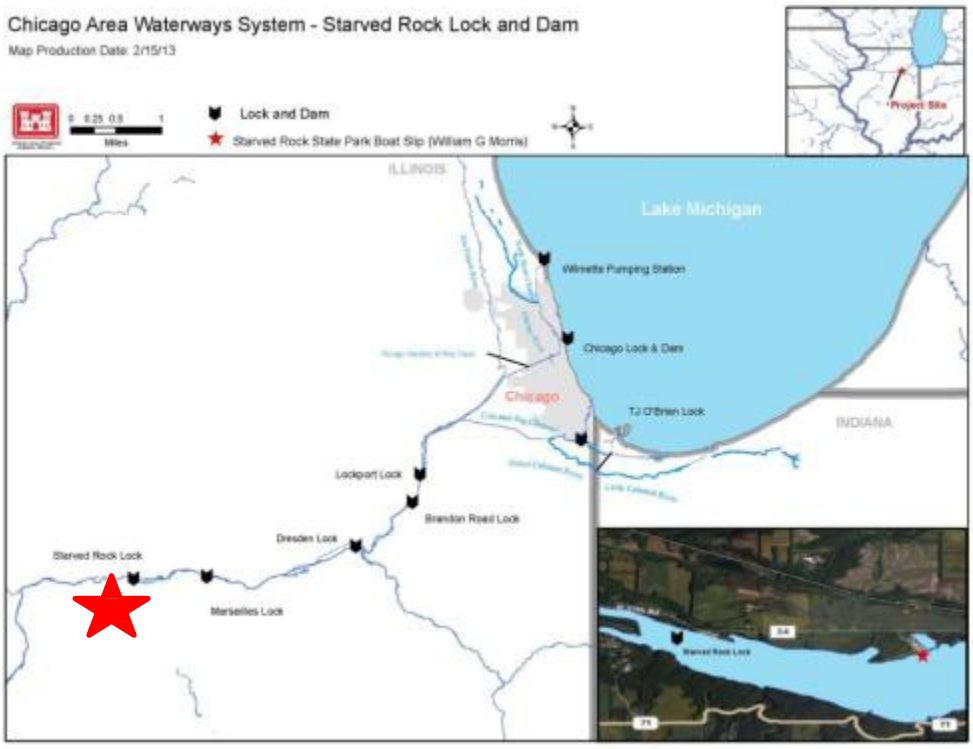
2.8 Sediment eDNA

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.

2.8.1 Sample Collection and Initial Screening

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 2.8.1), where both species of Asian carp are abundant. 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 2.8.1. 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 100 ml 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.

2.8.2 Sediment Sorption Testing

Efforts pertaining to progress on the sediment testing have been documented in the 2013 ECALS milestone report. Although there may be some repetition with respect to ECALS background information here, the entire sediment milestone report is presented in its entirety, with minimal editing, for completeness.

Introduction

Understanding the contribution of environmental DNA (eDNA) in sediments to the signature of Asian carp (specifically, bighead carp and silver carp) in surface waters is a critical factor in determining the utility of eDNA as a real-time biomonitoring tool, with the capability of distinguishing between eDNA artifacts and live fish. Sediment testing was conducted to better understand the interaction and persistence of eDNA in sediments, and the likelihood of long term release contributing to a persistent Asian carp signature even in the absence of fish.

There are numerous questions regarding the transport and fate of eDNA in surface waters and sediments and therefore the utility of eDNA as a real-time indicator of Asian carp populations. Bench tests were designed to address those mechanisms relevant to sediment as a source of eDNA (as opposed to actual fish), and the short and long term potential for release and detection of eDNA as an artifact originating from the sediment. Specifically, the testing was focused on questions regarding the affinity of sediments for eDNA, the persistence of eDNA in sediments, and the contribution of sediment associated eDNA to concentra- tions in surface water. The specific objectives of this study were three-fold:

1. To identify potentially relevant sediment and environmental parameters affecting sorption of extracellular DNA to sediments

2. To evaluate the degree and reversibility of sorption/desorption taking place between sediment and extracellular DNA

3. To evaluate the influence of different sediment types and environmental conditions on eDNA sorption/desorption in sediments

Background

Asian carp, including bighead carp (*Hypophthalmichthys nobilis*) and silver carp (*H. molitrix*) are invasive species that have been steadily dispersing upstream through the Mississippi and Illinois Rivers since the 1990s. An electrical barrier was installed near Lockport to deter the advance of Asian carp into the Great Lakes, where they pose a serious threat to native fish populations. The Chicago Area Waterways System (CAWS), a network of over 100 miles of rivers and canals connecting Lake Michigan with the Mississippi River, potentially provides a direct route for introduction of Asian carp into the Great Lakes. Asian carp have reportedly been seen in the CAWS below the barrier, but only one has been captured above the barrier to date (personal communication Kelly Baerwaldt, October 2013). Effective monitoring tools are needed, however, in order to detect any advance in the Asian carp population front and prevent their movement into sensitive areas; eDNA has been proposed as such a monitoring tool. Little is known about the persistence of the eDNA signature in sediment, and the potential for sediment to serve as an eDNA "vector", resulting in false positives. A literature search was conducted to ascertain what is presently known about the persistence and behavior of eDNA in sediments; this information was used to design appropriate bench scale testing to further evaluate the nature of the interaction of eDNA with sediments and the potential contribution to Asian carp "signature" in affected water bodies. Previous literature reviews of extracellular deoxyribonucleic acid (DNA) in soil (Levy-Booth et al. 2007) and soil/sediment (Pietramellara et al. 2009) environments have been compiled. The current effort was focused on literature relevant to environmental DNA (eDNA) in sediment specific to Asian carp.

Extracellular DNA differs from eDNA in that it is outside the cell, whereas eDNA is any DNA that is no longer associated with, in this case, a living carp. There- fore, eDNA includes extracellular DNA, but also DNA associated with mucus, scales, feces etc. Extracellular DNA is thought to be the DNA phase most likely to interact with sediments on a particle to particle level, such that long term storage and release might result.

Based on the results of the limited literature search, physico-chemical and geotechnical parameters potentially relevant to sediment/DNA interaction include (Ogram et al. 1988):

- Mineralogy and grain size
- Cation Exchange Capacity
- Surface area
- pH (DNA and mineral surfaces have a negative charge above pH 5)
- Ionic strength (conductivity, salinity) – cations may bridge negatively charged DNA fragments and mineral surfaces (at pH > 5)
- Organic content and composition – carbon type, humic acids, etc.
- Presence of organic contaminants such as polycyclic aromatic hydrocarbons (PAHs) and other petroleum hydrocarbons
- DNA fragment length (small fragments, <500 base pairs, exhibit strong binding)

Extracellular DNA does not exist as a dissolved fraction, but the fragments of DNA are small enough to pass through a typical filter used to filter water samples taken in the field. Therefore, depending upon whether or not the DNA is associated with particulates, it could potentially be found either in the operationally defined "dissolved phase", in the solids retained on the filter, or both.

It would appear the DNA has three major fates in soils (Levy-Booth et al. 2007):

- The DNA may persist through binding to mineral surfaces or absorbing into organic fractions.
- DNA may be broken down and incorporated into organisms as a nutrient source.
- Transfer of DNA can occur in soil microorganisms.
- Kinetics of sorption/exchange are thought to be rapid (seconds to minutes). Other factors important to persistence/degradation include:
- UV light exposure, exposure time and wavelength (Ravanat 2001)
- Time following cell death (Shapiro 2008; Cai et al. 2006; Kang et al. 2010)

- Bacterial and fungi mediated breakdown processes (Shapiro 2008; Ficetola et al. 2008)
- Preservation facilitated by rapid post-mortem desiccation, cold temperatures (and high salinity) (Shapiro 2008; Ficetola et al. 2008; Matsui, K. et al. 2001)
- Sorption to minerals or organic contaminants (PAHs) that may protect from enzymatic degradation (Lorenz et al. 1981, Aardema et al. 1983, Romanowski et al. 1991)

Based on the results of the literature study, it is clear that detritus entrapped in the sediment matrix and containing cellular DNA could result in periodic releases to surface water occurring in response to sediment disturbances; the extent and duration of these releases which would be expected to be a function of degrada- tion rate, sediment type, and other site specific conditions. What is not clear is the potential signature associated with extracellular DNA sorbed to and subsequently released from the sediment during periods of resuspension.

A number of challenges were anticipated in the sediment sorption testing. DNA water analysis is customarily conducted on a small subsample – approximately 100 µl – of larger field samples, with DNA concentrations in the ng/µl ranges. Typically, only a small fraction of DNA in a sample is recovered by DNA extraction. Using available PCR markers for Asian carp DNA, a detection limit of 10^{-4} ng/µl is possible; to achieve quantifiable results, solution concentrations of two or more times the detection limit are necessary. Variations in the extraction procedure itself may also be problematic. While higher concentration solutions (200-300 ng/µl) of naked (genomic) DNA can be generated for use in the sorption testing, the volumes that can be produced are small, and sample to sample contamination is more likely when working with such high concentra- tions. Sediment extractions to date have been limited to very small samples (0.1g-0.2g). The sorption testing was therefore constrained by the sediment sample extraction size limitations as well as limitations in the volume and concentration of DNA solution that could be generated for the testing. Obtaining a sediment sample inclusive of all of the constituents present in the bulk sediment was also expected to be difficult with such small sample masses. However, humic acids and other compounds commonly found in sediments are thought to be inhibitory to detection/quantitation of DNA; smaller sediment samples could be advantageous in this regard. Due to inhibitory effects, DNA sorption onto organic matrices may be impossible to measure. Further, while qPCR (real-time quantitative polymerase chain reaction) is relatively specific for carp DNA in water, sediments will also contain the rest of the carp genomic DNA and genomic DNA from myriad other organisms (e.g. microbes, benthic invertebrates, etc.) that inhabit the sediment. Proof of concept testing was required to determine whether sufficiently quantitative

measurement of DNA in sediments could be achieved to support customary sorption testing. The losses of DNA due to bacterial degradation or other factors also had to be accounted for in interpreting the results obtained.

Study Objectives

The purpose of the initial bench testing was to determine whether or not sorption of extracellular DNA takes place at measurable levels, with the ultimate goal of deriving partitioning coefficients for a variety of sediments, and evaluating the reversibility of the sorption processes. The interaction of the critical factors identified in the literature search will require further investigation in future studies.

Materials and Methods

Important sediment characteristics and environmental parameters were identified by means of a relatively extensive literature search. Subsequent laboratory testing was planned to be conducted in phases, with "go-no go" decision points based on the success of earlier phases. The planned testing phases were as follows:

Phase I: Evaluate the sorption/desorption behavior of eDNA with "clean" representative sediments from the affected river basin.

Phase II: Evaluate the magnitude and duration of the release of eDNA from eDNA containing sediments occurring under quiescent conditions and as a result of sediment resuspension.

Phase III: Evaluate the effect of various parameters, such as water depth, light, pH, and temperature, on the persistence of eDNA in sediments, and the magnitude and duration of release of eDNA from sediments.

Results obtained from the Phase I and II testing are contained in this report.

Sediment and Water Sampling

Sediment samples from areas potentially affected by Asian carp were collected in order to work with representative sediment types and, ultimately, to evaluate the effect of sediment characteristics on eDNA persistence and release. Because eDNA is typically found in the top few centimeters of sediment, representative surficial sediment samples were used in the testing. Sediment samples contain- ing eDNA were not specifically required for the testing, however, samples were handled in a manner consistent with preservation of any existing eDNA in the sediment and this DNA was accounted for in interpreting test results.

Sediment samples and surface water were obtained in November 2012 from two sites, Lake Calumet and Lockport Pool. Sediment samples were taken from approximately the top 3-4-in of the sediment, using a standard ponar sampler, as specified in the Field Sampling Plan and Quality Assurance Project Plan, attached as Appendix B. Multiple grabs were combined in clean 5-gallon buckets for later homogenization in the laboratory; 20 gallons of sediment were obtained at each site. Surficial water samples were also taken from just below the water surface; 10L of water was obtained from each site. Water samples were obtained in large plastic carboys, pre-rinsed with site water. Both sediment and water samples were iced immediately. The temperature of the samples was taken upon receipt at ERDC; samples were then placed in a cooler for storage at 4 degrees C until needed for testing.

Sample Homogenization

Each individual bucket of sediment was homogenized in a Hobart mixer; for each site, equal aliquots of sediment were taken from each bucket, composited and homogenized. Subsamples of the composite were taken for characterization and for all subsequent testing.

Sediment and Water Characterization

Sediment samples were characterized as follows:

- ☐ Visual characterization
- ☐ Mineralogy (XRD, TGA, DSC, Sequential extraction)
- ☐ Morphology (Microscopy)
- ☐ Grain size (Coulter Counter)
- ☐ Cation exchange capacity (Sequential extraction)

☐ Surface area (BET)
☐ pH (Probe)
☐ TOC (TOC analyzer – unmuffled samples)
☐ Organic composition
☐ Surface charge vs. pH (Titration)
☐ Glassy vs. crystal components (DTA)
☐ Organic matter (Sequential extraction)
☐ Carbonates, Fe-oxides (XRF)
☐ Carp DNA marker

Sediment pore water and site water were evaluated for the following (metals were measured only in pore water):

☐ Heavy metals (Elemental Analysis)
☐ pH (YSI Model 556MPS probe Probe/Paper)
☐ Conductivity (YSI Model 556MPS probe Probe)
☐ Salinity (Sper Scientific - Refractometer Model 300035)

The pH of dried, composited sediment was measured by combining 10 grams of dry sediment with 50 ml of DI water (1:5 ratio). The slurry was shaken for 2-3 minutes and allowed to settle for 3 minutes. pH was then measured using a YSI Model 556MPS probe. To assess the pH change induced during sorption testing, sediment was spiked with synthetic DNA marker solution as was used in the sorption testing; 0.8 g dried sediment (the largest sediment mass used in the sorption testing) was combined with 500 µl of DNA marker solution. The sample was shaken for 24 hours, then centrifuged for 1 minute at 3000 RPM to separate pore water. pH of the pore water was then measured using the pH probe. The pH of the marker solution was measured using pH paper because the volume was too small to permit use of a probe.

Proof of Concept Testing

Several preliminary proof of concept tests were conducted to address uncertainties regarding appropriate experimental design. Results of the proof of concept testing were used to inform the design and specifics of the subsequent sediment sorption testing. Proof of concept tests were conducted to assess the following parameters:

- *Baseline degradation* - Determination of baseline degradation in sediment and water samples, such that degradation could be distinguished from sorption

- *Sediment/DNA solution concentration and ratios* - Determination of appropriate sediment/DNA solution ratios, and solution concentrations needed to achieve measurable changes in concentration during sorption testing, taking into account limitations in the volume and concentration of DNA marker solution that could be prepared
- *Centrifuging procedures* - Determination of necessary/allowable centrifuge speeds and duration, such that a clear supernatant was obtained but DNA particulates in suspension were not removed from the water column during sample processing
- *Moisture content measurement* – Determination of necessary/allowable drying temperatures and duration to preserve DNA in the sediments while minimizing variability and error in moisture content and subsequent dry sediment mass measurement.
- *Initial and final pH of synthetic DNA solution* – Given the potential importance of pH in sorption behavior, the magnitude of pH change occurring during the sorption studies was evaluated in separate testing, in which the pH of sediment pore water and synthetic DNA solution were measured before and after sorption testing.

Baseline Degradation

Sediment and solution controls were analyzed for DNA at designated time periods, in order to estimate DNA losses attributable to degradation and establish extraction efficiency for all matrices. Although site water was available for the testing, the presence of dissolved organics may be inhibitory to DNA detection. Proof of concept testing was therefore conducted with DI water. Based on the literature, the kinetics of DNA sorption is expected to be measurable in minutes, rather than hours. However, because organic compounds are known to undergo a rapid sorption stage followed by a slower sorption stage, extended sampling intervals were therefore also evaluated. No sorption testing was conducted in the initial proof of concept testing; samples were tested for DNA concentration according to the following matrix:

- Sampling intervals 0, 72, and 120 hours (0, 3, and 5 days)
- Unspiked homogenized sediment
- DNA spiked sediment
- DNA-DI base solution
- 4-6 replicates of each sample at each time interval

Also, unspiked and spiked site water (spiked at the same DNA concentration as the DI base DNA solution) was analyzed for DNA concentration at time zero to assess background water column concentrations and to determine the impact of constituents in the site water on extraction efficiency and resulting DNA concentrations. (DI was ultimately used for the remainder of the sorption testing, to avoid introduction of additional constituents that might be inhibitory to DNA analysis and quantification.)

Unspiked site water

- DNA spiked site water
- 4 replicates

Discrete replicates were prepared and measured at each time interval (as opposed to consecutive subsamples from the same sample). DNA was extracted from fish muscle and liver tissue using DNeasy Blood & Tissue Kit from Qiagen, producing a solution containing marker DNA at approximately 50 ng/µl; this solution was diluted with DDI water to a marker concentration of approximately 20 ng/µl. The prepared solution was stored at -20 deg C prior to use. The preparation of the amplicon is further described in Appendix B.

Sediment was spiked by placing 0.2 g of dried sediment in a sterile 1.5ml or 2ml eppendorf tube, and then adding 100 µl of the 20 ng/µl solution. The spiked sediment sample was then homogenized by vortex for 10 seconds, to distribute the solution throughout the sediment.

DI samples were spiked by placing 100 µl of DI water in a sterile 1.5ml or 2ml eppendorf tube, and adding 100 µl of the 20 ng/µl DNA solution.

Sediment and water samples were allowed to stand at room temperature, in the dark, for the specified contact times, and then extracted using the CTAB extraction procedure (Doyle & Doyle 1987).

A second degradation study was conducted for sediment and solution at the concentrations ultimately selected for the subsequent sorption studies, in order to establish a degradation baseline rate representative of the actual testing conditions. DNA solution (100 µl at 0.5 ng/µl as measured by Nanodrop, 0.025 ng/µl as measured by Qubit) was added to 0.2 g of dried sediment, and extracted after 0, 1, 4, 8, 12, 24, 48, 168, 336 and 504 hours (0-21 days). Aliquots of the DNA solution were also allowed to stand, in the dark, for the same intervals and then analyzed for DNA marker concentration. The results of the degradation testing provided a basis for

corrections to aqueous DNA concentrations; the corrections facilitated later discrimination between losses attributable to degradation and losses attributable to sorption. Differences in actual and measured concentrations at time 0 were used to estimate losses due to extrac- tion/PCR inefficiency.

Moisture Content Measurement/Effect of Sediment Sample Size

Larger sediment samples could be advantageous in minimizing the relative error in the sorption estimates, but 0.2g is the approximate maximum sample size extractable for the existing procedure and available equipment. Larger sediment samples would necessitate extraction of subsamples rather than the entire sample. In order to extrapolate the sorbed sediment concentrations on a dry weight basis, the dry weight of the extracted sample must be known. This could be determined by obtaining the wet weight of each subsample to be extracted, and determining the moisture content from another subsample. Testing was therefore conducted to assess the accuracy with which moisture content could be measured in small sediment samples.

Samples were dried to a constant weight at 60 deg C, according to the procedure outlined in Appendix B. Sample drying of soils for measurement of moisture content is normally done at temperatures ranging from 100 deg C to 110 deg C (Klute 1986); saline sediments may even be dried at 180 deg C to remove waters of hydration. However, partial oxidation of organic materials may occur at these temperatures; given the relative importance of organics in sediment sorption behavior of anthropogenic compounds, and the potential for DNA destruction at higher temperatures, drying the samples at 60 deg C was considered preferable.

Moisture content was compared for samples dried first at 60 deg C, and then at 100 degrees C, to ascertain the potential error and variability that might be introduced by using a lower drying temperature. To ensure that moisture content could be accurately measured in small samples, and to inform the sediment mass to be used for the subsequent sorption testing, the effect of sediment sample size on moisture content determination was also evaluated by comparing the variation in moisture content obtained for six different wet sample weights, ranging from 0.1 g to 1.0 g, dried at 60 deg C to constant weight.

Sediment/DNA Solution Ratio and DNT-DI Solution Concentration

Informal preliminary tests were conducted to assess the volume of DNA-DI solution required to load the sediment with an extractable amount of DNA marker, and to produce a sufficient volume of supernatant with measurable DNA in sorption testing. Initially, 0.1 g of sediment was spiked with 100 µl of DNA-DI water containing 20 ng/µl DNA. For the larger testing matrices, however, the volume of DNA solution required precluded working at such high concentrations. A desktop study was conducted to estimate the sorption capacity of the sediment and refine the solution concentration required to produce meaningful data from the sorption tests. To produce meaningful data, the DNA solution concentration must be high enough that a measurable amount of DNA remains in the solution following sorption; the amount of DNA sorbed must also be sufficient to produce concentration changes that can be distinguished from analytical variability. This is a particularly challenging aspect given that the extraction efficiency of DNA is only about 30% of the DNA present. A more dilute DNA solution would be advantageous where the sediment sorption capacity is small; a higher concentra- tion solution would be needed where sediment sorption capacity is large.

Sediment sorption capacity reported in the literature ranges from approximately 200 µg/g to 16,000 µg/g, with the latter value being reported for sorption to pure clay. Estimates based on these values suggested that marker solution concentra- tions up to 200 ng/µl might be needed. However, an alternative approach that takes into account the length of the target marker was also evaluated, based on the following relationship from the URI Genomics & Sequencing Center (http://cels.uri.edu/gsc/cndna.html):

$$ngDNA * \frac{1ug}{1000ng} * \frac{1Da}{1.66*10^{-18}ug} * \frac{1BP}{650Da} * \frac{1Copy}{108\,BP} = number\ marker\ copies \qquad (1)$$

Preliminary sorption testing conducted to verify that interferences produced by the sediment matrix would not prevent DNA quantification was conducted using nominal solution marker concentrations of 35 ng/µl and 200 ng/µl, with 0.2g sediment samples. The results of this testing also provided some insights into the expected sorption capacity of the sediment.

Sorption of DNA obtained from a tissue extraction (genomic DNA) is not measurable because the qPCR marker used to quantify the DNA is specific only to a single locus within the mitochondrial DNA of bighead carp (for the purposes of this study, we limit the definition of a DNA marker to a selected DNA sequence specific to a target species; in this case, bighead carp. The present marker, USGS-SC qPCR marker, contains 108

base pairs per copy). Solutions and sediments were therefore spiked with purified stocks of PCR amplicon; the amplicon was the product of PCRs of genomic DNA from bighead carp, using select qPCR primers (sans fluorophore-labeled probes) developed for TaqMan® assays of this species.

Centrifuging Procedures

Extracellular DNA in solution exists as a suspension of particulates, rather than a true, dissolved moiety. Tests were conducted to determine whether or not DNA became stratified in supernatant samples following centrifuging, such that separation of the operationally defined "dissolved phase" of DNA could not be completely separated from the sediment solids for analysis. Test samples were centrifuged at different speeds and times to achieve a clear supernatant; the supernatants were then sampled from different levels in the fluid and analyzed for DNA to determine whether the solution/suspension was homogeneous.

Equilibrium Testing

The kinetics of DNA sorption was evaluated through carefully structured equilibrium testing (Table 2.8.1); this data was used to establish the contact time required for the subsequent batch sorption tests. Dry sediment (prepared according to the procedure in Appendix B) was weighed into sterile, tared and labeled mini-centrifuge tubes (Figure 2.8.2), and the weight recorded to three decimal places. Digital pipetters were used to add 500 µl of DNA marker solution to each sediment sample (Figure 2.8.2). To minimize differences in actual contact time, sediment was weighed into centrifuge tubes for all 5 replicates for a single contact time; DNA marker solution was then quickly added to each tube, the tubes were sealed, and placed inside large, sterile sample containers (Figure 2.8.2). These containers were fastened to an oscillating table and samples were agitated for the specified contact time. At the end of the contact time, samples were immediately transferred to the mini-centrifuge (Figure 2.8.2), and centrifuged at 3000 RPM for 12 minutes. Samples were then removed from the centrifuge and 400 µl of clear supernatant pipetted off each sample to sterile, labeled centrifuge tubes; only 400 µl of the supernatant was removed in order to avoid pulling sediment solids into the pipette. The sediment and 100ul residual solution was submitted as the "sediment" sample; the 400 µl supernatant was submitted as the "supernatant" sample for each sample pair (Figure 2.8.2).

Samples were frozen immediately to preserve them for analysis. Each replicate was analyzed in triplicate.

When the solution concentration stabilized (a plot of solution concentration vs. time became asymptotic), the system was assumed to have achieved equilibrium. Based on the literature results, sorption was expected to be relatively rapid (minutes to hours). Based on the preliminary degradation testing, long contact times could have unacceptable levels of DNA losses due to degradation, preventing the collection of meaningful data. Equilibrium testing was therefore conducted over a period of 24 hours.

The concentration range and solution/sediment ratio selected for the equilibrium testing was determined considering 1) the results of the proof of concept testing, 2) sediment sorption capacities reported in the literature and 3) environmentally relevant sediment DNA concentrations.

A conceptual plot of the expected data from the equilibrium testing is given in Figure 2.8.3.

Selected Equilibrium Testing Conditions

Based on the preliminary testing conducted, the equilibrium testing was conducted using 0.2 g sediment samples (dry weight), and 500 µl of DNA marker solution at a nominal concentration of 0.5 ng/µl (apparent interferences introduced by the gel (2% size select gel from Life Technologies) used in isolating the marker DNA for use in solution resulted in overestimates of the actual solution concentrations). Control samples were run on the solution itself, and a sample of the solution taken through the extraction process; the solution controls provided insights on the extraction efficiency (about 30%), and the resulting copy numbers were compared to the expected copy numbers based on the documented DNA mass in the samples (based on Nanodrop), to obtain a correction factor for the initial solution concentrations. Each batch of sediment and solution had to be treated separately (initial solution concentrations, though nominally equal for each batch, were subject to different correction values due to differences in solution age, pipetting error and diluting error). Five replicates were prepared for each of the 7 contact times specified in Table 2.8.1. Dry sediment samples were weighed, spiked with DNA, packaged, shaken, agitated, processed and preserved as described in the preceding section.

Table 2.8.1. Equilibrium testing matrix.

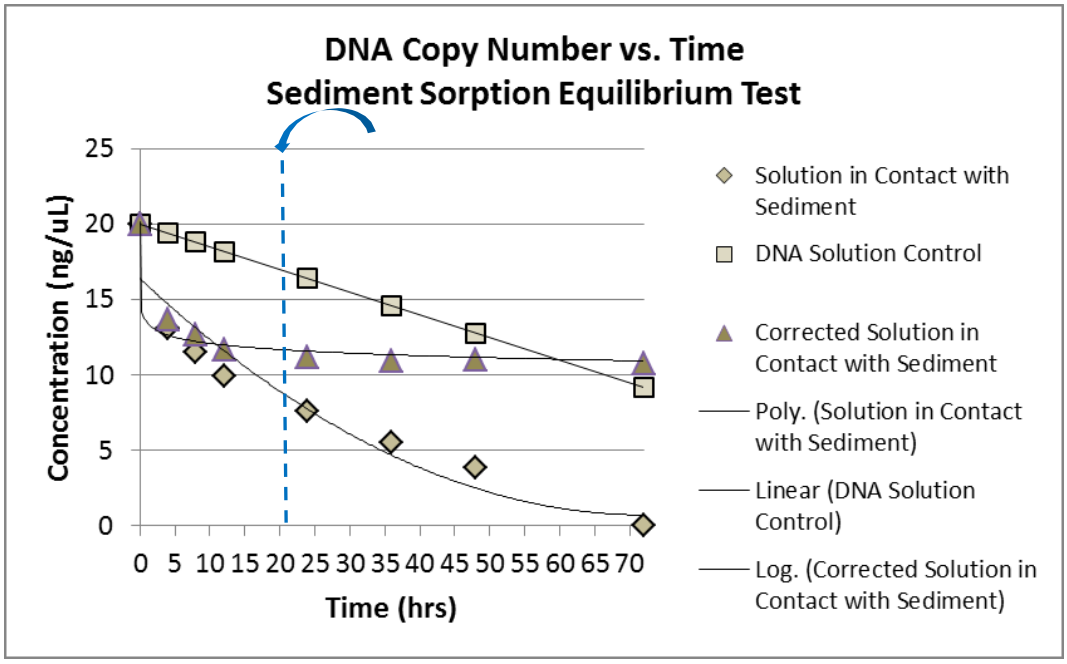
Sample ID	**Time (hrs)**
Initial Solution Concentration - (Ci)	0
Solution conc at time t = 4 (C4)	.5
C8	1
C12	2
C24	4
C36	8
C48	12
C72	24

Sorption Testing

Batch sorption tests were conducted for the purpose of establishing a partitioning coefficient for the DNA marker and the sediments, under a single set of conditions. Five dry sediment masses, ranging from 0.2g to 0.8g, and a single DNA concentration (0.5 ng/L as measured by Nanodrop) were used; initial solution concentration was verified using Qubit, resulting in a concentration of 0.025 ng/µl, but with comparable copy numbers. Five replicates were prepared for each sediment mass (Table 2.8.2), for each sediment. A 24 hour contact time was selected based on the results of the equilibrium testing; this was considered to be the minimum contact time required to establish equilibrium yet limit DNA losses attributable to degradation.

Figure 2.8.2. Mini-centrifuge tubes; adding DNA solution to sediment samples; sterile "overpack" containers fastened to shaking table; mini-centrifuge; and sample set ready for analysis.

Figure 2.8.3. Conceptual result from equilibrium testing.



Samples were prepared and processed in exactly the same manner as for the equilibrium study. 500 µl of DNA-DI solution was added to the specified sediment mass, and the samples shaken for 24 hours. Buffering was not used due to concerns regarding potential analytical interferences; pH was measured in representative samples before and after sorption in order to document pH changes that might affect sorption. Sediment and supernatant samples were then separated as before, and frozen immediately to preserve them for analysis; supernatant volumes varied from sample to sample, with smaller volumes recoverable for the larger sediment masses. Sorption is typically evaluated based on changes in solution concentration. Because of the potential for analytical inhibition resulting from sediment constituents, concentrations of DNA were measured in both supernatant and sediment, in order to facilitate mass balance calculations (Appendix B).

Table 2.8.2. Batch sorption testing matrix.

Testing matrix	Dry sediment mass	Replicates	Solution Concentration	Solution Volume	Contact Time
	(g)		(ng/µl)	(µl)	(hrs)
Lockport Pool	0.2	5	N/A	Baseline DNA Analysis	
	0.2	5	0.5	500	24
	0.3	5	0.5	500	24
	0.45	5	0.5	500	24
	0.68	5	0.5	500	24
	0.8	5	0.5	500	24
Lake Calumet	0.2	5	N/A	Baseline DNA Analysis	
	0.2	5	0.5	500	24
	0.3	5	0.5	500	24
	0.45	5	0.5	500	24
	0.68	5	0.5	500	24
	0.8	5	0.5	500	24

Quiescent and Re-Suspension Release Testing

Batch testing was also conducted to assess the potential for release of DNA to the water column from the sediment, either under quiescent conditions, or following re-suspension events. The testing matrix is given in Table 2.8.3. In order to determine whether release persists over time once DNA is "stored" in the sediment, testing was conducted over multiple time intervals. Because relatively long testing periods were involved (1 - 21 days) during which time DNA degradation could occur (even though the marker solution is relatively stable), both sediment and supernatant were analyzed at the end of the specified testing interval, to facilitate construction of a DNA mass.

Five replicates were prepared for each contact time (5), for each sediment (2), and for each testing condition (2 – quiescent and re-suspension). An additional 5 sediment samples were loaded with DNA (0.2g sediment plus 100 µl DNA-DI solution at a concentration of 0.5 ng/µl), and analyzed at 24 hours to verify initial DNA marker mass (without the subsequent addition of DDI water). Another 5 dry 0.2g sediment samples were analyzed for DNA to establish the background DNA concentration in each of the sediments.

The specified dry sediment was placed in sterile centrifuge tubes. To load the sediment with a known amount of DNA marker, 100 µl of DNA marker solution at a concentration of 0.5 ng/µl was pipetted onto the dry sediment. After 24 hours had elapsed, allowing the DNA in the solution to equilibrate with the sediment, 500 µl of DDI water was added to all but 5 of the DNA-loaded sediment samples, taking care not to disturb the surface of the sediment. This was to simulate the overlying water column; release of DNA to the supernatant (water column) was measured after the specified contact times. The remaining 5 loaded sediments were extracted without further solids/water separation to determine the measurable DNA marker concentration of the loaded sediment samples at the beginning of the experiment.

For the quiescent testing, the samples were maintained at room temperature in a dark cabinet to minimize UV-associated DNA degradation. At the end of each specified contact time, 5 samples were removed, and the sediment and water phases separated as for the equilibrium testing (centrifuge, pipette off 400 µl of supernatant, package in a separate sterile centrifuge tube, label supernatant and sediment/residual solution, respectively). Samples were frozen immediately to preserve them for later analysis of marker DNA.

For the re-suspension testing, the samples were maintained at room temperature in a dark cabinet, as for the quiescent testing. At the end of each specified contact time, five samples were removed, agitated gently to re-suspend the solids completely, and then allowed to settle in the dark for another 24 hours. Sediment and supernatant samples were then separated and processed as for the quiescent samples.

Table 2.8.3. Quiescent and re-suspension release testing matrix. *All sediment dry mass = 0.2 g, 5 replicates. ** Analyze entire sample as loaded sediment sample (no solid/water separation). *** After addition of DDI to samples.

Testing matrix*	Conditions	DNA Soln. Conc. (ng/µl)	DNA Soln. Vol. (µl)	DDI Vol. (µl)	Sampling Time*** (days)
Lockport Pool	Baseline DNA	N/A	0	0	1
Lake Calumet	Baseline DNA	N/A	0	0	1
Lockport Pool	Quiescent	0.5	100**	0	1
Lockport Pool	Quiescent	0.5	100	500	1
Lockport Pool	Quiescent	0.5	100	500	3
Lockport Pool	Quiescent	0.5	100	500	7
Lockport Pool	Quiescent	0.5	100	500	14
Lockport Pool	Quiescent	0.5	100	500	21
Lake Calumet	Quiescent	0.5	100**	0	1
Lake Calumet	Quiescent	0.5	100	500	1
Lake Calumet	Quiescent	0.5	100	500	3
Lake Calumet	Quiescent	0.5	100	500	7
Lake Calumet	Quiescent	0.5	100	500	14
Lake Calumet	Quiescent	0.5	100	500	21
Lockport Pool	Re-suspension	0.5	100**	0	1
Lockport Pool	Re-suspension	0.5	100	500	1
Lockport Pool	Re-suspension	0.5	100	500	3
Lockport Pool	Re-suspension	0.5	100	500	7
Lockport Pool	Re-suspension	0.5	100	500	14
Lockport Pool	Re-suspension	0.5	100	500	21
Lake Calumet	Re-suspension	0.5	100**	0	1
Lake Calumet	Re-suspension	0.5	100	500	1
Lake Calumet	Re-suspension	0.5	100	500	3
Lake Calumet	Re-suspension	0.5	100	500	7
Lake Calumet	Re-suspension	0.5	100	500	14
Lake Calumet	Re-suspension	0.5	100	500	21

Results

Sediment Characterization

Visual Characterization

Samples of both sediments used in the study are pictured in Figures 2.8.4. Visual examination of the Lockport Pool sediment revealed the following characteristics:

- ☐ Dark color due to abundant coal fragments
- ☐ Slag and other anthropogenic debris
- ☐ Petroleum odor
- ☐ Predominantly granular/silt

Visual examination of the Lake Calumet sediment revealed the following characteristics:

- ☐ Lighter color than Lockport Pool
- ☐ Asiatic clams and Zebra mussels
- ☐ No petroleum odor
- ☐ Predominantly granular/silt

Mineralogy

The results of x-ray diffraction studies conducted on both sediments are pictured in Figure 2.8.5. Quartz was the predominant component in both sediments, followed by feldspars (K and Na), dolomite, calcite and phyllosilicates in the Lockport Pool sediment. In Lake Calumet, after quartz, dolomite was the next most abundant constituent, followed by the phyllosilicates, feldspars, and calcite. Muscovite and chlorite were phyllosilicates identified in both sediments; no expansive clay minerals were identified.

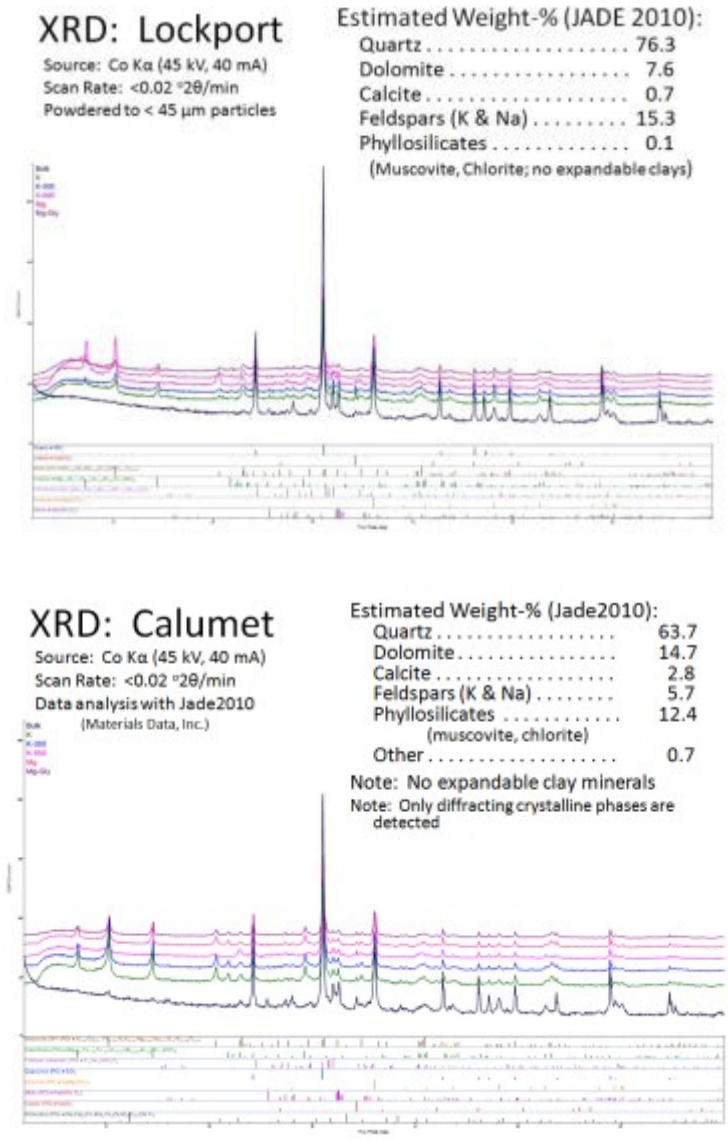
Thermo-gravimetric analysis (TGA) produced the following findings for both sediments:

- ☐ Gradual mass loss over a temperature range of 20 to 1450 degrees C
- ☐ Marked mass loss between 720 and 850 degrees C

Results of TGA and differential scanning calorimetry (DSC) are given in Figure 2.8.6. The following observations can be made from the graphs:

Figure 2.8.4. Lockport Pool sediment and Lake Calumet sediment, respectively.

Figure 2.8.5. Lockport Pool sediment and Lake Calumet XRD analyses.



Lake Calumet

☐ TGA ~9% weight loss 750-850 degrees C
☐ Main DSC peak (endothermic dip) at ~830 degrees C, due to dehydroxylation of multiple phases (e.g. clays, dolomite)

Lockport Pool

☐ TGA~7% weight loss 710-820 degrees C
☐ DSC peak (dehydroxylation) at ~810 degrees C

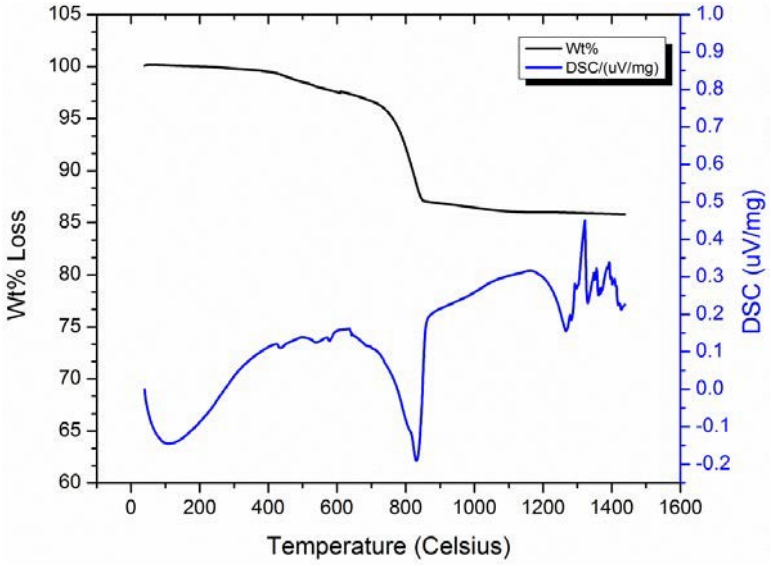
Carbonates/Iron Oxides

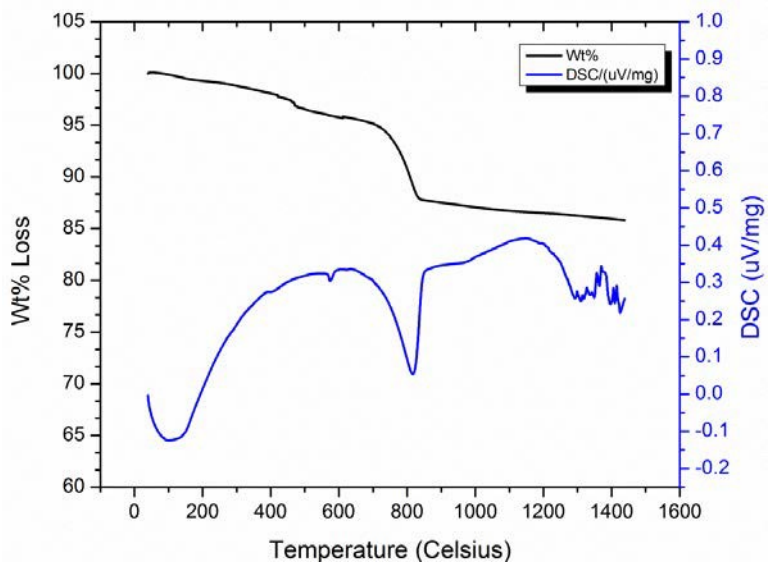
Table 2.8.4 summarizes the results of the X-ray fluorescence (XRF) analysis conducted on both sediments. The results represent calculated XRF based on best-fit XRD data. The predominant constituent in both sediments is SiO_2. The Lake Calumet sediment contains approximately two times the carbonate present in the Lockport Pool sediment.

Table 2.8.4. XRF sediment analysis.

	Weight-Percent Oxides	
Oxide	**Calumet**	**Lockport**
Fe_2O_3	0.3	0
BaO	0	0.1
TiO_2	0.1	0
CaO	6	2.7
K2O	1.3	2.1
SiO_2	74.1	86.3
Al_2O_3	5.7	2.9
MgO	3.5	1.7
Na_2O	0.7	0.3
CO_2	8.3	3.9
Totals	100	100

Figure 2.8.6. Lake Calumet and Lockport Pool TGA-DSC results, respectively.





Surface Area

Surface area estimates were obtained for both sediments by BET analysis, using a Quantachrome NOVA 3200e surface area and pore size analyzer. Surface area of the Lake Calumet sediment was estimated to be approximately 7.49 +/- 0.79 m^2/g; surface area of the Lockport Pool sediment was estimated to be approxi- mately 3.08 +/- 0.39 m^2/g.

Sequential Extraction

Cation exchange capacity and major and minor sediment fractions were determined using the sequential extraction procedure (modified Tessier sequence), described as follows:

- ☐ Exchangeable: readily desorbed with 1M $MgCl_2$
- ☐ Carbonate: dissolved in 1M sodium acetate
- ☐ Fe/Mn Oxyhydroxides: reductive dissolution with dithionite
- ☐ Organics: wet, heated oxidation with strong H_2O_2-HNO_3-NH_4OAc
- ☐ Residual: digestion in concentrated HF-$HCLO_4$ acids

The procedure was modified to determine mass loss with each step. Extractions were performed in triplicate with analysis by ICP-MS (Inductively Coupled Plasma Mass Spectrometry). Results of the sequential extraction for Lake Calumet are given in Figure 2.8.7. Results for Lockport Pool are given in Figure 2.8.8.

Quantitative measures obtained with sequential extraction:

- ☐ Cation Exchange
  - o Based on displacement by Mg^{2+}
  - o Calumet: 3.54 ± 0.08 meq / 100 g sediment
  - o Lockport: 6.66 ± 0.12 meq / 100 g sediment
- ☐ Carbonate Content (dolomite, calcite/aragonite):
  - o Calumet: 6.91 ± 0.33 wt-% (~2x Lockport)
  - o Lockport: 3.46 ± 0.51 wt-%
- ☐ Fe/Mn-Oxyhydroxides
  - o Calumet: 2.83 ± 0.41 wt-%
  - o Lockport: 3.75 ± 0.46 wt-%
- ☐ Organic
  - o Calumet: 12.06 ± 0.71 wt-% (peroxide extraction), 4.76 ± 0.16 wt-% (combustion)
  - o Lockport: (no viable peroxide extraction data), 7.91±0.5 wt-% (combus- tion), evidence of significant coal content

Figure 2.8.7. Sequential extraction results – Lake Calumet.

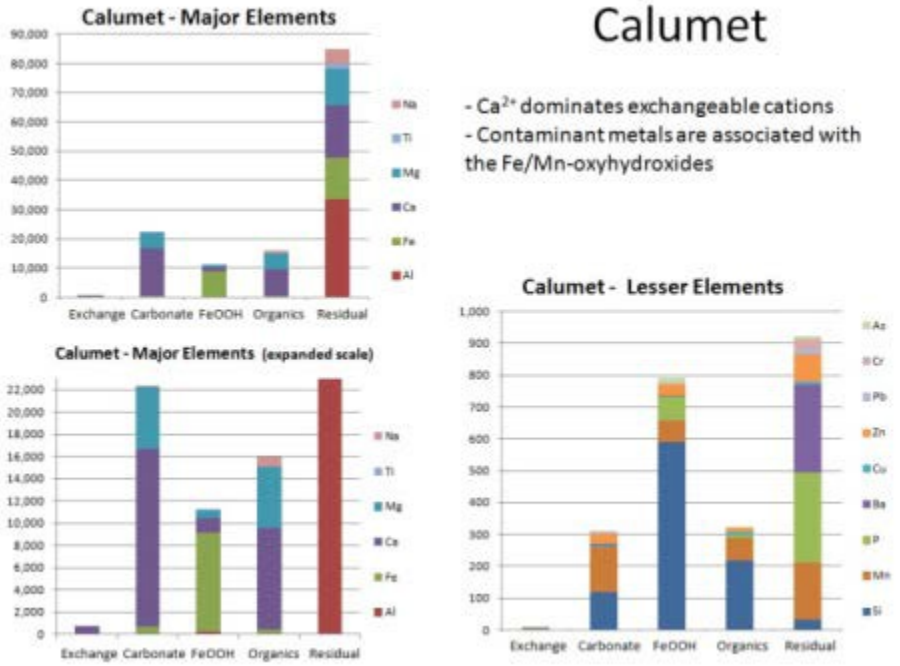
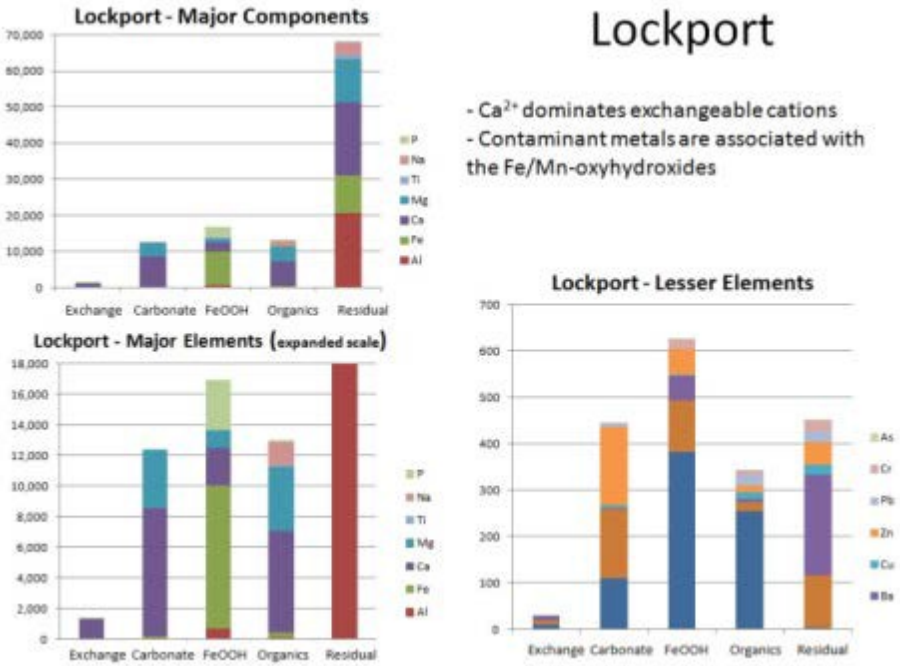


Figure 2.8.8. Sequential extraction results – Lockport Pool.



Pore Water Chemistry

Results of the pore water elemental and metals analysis are given in Table 2.8.5.

Table 2.8.5. Pore water analysis.

Element	**Calumet**	**Lockport**
Calcium	41.8	75.6
Magnesium	46.5	25.1
Sodium	53.8	89.9
Potassium	11	8.42
Phosphorus	0.0678	2.07
Silicon	5.41	12.4
Sulfur	0.584	31.5
Arsenic	0.0172	0.0174
Barium	0.04	<0.0100
Boron	0.175	0.215
Copper	<0.0100	0.0122
Iron	0.0682	<0.0100
Selenium	0.0412	0.0752
Strontium	0.405	0.288
Zinc	<0.0100	0.0143
Aluminum	<0.0100	<0.0100
Cadmium	<0.0100	<0.0100
Chromium	<0.0100	<0.0100
Cobalt	<0.0100	<0.0100
Lead	<0.0100	<0.0100
Lithium	<0.0100	<0.0100
Manganese	<0.0100	<0.0100
Nickel	<0.0100	<0.0100
Tin	<0.0100	<0.0100
Titanium	<0.0100	<0.0100

Grain Size Distribution

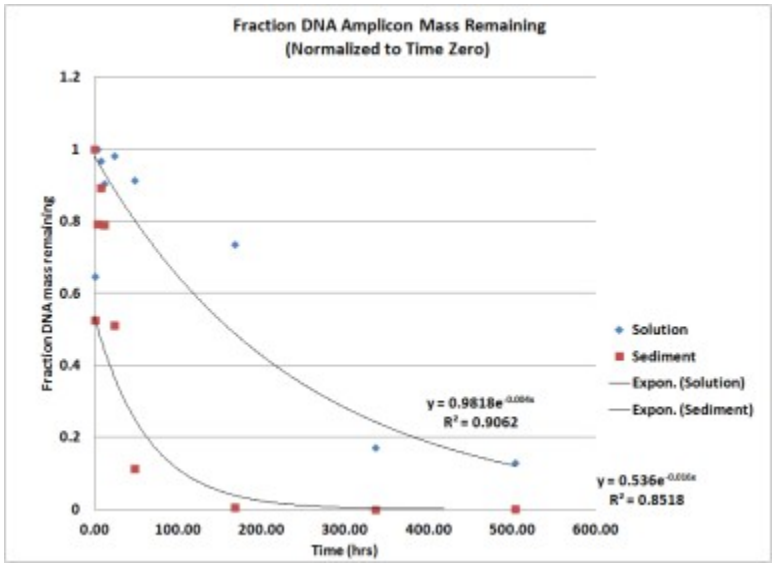
Cumulative and differential particle size distributions were plotted from the Coulter Counter data for both sediments. Plots and grain size distributions are provided in Appendix B. Results obtained with the Coulter Counter are reported in "percent by volume" and will vary somewhat from "percent by mass", depending upon the specific gravity of the particles. The grain size distribution by volume is nevertheless useful in determining the general character of a sediment (fine vs. coarse). Approximately 75% of the Lake Calumet sediment would be considered fined grained (particles < 75 um in diameter), with approximately 20% of the sediment being less than 5 um in diameter. Of the coarse grained material, the largest particle size measured in the Lake Calumet sediment was approximately 282 um. Approximately 50% of the Lockport Pool sediment was <75 um in diameter, with approximately 17% being less than 5 um in diameter. The largest particle size measured in the Lockport Pool sediment was approxi- mately 864 um in diameter.

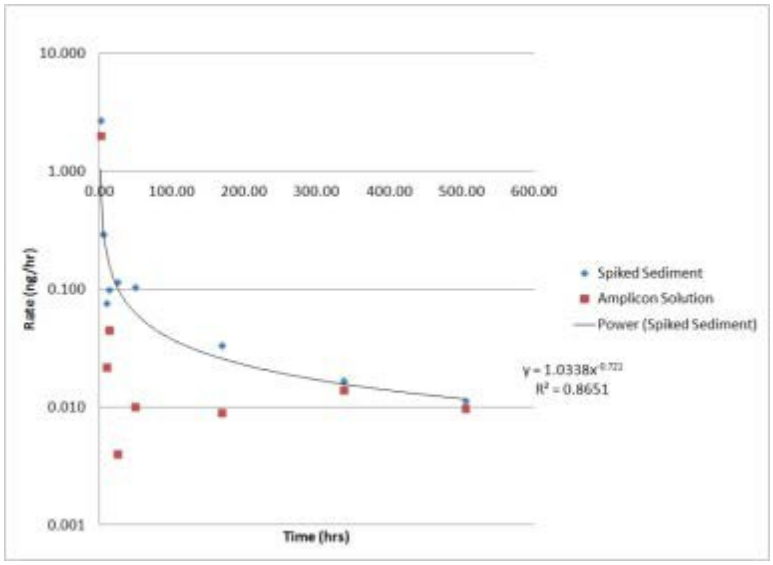
Proof of Concept Testing

Baseline Degradation

Results of the degradation study for the 0.5 ng/µl DNA marker solution and sediment spiked with 100 µl of the same solution, are illustrated in Figure 2.8.9. For the sediment samples, the amount of DNA added to the sediment recoverable after 24 hours (Time 0 for the purposes of the degradation study) ranged from 21% to 45%, with a mean value of approximately 32%. For the DI samples, the amount of added DNA recoverable at Time 0 ranged from 1% to 28%, with a mean value of approximately 8%. DNA concentrations were normalized to the Time 0 concentration, to distinguish subsequent losses potentially attributable to degradation from extraction/analytical inefficiencies. The rate of DNA mass loss from the sediment was relatively well described by a power function; the rate of mass loss from the solution showed no significant correspondence to time, although a generally declining trend was observed when comparing the magnitude of loss during the first 48 hours with that of the remainder of the testing.

Figure 2.8.9. Fraction and rate of DNA marker losses over time from sediment and solution.





Sediment/DNA Solution Concentration and Ratios

A sediment/DNA solution ratio of 100 µl was found to fairly uniformly wet the entire sediment sample for a dry sediment mass of 0.1 g. Based on Equation 1, a solution concentration of 0.5 ng/µl was calculated to provide sufficient DNA to

yield measurable residual concentrations in sorption testing conducted with 0.2g dry sediment.

Centrifuging Procedures

Centrifuge speeds of 3000 RPM and periods of 12 minutes were required to produce a clear supernatant, free of sediment solids. The DNA suspension in the resulting supernatant produced using this procedure was determined to be essentially homogenous (no gradient was detectable). Centrifuging according to this protocol was therefore determined to be acceptable.

Moisture Content Measurement/Sediment Sample Size

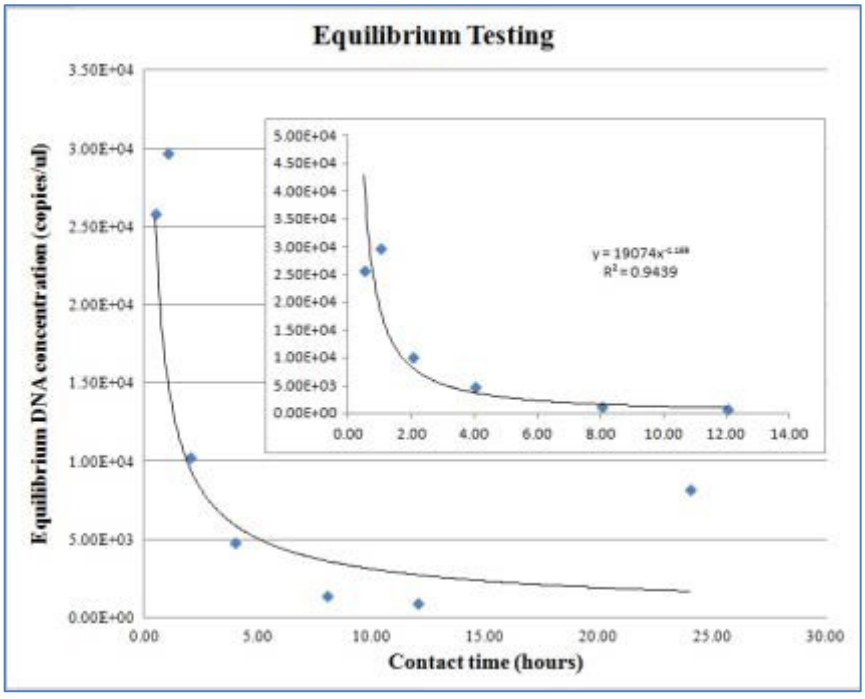
Moisture contents obtained for samples dried at 60 deg C were compared to aliquots of the same samples that were subsequently dried at 100 deg C, to assess the potential error in moisture content determination. The additional weight loss occurring at the higher temperature was small, suggesting the error in moisture content determined by drying at 60 deg C ranged from only 0.3% to 2.6%. A drying temperature of 60 deg C was therefore selected for all sediment sample preparation.

Results of the sediment moisture content determination on six different wet sample masses were evaluated statistically. Standard deviation of the individual moisture content values for the Lockport Pool sediments ranged from 2.37 to 6.31%. The smallest variability in moisture content for the Lakeport Pool sediments was obtained for the 0.2 g samples (COV =0.041). The smallest variability in moisture content for the Lake Calumet sediments was obtained for the 0.5g samples (COV=0.04), followed by the 0.4g samples (COV=0.07). The 0.2g Lake Calumet samples had the highest COV (0.11), the highest value obtained for all the sediment sample masses measured. Overall, the variability in the moisture content measurements was high enough to suggest that less error would be introduced by conducting the sorption sampling with samples small enough to be extracted in their entirety, rather than larger samples for which subsamples would need to be extracted and dry sediment mass extrapolated based on water content. The selected sediment mass for the sorption testing was therefore 0.2g dry weight. A consistent sediment/extractant ratio was also maintained in order to avoid concentration of sediment constituents that might be inhibitory to the DNA analysis.

Equilibrium Testing

Results obtained from the equilibrium testing are illustrated in Figure 2.8.10. Although there was a single spike in equilibrium concentration at 24 hours, it appears that the system was nearing equilibrium after 12 hours. The result obtained using the regression equation obtained excluding the 24 hour data point gives a predicted concentration that is consistent with several of the individual replicate analyses obtained for that time period. The 24 hour data point was excluded from the regression to enable a clearer understanding of the sorption behavior up to this point, where the solution concentration was clearly and consistently decreasing. The significance of the 24 hour spike remains to be resolved; the spike may have been a data outlier or it may be evidence of subsequent desorption of DNA from the sediment. Given the general trend of the data and the sorption kinetics reported in the literature, the 24 hour spike was considered likely to be an anomaly; the system was assumed to be at equilibrium after 24 hours, and this was selected as the contact time for subsequent batch testing.

Figure 2.8.10. Equilibrium test results.



Batch Sorption Testing

Partitioning data obtained from the batch sorption data showed that virtually all the DNA present in solution was taken up by the sediment for all sediment masses tested. From an initial DNA marker concentration in solution of 0.5 mg/l, equilibrium concentrations in the supernatant were reduced by many orders of magnitude, ranging from 2.1E-09 mg/l to 1.03E-07 mg/l for the Lake Calumet sediment, and from 2.74E-09 mg/l to 1.58E-06 mg/l for the Lockport Pool sediments. The data was plotted using both Freundlich and Langmuir isotherms [1]. Neither the Freundlich nor the Langmuir well described the Lake Calumet data, which displayed no definite trends, indicating that the amount sorbed was not correlated with the equilibrium concentration. Both the Freundlich and Langmuir appeared to well describe the Lockport Pool sorption data, but the model is suspect, displaying the inverse of the expected trends. This is likely because there was very little difference in the amount of DNA sorbed from sample to sample, such that the numerator of the capacity parameter (X/M), was essentially constant, producing the result in Figure 2.8.11 when plotted against the corresponding sediment masses. Initially, it appeared that the sorption capacity of the sediment exceeded preliminary estimates and there was insufficient DNA in solution to saturate the sorption sites on the sediment. However, there was also an issue with the accuracy of the instruments measuring the DNA concentration in the marker solution. This was discovered and reported by the genomics lab after the equilibrium and partitioning tests had been completed. Based on comparison of analytical results obtained using two different instruments (Nanodrop and Quibit), the concentration by mass reported by these instruments was inaccurate. The copy numbers obtained using qPCR corresponded well with a 0.025 mg/l solution; average concentration calculated from reported copy number was approximately 0.05 mg/l for solutions used in the equilibrium, partitioning and release testing. In either case, it appears that a much lower than desired solution concentration likely contributed to the failure to saturate the sorption sites on the sediment. This serves to illustrate the technical difficulty in accurately quantifying DNA in solution.

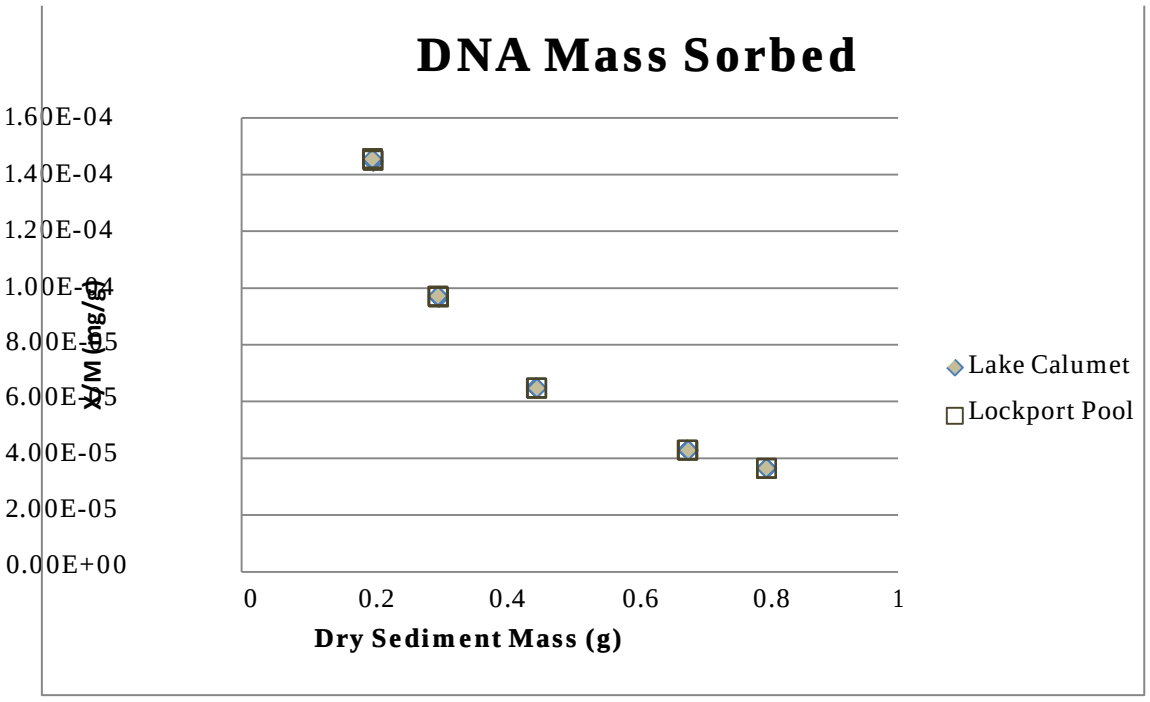
Because the DNA concentration of the solution was insufficient to saturate the available sorption sites on the sediment, no partitioning coefficient could be

[1] Freundlich and Langmuir sorption isotherms are commonly used to evaluate the relationship between sorbent and sorbate, or solute. Although the mathematical form of the isotherms differs, the procedure essentially involves plotting the amount of solute sorbed against the amount of solute still in solution at a given time. , When the isotherms produced conform to the expected trend for the specific model used, they are considered a good “fit” or “to well describe the data”, and may inform the type of sorp- tion occurring (monolayer or multilayer). Distribution coefficients can also be extrapolated from the plots.

established from these data. Based on the available data, however, the magnitude of DNA mass sorbed was roughly 1.0E-05 to 1.5E-04 mg/g (8.58E10 to 1.29E12 copies/g). Higher sorption would be expected if the test were repeated with higher initial solution concentrations. By comparison, literature values for sediment sorption capacity ranged from approximately 2.0E-01 mg/g (1.72E15 copies/g) to 16 mg/g (1.37E17 copies/g), for a soil containing montmorillonite (Ogram et al 1988) and 41 mg/g (3.51E17 copies/g) for kaolinite (Cai et al 2006).

The pH of the materials used in the sorption testing was measured in order to evaluate the potential influence pH changes may have had on the amount of DNA sorbed. Results obtained were reported in Table 2.8.6. With the exception of the site water, which was not used in the testing, pH values of all materials both before and after sorption testing were between 6.0 and 7.8. The pH of the dried sediment used in the sorption testing was 6.47 and 6.81 for Lake Calumet and Lockport Pool, respectively. Following sorption, the pH of the water extracted from the sediment was approximately 6.8, which was also the pH of the DNA solution prior to the sorption testing.

Figure 2.8.11. DNA concentration on sediment vs. dry sediment mass.



Release Testing

The concentration of DNA marker was measured in spiked sediment and overlying supernatant at 5 different time intervals, from 1 to 21 days, for two test conditions (quiescent and resuspended). The number of DNA marker copies measurable in sediment and supernatant were adjusted to correct for extrac- tion/analytical inefficiency based on DNA recovery from spiked sediment and marker solution at Day 1. The adjusted results were then plotted as a function of time, expressed as percent of DNA added to the sediment samples, for both sediments and both testing conditions (Figures 2.8.12 and 2.8.13). The residual DNA mass in the sediment was in several cases greater than 100% of the added DNA; this is an artifact of the variability in the percentage of DNA recoverable by the extraction procedure, magnified by the extraction correction procedure.

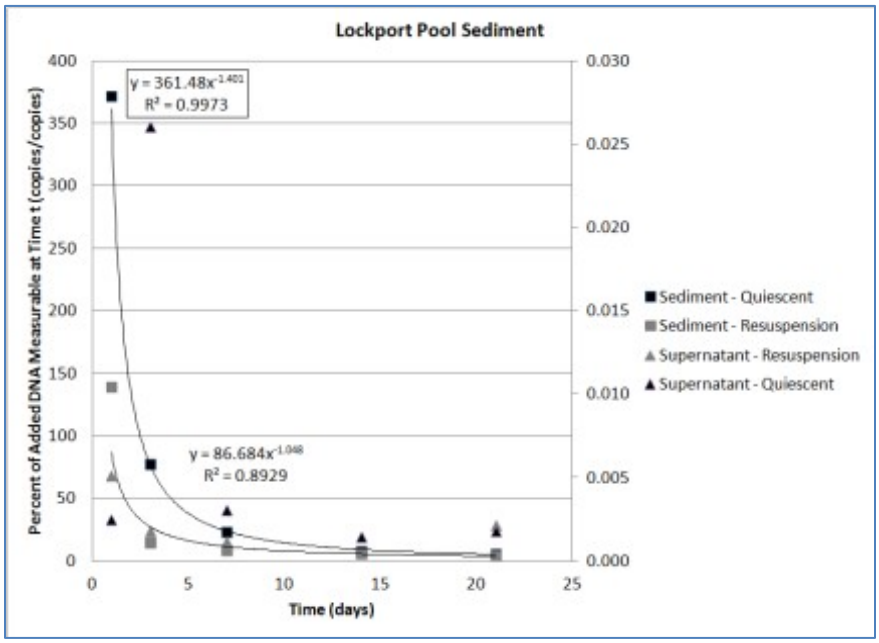
The DNA content of the sediment was shown to be strongly a function of time for quiescent testing conditions and was well described by a power function for both Lockport Pool (R^2=0.9973) and Calumet Lake (R^2=.8048). DNA content of sediment samples subjected to resuspension conditions was also well correlated, although R^2 values were slightly lower for resuspended sediment than for quiescent sediment, for both sites (Lockport Pool R^2 =.8929, Calumet Lake R^2 =.7261). The percentage of added DNA detectable in the supernatant was never greater than 0.025% for either site, and was in most cases significantly less than this. The rate of DNA loss from sediment was well correlated to time for both sediments and both testing conditions (Figure 2.8.14), but not for supernatants. Supernatant concentration for resuspended sediments showed little correlation to time for Lockport Pool, and no correlation for Lake Calumet.

Table 2.8.6. pH, conductivity, and salinity of testing materials.

Site	Matrix	pH	Temp. (C)	Cond. (mS/cm)	Salinity (ppt)
Lake Cal-umet	Supernatant/pore water post sorption	7.82	20.43	0.632	0
	Site water	8.04	21.97	0.538	0
	Wet sediment	7.00	19.87	0.464	0
	Dried sediment	6.47	19.89	0.291	0
	Sediment post sorption	6.80*	----	----	----
Lockport Pool	Supernatant/pore water post sorption	7.09	21.22	1.288	0
	Site water	7.44	21.16	0.957	0
	Wet sediment	6.86	20.02	0.314	0
	Dried sediment	6.81	20.06	0.356	0
	Sediment post sorption	7.00*	----	----	----
	DNA solution @ 0.5 ng/ul	6.80*	----	----	0

*pH measured with pH paper due to limited sample volume.

Figure 2.8.12. Lockport Pool and Lake Calumet sediment release testing – percentage of DNA detectable in sediment.



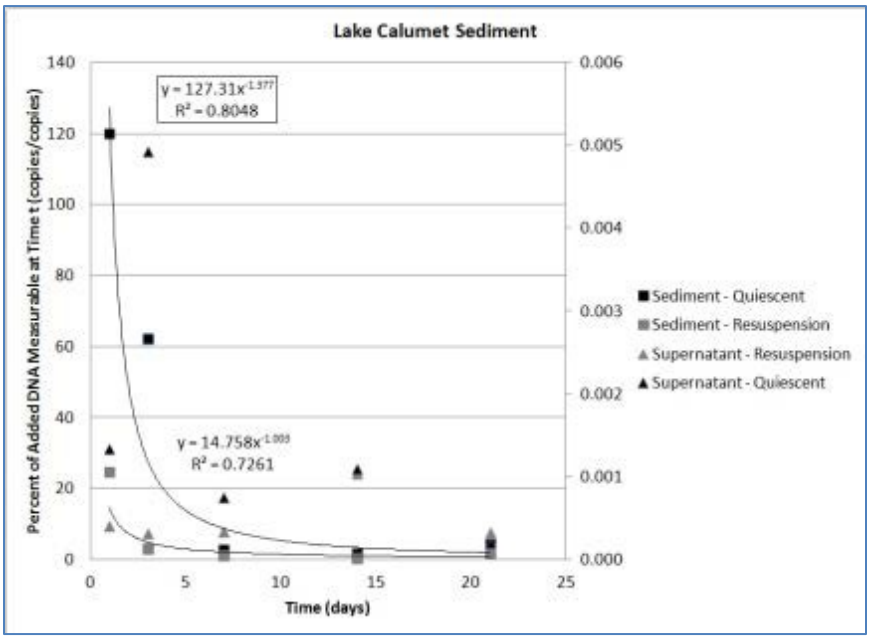
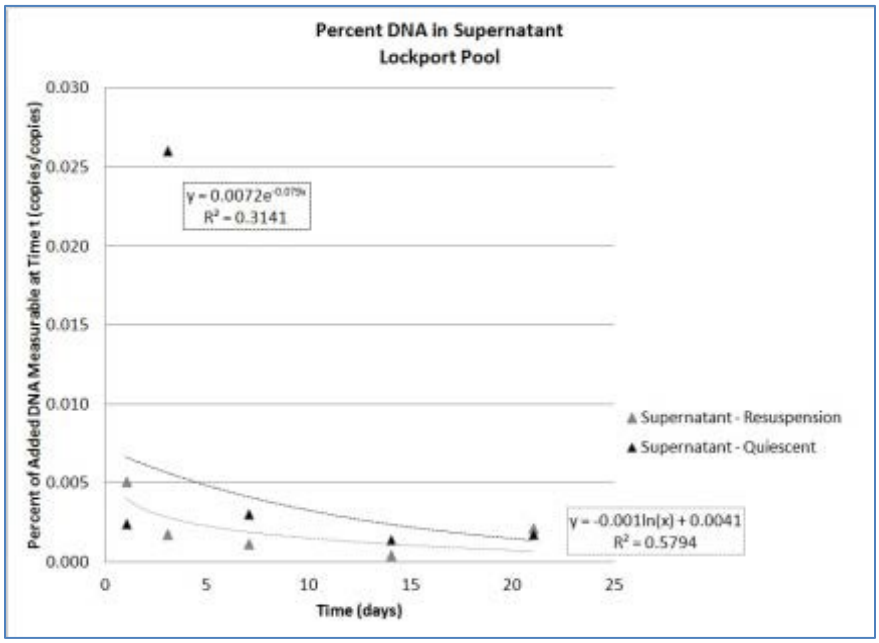


Figure 2.8.13. Lockport Pool and Lake Calumet release testing – percentage of DNA detectable in supernatant.



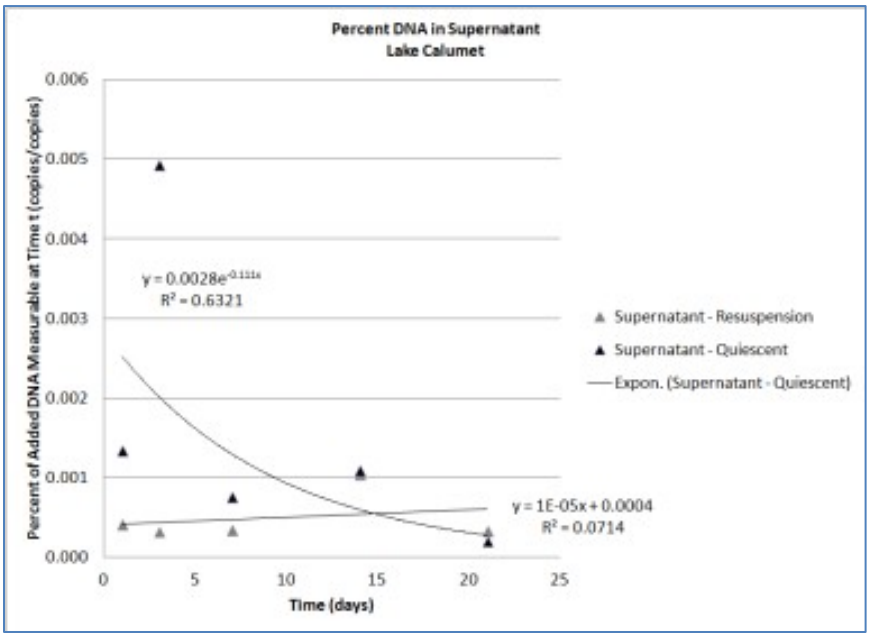
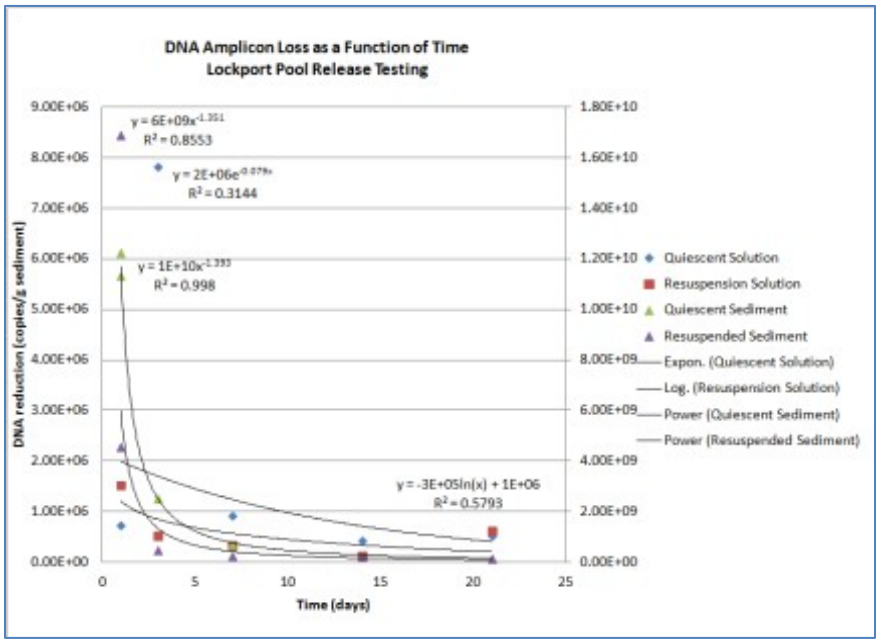
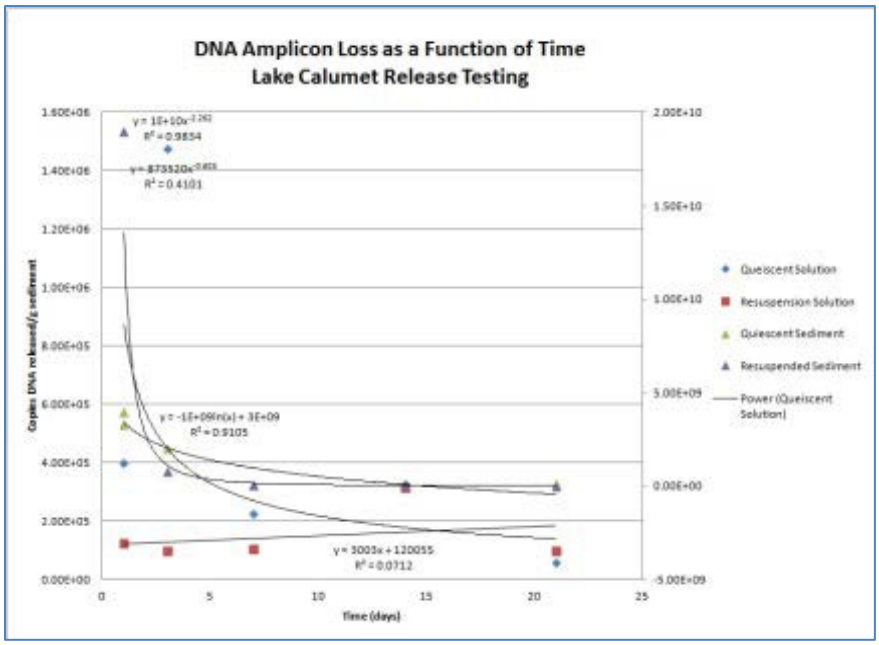


Figure 2.8.14. Rate of amplicon loss in sediment and supernatant – Lockport Pool and Lake Calumet release testing.





Discussion

Environmental parameters reported to be influential in the sorption of DNA included:

- Mineralogy and grain size
- Cation Exchange Capacity
- Surface area
- pH (DNA and mineral surfaces have a negative charge above pH 5)
- Ionic strength (conductivity, salinity) – cations may bridge negatively charged DNA fragments and mineral surfaces (at pH > 5)
- Organic content and composition – carbon type, humic acids, etc.
- Presence of organic contaminants such as polycyclic aromatic hydrocarbons (PAHs) and other petroleum hydrocarbons
- DNA fragment length (small fragments, <500 base pairs, exhibit strong binding)

The sediments characterized for this testing effort were largely coarse grained, will little clay, evidencing relatively low surface area and cation exchange capacity values. This suggests that these sediments would have relatively low sorptive capacity as compared to finer and higher clay content sediments. Organic content ranged from approximately 5 to 8 percent by weight (as measured by combustion) for these sediments, which is relatively low. The sediment from Lockport Pool evidenced a rather distinct petroleum odor, suggesting the present of PAHs which could also enhance the DNA sorption observed in these sedi- ments. Chemical analysis of the sediment was not conducted as part of this study however, and no inferences can be made regarding the influence of PAHs on DNA sorption behavior.

The relative conductivity of the pore water and site water was similar at both sites. Lake Calumet site and pore water conductivity was measured at 0.538 and 0.632 mS/cm respectively. Lockport Pool site and pore water conductivity was 0.957 and 1.29 mS/cm respectively. Conversion of conductivity to ionic strength is somewhat site specific, however, the salinity was too low to be measured in site water or pore water from either site. Low salinity is consistent with a low ionic strength solution with few cations available to aid sorption between DNA and the sediments.

Solution pH above the point of zero charge of the minerals present is most favorable for sorption of DNA. For the minerals identified in these sediments (quartz, dolomite, calcite, K and Na feldspars, muscovite and chlorite), the point of zero charge ranges from approximately 2 (quartz and some feldspars) up to approximately 7 (dolomite) (Zhao et al 2008; Alvarez-Silva et al 2010; Prasanphan and Nuntiya 2005; Somasundarin 2006). Generally, increasing (negative) surface charge is seen on these mineral surfaces with increasing pH, although some surface charges appear to be independent of pH (e.g. clay basal planes as reported by Zhao et al (2008). Correspondingly, charge dependent sorption could be expected to increase with increasing pH; Romanowski et al. 1991 reported an 8 fold increase in DNA sorption to sand with pH increase from 5 to 9. Large pH changes were not seen in the sorption testing conducted as a part of this study. The pH of the DNA amplicon solution prior to testing was 6.8. Supernatant/pore water pH post-sorption was 7.09 to 7.82, for Lockport Pool and Lake Calumet, respectively. The pH of the system was therefore within a range that should have been favorable for sorption of the DNA by these sediments.

The length of the DNA marker used in the sorption testing was relatively small (108 base pairs) also suggesting that relatively strong sorption could be expected under favorable conditions.

The amount of DNA in the solution used for the sorption testing was insufficient to permit determination of maximum sorption capacity of these sediments. Equilibrium DNA concentrations in solution were very low for all of the batch tests. Losses potentially attributable to degradation over a 24 hour period were estimated, based on the degradation controls, to be roughly 60% of the initial DNA mass added to the sediment. Similarly, losses measured in the Amplicon solution control represented approximately 12% of the initial DNA mass present over a 24 hour period. Losses in excess of these percentages were assumed to be attributable to sorption. Observed sorption for the test sediments was at least 3 to 5 orders of magnitude lower than the capacity values reported in the literature for DNA sorption on sediments. Differences between the sediments and testing procedures used in this study to those used by Ogram et al (1988) and Cai et al (2006) were potentially responsible for the differences in sorption capacity obtained in the sorption testing. However, the results obtained by these researchers suggest that higher sorption could be expected if the study sediments were appropriately challenged with a higher concentration of DNA in solution.

More important to the questions of most relevance to sediment as a source of DNA in water column samples are the results of the release testing. Desorption, as reflected by the percent of DNA added to the sediment measurable in the supernatant, was observed for both quiescent and sediment samples. The percentage of DNA measureable in the sediments as a function of time was higher for the quiescent samples than for the resuspended samples, but the trends over time were quite similar, and the DNA content of the sediments was comparable at the end of the testing period. A higher percentage of added DNA was measurable in the supernatant of the quiescent samples initially; as much as one to two orders of magnitude higher than for the resuspended sediment samples (with the exception of the very first data point – time zero – for Lockport Pool). The magnitude of DNA measured in the supernatant samples was quite low (maximum of 0.025 percent for Lockport Pool and 0.005 percent for Lake Calumet) for all sampling times. The fraction of DNA measurable in the supernatant diminished over time for both quiescent and resuspended samples, but the trend for the resuspended sediment samples was much more flat (Figures 22 and 23). The persistent, low level signature in both sediment and supernatant suggests the potential for a persistent, low level signature in the water column due to dispersion or resuspension, although no inferences can be made beyond the 21 day testing period.

Conclusions

The results of the sorption and release testing appear to support the conclusion that DNA sorption does occur in these sediments, although the maximum capacity of the sediments could not be estimated from the available data. In addition, the data suggests that measurable amounts of DNA could be measured in overlying water for up to 21 days following loading of the sediment with DNA marker, with or without a resuspension event. The magnitude of the DNA signature in the overlying water diminished to a very low value over a period of three to seven days, however, the presence of measurable DNA in the sediments throughout the testing period suggests that a long term low level signature may persist under similar conditions beyond the 21 day period tested. The rate of DNA loss from the sediment appears to be very rapid for the first 3 to 5 days, slowing to a near asymptotic level thereafter.

3 Development of Additional Asian Carp eDNA Markers

Efforts pertaining to progress on marker development have been documented in the 2013 ECALS milestone report. Although there may be some repetition with respect to ECALS background information here, the entire marker development milestone report is presented in its entirety in the following pages, with minimal editing, for completeness.

Invasive aquatic nuisance species pose a major threat to aquatic ecosystems worldwide. Invasive Asian carp species, 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), and possibly Lake Michigan and the Great Lakes ecosystem, electrical barriers have 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 river mile (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 self-sustaining Asian carp population become established in the Great Lakes, populations of native fishes and many threatened or endangered plant and animal species could be negatively affected. 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 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 because it can detect the presence of Asian carp DNA in water when fish populations are at very low abundance (though other vectors, such as piscivorous birds may deposit Asian carp DNA into a system). The current genetic markers used for these assays were developed for silver and bighead carp by researchers at the University of Notre Dame (*e.g.* Jerde *et al.* 2011). These methods are based on conventional polymerase chain reaction (cPCR) analysis in which the presence or absence of eDNA is determined by PCR amplification (repeated copying) of a target DNA fragment called a

"genetic marker". The PCR-amplified product is then isolated by gel electrophoresis and the DNA is sequenced to confirm the species of origin. The Quality Assurance Project Plan (QAPP) for the Environmental DNA (eDNA) Monitoring of Invasive Asian Carp in the CAWS outlines the detailed procedures for the current planning, collection, filtering and processing of eDNA samples. However, the current eDNA monitoring protocol uses only a single genetic marker to detect the presence of DNA for each carp species, which provides very little information about the nature of the carp DNA detected in a sample. Use of alternative genetic markers that provide more information about the carp DNA in an eDNA sample, such as concentration of carp DNA, would increase the utility of the information gained from the Asian carp eDNA monitoring program. The co-application of multiple markers could also increase likelihoods of detection for Asian carp eDNA at low concentrations.

What follows here is a pre-print of an article submitted to the journal Molecular Ecology Resources, which succinctly describes the ECALS efforts to develop additional Asian carp eDNA markers. The article has been modified slightly to reflect the format of this report.

3.1 Development and Testing of New Genetic Markers for the Detection of Invasive Bighead and Silver Carp DNA in environmental water samples from North America

Summary

Invasive Asian bighead and silver carp (*Hypophthalmichthys nobilis* and *H. molitrix*) pose a substantial threat to North American waterways. Recently, environmental DNA (eDNA), the use of species-specific genetic assays to detect the DNA of a particular species in a water sample, has gained recognition as a tool for tracking the invasion front of these species toward the Great Lakes. The goal of this study was to develop new species-specific conventional PCR (cPCR) and quantitative (qPCR) markers for detection of these species in North American waterways. We first generated complete mitochondrial genome sequences from 33 bighead and 29 silver carp individuals collected throughout their introduced range. These sequences were aligned with other common and closely related species to identify potential eDNA markers. We then field tested these genetic markers for species-specificity and sensitivity in environmental samples. Newly developed markers performed well in field trials, had low false positive rates and had comparable sensitivity compared to current markers. The new markers developed in this study greatly expand the number of species- specific genetic markers available to track the invasion front of bighead and silver carp, and can be used to improve the resolution of these assays. Additionally, the use of the qPCR markers developed in this study may reduce sample processing time and cost of eDNA monitoring for these species.

Introduction

Invasive aquatic nuisance species pose a major threat to aquatic ecosystems worldwide. In North America, invasive Asian carps, particularly bighead carp (BHC; *Hypophthalmichthys nobilis*) and silver carp (SC; *H. molitrix*), have been very problematic in freshwater ecosystems. Asian carps were imported into the U.S. in the 1970s to control algae in Arkansas fish farms (Freeze and Henderson 1982). Flooding allowed them to escape and establish reproducing populations in the wild by the early 1980s. They have since been steadily dispersing upstream throughout the Mississippi River watershed (Freeze and Hendersen 1982; Tucker et al. 1996). At present, BHC and SC have been found in 23 states, and they have rapidly expanded their population sizes, with BHC and SC representing over 60% of the biomass in some portions of their North American Range (Garvey et al. 2012). These filter-feeders cause significant ecological impacts by altering plankton communities at the base of the food chain and outcompeting native species for resources. There is considerable concern that these species will enter the Great Lakes through man-made shipping, sanitation and flood control canals, such as those of the Chicago Area Waterways System (CAWS). Should self- sustaining BHC or SC populations become established in the Great Lakes, these species could potentially cause dramatic ecosystem alterations, leading to negative effects on populations of native fishes and many threatened or endangered plant/animal species (Asian Carp Regional Coordinating Committee 2013). The impact of this invasion on Great Lakes fisheries is of particular concern.

Aquatic organisms shed biological materials (*e.g.*, scales, epithelial cells, slime coats, waste) containing DNA into their environments. This environmental DNA (eDNA) can persist in aquatic environments for extended periods (Dejean et al. 2011, Thomsen et al. 2012), and the eDNA in water samples can be assayed using species-specific genetic markers to determine whether a species of interest may be present. Because eDNA can be detected in water when target species' populations are at low abundances, eDNA techniques may be particularly helpful in tracking changes in the distributions of aquatic invasive species (Ficetola et al. 2008; Dejean et al. 2012; Jerde et al 2011, Goldberg et al. 2013, Lance and Carr 2012) or identifying locations where threatened or endangered species may occur (Goldberg et al. 2011; Olson et al. 2012; Farrington and Lance, in prep).

Since 2009, eDNA monitoring has been used to track the invasion front of BHC and SC throughout the CAWS, Des Plaines River, and near-shore waters of Lake Michigan. The current eDNA monitoring program employs a single, species- specific genetic marker to detect each species (Jerde *et al.* 2011). The program utilizes conventional polymerase chain reaction (cPCR) analysis, whereby the presence or absence of eDNA is determined by PCR amplification of a target DNA fragment. The PCR-amplified product is then isolated by gel electrophoresis and the DNA is sequenced to confirm the species of origin.

The QAPP outlines the detailed procedures for the current planning, collection, filtering and processing of eDNA samples (USFWS 2014).

The development of additional BHC and SC eDNA markers could provide a suite of assays to provide multiple lines of evidence or secondary verification for eDNA detections. In addition to cPCR markers, quantitative PCR (qPCR) may be used as an eDNA monitoring tool. The use of qPCR has several potential advantages relative to cPCR, including, typically, more rapid PCR thermal-cycling programs, which can be important for large-scale sampling efforts, a reduced sensitivity in some cases to PCR inhibitors (personal observation; Barnes et al. 2014), and the ability to quantify, to some degree, the amount of DNA in a sample (taking into account inherent variations in DNA extraction recoveries and qPCR-based copy number estimates). Also, while conventional PCR requires specific oligonucleo- tide binding at *two* locations (the forward and reverse primers) in order to produce a PCR product, hydrolysis probe-based qPCR, which is one of two common qPCR methodologies, may often be a more stringent assay because it requires specific oligonucleotide binding at *three* locations (forward and reverse primers, as well as the internal hydrolysis probe) in order for the reaction to produce a product that emits a fluorescent signal.

Our objectives in this study were to: 1) sequence full mitochondrial (mtDNA) genomes from multiple BHC and SC throughout their North American range to represent the intraspecific genetic variation of each species, 2) use multiple sequence alignments of BHC, SC and other closely related species that may be present in aquatic ecosystems in the Midwestern U.S.A. to design species-specific cPCR and qPCR markers for the detection of BHC and SC in eDNA monitoring programs, and 3) test the specificity and sensitivity of these new markers in detecting BHC and SC in laboratory and eDNA field trials.

Methods

Sample Collection, DNA sequencing, and Alignment

Tissue samples (fin clips or livers) were collected from silver and bighead carp populations throughout their introduced range within the Mississippi River watershed (Figure 3.1.1; Table 3.1.1). Total genomic DNA was extracted using DNeasy Blood and Tissue Kits (QIAGEN Inc.) according to the manufacturer's instructions. DNA extractions were enriched for mitochondrial DNA using long PCR to amplify the mitochondrial genome as a single 16.6 kb fragment. Primer sequences were S-LA-16S-L 5'-CGATTAAAGTCCTACGTGATCTGAGTTCAG-3' and S-LA- 16S-H 5'-TGCACCATTAGGATGTCCTGATCCAACATC-3' (Miya and Nishida 2000).

QIAGEN LongRange PCR Kit reagents were used to formulate a 25 µL PCR reaction mixture containing 1× LongRange PCR buffer, 500 µM dNTPs, 1.25 U LongRange PCR Enzyme mix, 0.4 µM of each primer, and 1 µL of DNA template. Temperature cycling

conditions began with an initial denaturation step of 93°C for 3 min, followed by 10 cycles of 93°C for 15 sec, 62°C for 30 sec and 68°C for 18 min. An additional 29 cycles were then run adding 20 sec to the extension step for each cycle. Because amplification of a single fragment was not successful for all samples (likely due to degraded template DNA), we also attempted to amplify the mitochondrial genome in three shorter, overlapping fragments, using the same PCR chemistry and cycling conditions described above. Primer sequences were designed using Primer3 software (Rozen and Skaletsky 2000) based on BHC and SC complete mitochondrial genome sequences available on GenBank (accession numbers NC_010194, EU343733, JQ231114, HM162839, EU315941, NC_010156). The following primers were designed to amplify fragments of approximately 7.4, 7.0 and 3.0 kb, respectively: LC1-F and R (5'- GAATGGGCTAAACCCCTAAA -3' / 5'- TCGTAGTGAAAAGGGCAGTC -3'); LC2- F and R (5'- CAGGATTCCACGGACTACAC -3' / 5'- TTGGGGTTTGACAAGGATAA -3'; LC3-F and R (5'- CATGCCGAGCATTCTTTTAT -3' / 5'- CAACATCGAGGTCGTAAACC -3'). When agarose gel electrophoresis revealed that all three of the shorter PCR reactions produced bands of the expected sizes, the reaction products were pooled for sequencing.

PCR products were purified using ZR-96 DNA Clean and Concentrator- 5 kits (Zymo Research) and prepared for next-generation sequencing using Nextera DNA Sample Preparation Kits (Illumina, Inc.); Nextera Index Kits were used to pool up to 96 libraries into a run for sequencing. Sequencing was performed on the Illumina MiSeq system, using 150 bp paired-end reads. MiSeq Reporter Software was used to sort the resulting pool of sequences by the indices to identify the sequences arising from each sample. Mitochondrial genomes were assembled by aligning the reads of each individual to a reference sequence of the appropriate species from GenBank (see above for accession numbers) using Geneious software v.6 (Biomatters Ltd., Auckland, New Zealand). Consensus sequences generated for each individual were exported and aligned, along with sequences of some related cyprinid fish species that may be present in the same North American regions as BHC and SC (common carp, grass carp and black carp; GenBank accession numbers NC_010288.1, NC_018035.1, NC_018039.1, NC_018036.1, NC_011141.1). Alignments were carried out using the default settings in MUSCLE (Edgar 2004) as implemented in Geneious V6.

Figure 3.1.1. Geographic distribution of sample collection for mitochondrial DNA sequencing.

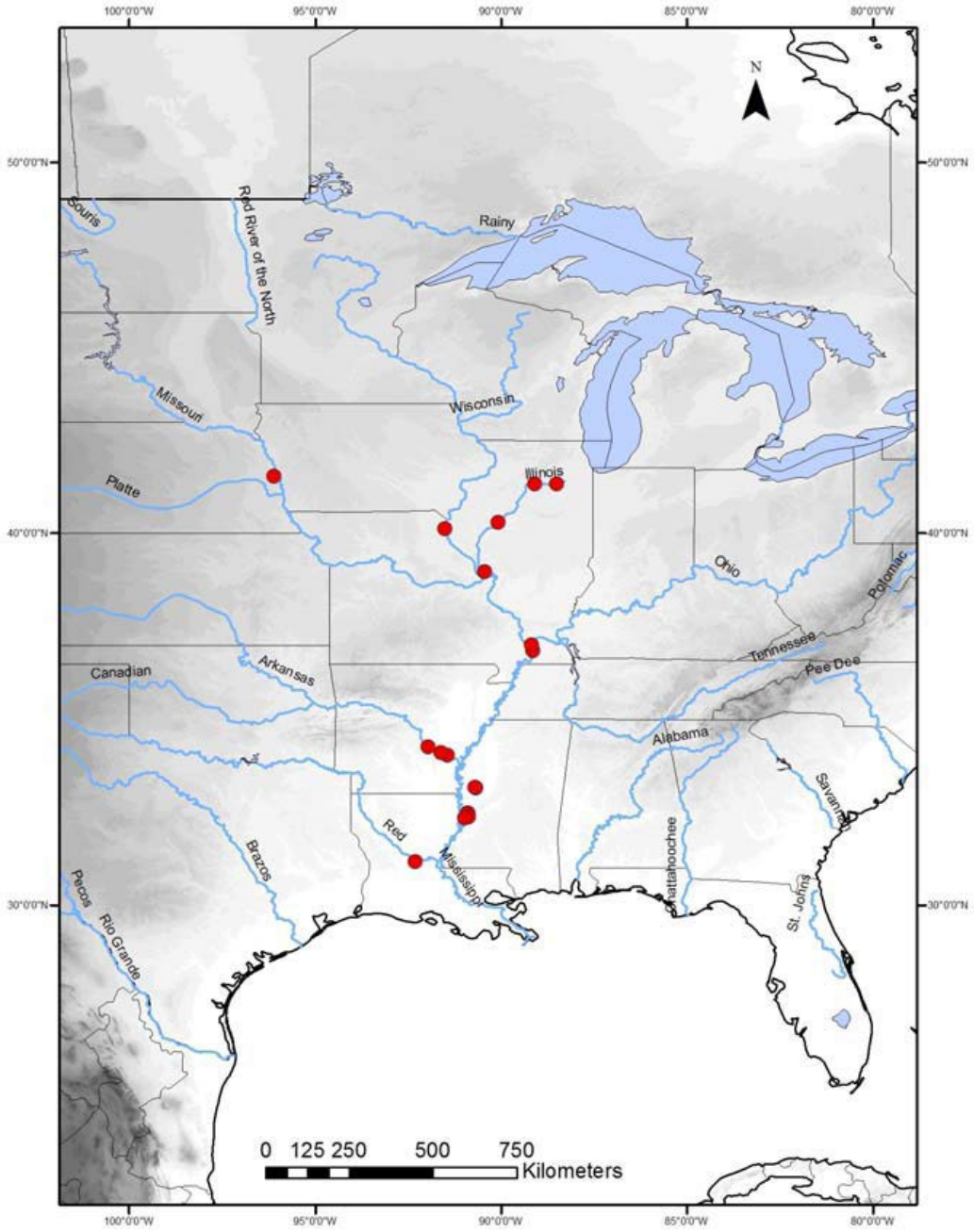


Table 3.1.1. Origins of silver and bighead carp samples included in mtDNA genome sequencing and alignment.

Location	Silver Carp (n)	Bighead Carp (n)
East Lower Mississippi (Yazoo River, Steele Bayou, Big Sunflower River)	3	5
West Lower Mississippi (Red River, Atchafalaya River)	3	6
Arkansas River	3	3
Ohio River (at junction to Mississippi River)	3	3
Mississippi River (Knowlton Lake)	2	-
Illinois River (LaGrange Reach)	-	3
Illinois River (Marseilles Reach)	3	3
Illinois River (Starved Rock)	2	3
Mississippi River (Laketon, KY)	3	1
Upper Mississippi River (Pool 20)	1	3
Upper Mississippi River (Pool 26)	3	3
Missouri River (north of Omaha)	3	-

Marker Design

Marker loci were designed using the multiple sequence alignment of complete mitochondrial genomes of bighead carp, silver carp, and several related species (listed above). Potential PCR primer sites were chosen by identifying sequence regions that demonstrated no mismatches within the target taxa and that maximized differences between target and non-target taxa. Because eDNA may experience rapid degradation by environmental conditions, marker loci were designed to be short (<400 bp) to increase amplification probability. Primer3 (Rozen and Skaletsky 2000) was used to design cPCR primers and qPCR primer/probe sets with preference for primers that contained 3'-end mismatches to homologous DNA in non-target species. All qPCR probes were labeled with 6FAM as the 5' fluorescent tag, and TAMRA as the 3' quencher. Due to the limited genetic divergence between bighead and silver carp, we also developed a series of general BHC/SC markers that may detect both species.

Marker testing for specificity and efficacy in eDNA field trials

Unless otherwise noted, newly designed cPCR markers were tested using 25 µL reactions containing 1× Platinum® *Taq* PCR buffer (Invitrogen), 200 µM dNTPs, 1.5 mM MgCl2, 0.2 µM of each primer, 1.25 U Platinum® *Taq* polymerase (Invitrogen), and 1 µL DNA template. Temperature cycling conditions began with an initial denaturation step of 94°C for 10 min, followed by 45 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min 30 sec, with a final elongation step at 72°C for 7 min. Amplification products of cPCR assays were purified using E165 Gel SizeSelect Gels (Life Technologies) and sequenced using an ABI 3500XL Genetic Analyzer with BigDye chemistry and standard sequencing protocols. Resulting sequences were compared against BHC and SC reference DNA sequences and subjected to GenBank BLAST searches to identify the source species of the amplification product.

All qPCR reactions were run in 20µl volumes containing 1X TaqMan® Environ- mental Master Mix, 0.54 µM of each primer, 0.125 µM of the probe, and 1µL of DNA template. Temperature cycling began with an initial denaturation step at 95°C for 10min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. qPCR reactions were run on a ViiA™ 7 Real- Time PCR System (Applied Biosystems). qPCR reactions were considered positive if the amplification curve crossed the fluorescence detection threshold by the end of the 40 cycle qPCR run. Both cPCR and qPCR markers were tested for: 1) species-specificity, 2) ability to amplify target species DNA from eDNA samples collected in areas of known BHC and SC presence, 3) false-positive amplification from eDNA samples that likely do not contain Asian carp DNA, and 4) limits of detection, or sensitivity, in targets species (*i.e.*, the minimum amount of starting DNA that can result in a detectable cPCR or qPCR product).

Species-specificity of both cPCR and qPCR assays was tested using a panel of genomic DNA (1 ng/µL) from individuals of the target species and 29 additional species likely to be present in the CAWS. This panel included closely related, non- target species such as shiners, common carp, goldfish, and grass carp (Table 3.1.2). If cPCR markers amplified non-target species, annealing temperatures were adjusted in an attempt to eliminate non-target amplification. The cPCR and qPCR markers that amplified the target species and showed little cross- amplification in non-target species were further tested using field-collected water samples from Steele Bayou, a backwater flood control area near the Yazoo River's confluence with the Mississippi River near Vicksburg, MS. U.S.A. Steele Bayou is locally known to have well-established BHC and SC populations with high densities (pers. commun., A. Katzenmeyer). We also tested for amplification of BHC and SC in water samples collected from a small tributary of Fishing Creek (Clinton County, PA, U.S.A), an area outside the introduced range of BHC and SC. These samples have all the typical components of environmental water samples, but were free of target DNA, and thus provided test cases to detect potential non-target amplification within naturally occurring DNA pools. In all cases, surface water samples were collected in 50 mL conical tubes. In the laboratory, tubes were centrifuged at maximum speed (4000 g)

for 30 min at 4°C. The supernatant was poured off and DNA was extracted from the remaining pellet of material using a modified cetyltrimethyl ammonium bromide (CTAB) chloroform protocol (Doyle and Doyle 1987). Each cPCR and qPCR marker was tested on a panel of 44 Steele Bayou and 44 Fishing Creek samples, with 4x replication of PCR reactions to evaluate the detection rate of these species from areas of known presence and the potential false positive rate from waters where they are absent. The performance of all new markers, as measured by rate of detection, was compared to the cPCR markers for BHC and SC from Jerde et al. 2012 (primers HN203-F & HN498-R and HMF-2 & HMR-2, respectively), which are currently used in the Asian carp monitoring QAPP (USACE 2012). We refer to these markers here as QAPP-SC and QAPP-BHC.

Table 3.1.2. Panel of 29 non-target fish species collected from the CAWS used for testing of primers for cross-species amplification.

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

Markers with high detection rates and low false-positive rates in environmental samples were subjected to sensitivity testing. Genomic DNA of SC and BHC was extracted and the concentration of each was normalized to 1 ng/μL. A serial 1:10 dilution series was prepared and markers were tested across the concentration range of 0.1 ng/μL (10^{-1}) through 10^{-7}, with four replicate cPCRs or qPCRs at each concentration. A limitation to the use of genomic DNA in sensitivity testing for cPCR markers is that the number of marker copies present in the normalized DNA extractions, and therefore available for PCR amplification, is unknown. To estimate starting copy number in qPCR reactions, each qPCR marker was cloned into a bacterial plasmid vector using TOPO® Cloning kits (Life Technologies) as per the manufacturer's instructions. Successfully cloned bacterial colonies were cultured and plasmids extracted using Qiagen Miniprep plasmid extraction kits. The estimated number of plasmids in the resulting elutions was calculated using the combined base pair length of the plasmid and marker insert, a standard DNA base-to-Daltons conversion for double-stranded DNA (650 Daltons/base; Roche Applied Science 2011), a Daltons-to-nanograms conversion, and DNA mass quantification of elutions using a NanoDrop 1000. A dilution series of the plasmid elution was then used to generate a standard curve for estimation of copy number in the qPCR reactions.

To test whether the throughput of eDNA screening methods could be increased by assaying for multiple markers simultaneously within single PCRs, several markers were combined in pairs for multiplex cPCR or qPCR reactions. For cPCR, the QAPP-SC and SC-1 markers were combined. For qPCR, three primer sets were tested: BH-TM1/BH-TM2, SC227 TM4/SC-TM5, and AC-TM1/AC- TM3. For qPCR multiplexing, the two markers utilized probes with different fluorescent labels (FAM or VIC). A genomic DNA dilution series and plasmid standards were again used for testing, with markers and standards run both individually and in combination in order to directly compare sensitivity in single versus multiplex reactions. qPCR reactions were prepared as described above, with both sets of primers and probes added to the reaction, and the same temperature cycling conditions.

Results

We generated complete mtDNA sequences for 33 BHC and 29 SC individuals (Table 3.1.1); all DNA sequences were submitted to GenBank (Accession numbers to be provided in forthcoming publication). Average whole genome sequence coverage for the 62 haplotypes sequenced was 1595X (range 3-11971X coverage). Total length of the aligned BHC and SC genomes was 16620 bp. There was very little sequence variation within species, with only 40 (0.24%) and 34 (0.20%) variable sites for BHC and SC, respectively. When species alignments were combined, there were a total of 823 (4.95%) variable sites across the mitochon- drial genome. These genomes were aligned with

mtDNA genomes from closely related species obtained from GenBank, including common, grass and black carp for identification of potential species-specific eDNA markers.

Based on the alignment of mitochondrial genomes, we initially designed 12 SC, 11 BHC and 16 general BHC/SC cPCR markers. For TaqMan® qPCR, we initially designed five markers for BH, six for SC, and three general BHC/SC markers. Based on results from the initial cross-species screening, several markers amplified non-target species and were not further investigated, reducing the number of potential markers for testing to six for cPCR and eight for qPCR (Table 3.1.3). We focused all subsequent field and sensitivity testing on the markers with high affinity for the target species and little or no amplification of other species.

Assays of the 44 Steele Bayou samples with the established markers QAPP-SC and QAPP-BHC resulted in 28 (64%) positive SC detections and 0 (0%) positive BHC detections (Table 3.1.4). All of the newly designed BHC markers performed better than the QAPP-BH marker, with the highest detection rate from the qPCR marker BH-TM2 (9 of 44, 20%). In comparison to the QAPP-SC marker, all newly-designed SC markers had similar or higher numbers of detections, with the highest detection rates from cPCR marker SC-1 and qPCR maker SC-TM5, both with 32 positive samples (73%). Positive detection rates were 57-68% for the general (BHC/SC) markers. For the Fishing Creek samples, none of the new cPCR markers produced bands in the same size range as target species (or close enough in size to have warranted sequencing confirmation in an actual eDNA monitoring sample) and none of the qPCR markers produced quantifiable fluorescence.

All the tested markers consistently yielded positive results from genomic DNA down to at least the 10^{-3} dilution (0.001 ng/µL). Three SC (SC-5, SC-7 and SC- TM4) and one BHC/SC marker (AC-TM2) had consistent detections at 10^{-4}, and nearly all markers had >50% detection rates among the four replicates at the 10^{-4} dilution level, which is estimated to have copy numbers in the single digits (Table 3.1.5). Detections became more stochastic at concentrations below 10^{-4}, as expected for samples with extremely low copy number (average of ≤1 marker copy per reaction). Sensitivity of the new BHC and SC markers was comparable to the QAPP markers.

Multiplexing of cPCR markers was found to be unfeasible for high throughput processing and analysis using standard gel electrophoresis equipment in our lab. The new cPCR markers were all designed to be in the size range of 200-300 base pairs to increase the potential for amplification of degraded eDNA, therefore, amplicon base pair lengths were too similar for clear differentiation of bands on 2% agarose gels; gel-based isolation of fragments for sequencing would also be infeasible with this combination of amplicon lengths and electrophoresis equipment. Longer-running or higher density gels

may have allowed more reliable separation of different cPCR marker bands but may somewhat negate the cost and time savings gained from multiplexing. Trials of multiplexed qPCR markers were successful for all combinations of markers tested, with no substantial reduction in marker sensitivity (limits of detection; Table 3.1.6).

Table 3.1.3. Primer sequences of markers used for field and sensitivity testing. For targeted species, BH = bighead carp, SC = silver carp, Both = primers that could potentially be used for non-specific detection of both BHC and SC. Names containing TM indicates TaqMan® qPCR markers.

Name	Target species	Forward	Reverse	Probe (TaqMan® markers only)	Length (bp)
SC-1	SC	GGACCCAGTACTATTAACTGCTCTA	TCCTAGGGCAAGGAGGGTA		171
SC-5	SC	TCCGATTACCGCCACAATTATAGCCTTAG	GATAGGGTTAGTGGAAGAGAGGAC		161
SC-7	SC	ACTGAATAAACACACACATGTTCGAT	ATCATCACCCGATTAGTAAAAATG		275
SC-TM4	SC	CCACTAACATCACCACGCAA	AGCCTTTTCCAGAGGCTTGG	TAACCCAGCTGCCAATACAA	168
SC-TM5	SC	CCACAACTTACCCTCCTTGCC	AAGGGTATTAATTTTTGTGGTGGA	TCATGACATCCGCAGCATTCCTC	98
BH-6	BH	CAATACCCTAGCAATTATTCCCTTA	TGTAATTCCAAGGGCGGTTAG		375
BH-8	BH	GATGTAAACTATGGCTGGCTTATT	TGTAGAAAGAGGAGGTGTAGGAA		388
BH-TM1	BH	TAGACCTTCTAACAGGACTAATTC	AATCCACCTCATCCTCCAAC	CCGCCCTTGGAATTACATCCACA	144
BH-TM2	BH	CCTTCGTCAAACAGACCTTAAATCC	CCCTCATGGGGTTTGGATTAGA	CCACATAGGACTTGTAGCGGGTGGA	96
BH-TM4	BH	CCACTAACATCGCCACGTAG	AACCTTTTCCAGAAGCTTGG	TAGCCCAGCCGCCAACACAA	168
AC-6	Both	GTTCCTAATCAGCACCTTAGTACTCT	AATTCGAAGGGATGGCAAG		156
AC-TM1	Both	GGCCGGAACAGGATGAACAGTT	TAATAGTTGTGGTGATGAAGTTAATTG	CACGCAGGAGCATCCGTAGACCT	145
AC-TM2	Both	CAATTAACTTCATCACCACAACTATTA	TCCAGCAGCTAAAACTGGTAAGG	AAACACCTCTCTTTGTTTGAGCTGTGC	133
AC-TM3	Both	TTCATCGGCGTAAATCTTACAT	AGGGAAATAAGAGATCCGATAGA	ACCCAGATGCCTACGCCCTG	133

Table 3.1.4. Number of positive detections noted for each marker tested using 44 eDNA field samples. Steele Bayou samples were collected from an area of high concentrations of both BHC and SC, whereas Fishing Creek samples were collected from an area where carp are absent. QAPP-SC and QAPP-BH are the markers currently used for eDNA testing. Names containing TM indicate TaqMan® qPCR markers.

Marker	Steele Bayou	Fishing Creek
Silver Carp:		
QAPP-SC	28	0
SC-1	32	0
SC-5	25	0
SC-7	23	0
SC-TM4	26	0
SC-TM5	32	0
Bighead Carp:		
QAPP-BH	0	Not tested
BH-6	6	0
BH-8	6	0
BH-TM1	9	0
BH-TM2	7	0
BH-TM4	6	0
Bighead and Silver		
AC-6	30	0
AC-TM1	25	0
AC-TM2	30	0
AC-TM3	28*	0

* Tested with only 42 samples

Table 3.1.5. Sensitivity testing. Estimated marker copy numbers per dilution are based on averages calculated across all replicates of qPCR sensitivity trials using a plasmid DNA standard. AC markers were tested using SC dilutions. The number of positive detections out of four replicates is noted for each marker and dilution level. U = Undetermined copy number. Amplifications at these levels are likely due to stochasticity of PCR at such low DNA concentrations.

Dilution:	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**
Silver Carp:							
Estimated Copy Number	6051	508	46		1.6	U	U
QAPP-SC	4	4	4	4	0	0	0
SC-1	4	4	4	3	0	0	0
SC-5	4	4	4	4	0	2	0
SC-7	4	4	4	4	0	0	0
SC-TM4	4	4	4	4	1	1	0
SC-TM5	4	4	4	3	2	1	1
Bighead Carp:							
Estimated Copy Number	2193	230	16	2.2	1.1	U	U
QAPP-BH	4	4	4	4	1	1	0
BH-6	4	4	4	1	0	0	0
BH-8	4	4	4	3	0	1	0
BH-TM1	4	4	4	3	1	0	0
BH-TM2	4	4	4	3	0	0	0
BH-TM4	4	4	4	3	0	1	0
Bighead and Silver Carp:							
Estimated Copy Number	6051	508	46	3.4	1.6	U	U
AC-6	4	4	4	2	0	1	0
AC-TM1	4	4	4	2	2	0	0
AC-TM2	4	4	4	4	2	0	0
AC-TM3	4	4	4	3	0	0	0

Discussion

The large number of mtDNA haplotypes generated in this study allowed us to capture inter- and intra-species genetic variation in SC and BHC across their introduced, North American range. This information, along with comparisons to DNA sequences from related species found in the central United States, aided in the design of cPCR and qPCR markers specifically for eDNA testing for SC and BHC in their introduced range. Effective design of PCR-based assays for the differential or discriminatory detection of species requires that sequence differences among taxa be clustered so that multiple differences among taxa are grouped into the length of a PCR primer and two or more of these areas are grouped within a few hundred base pairs. Because SC and BHC are closely related and have very low levels of sequence divergence across their mitochondrial genomes, a very limited number of sites demonstrated a sufficient number of clustered polymorphisms to develop effective species-specific markers. Despite careful selection of markers to maximize differences among species, cross-amplification with either the non-target Asian carp species or another non-target fish was observed for many markers, resulting in the elimination of nearly 70% of the originally designed markers. Despite these difficulties, we were able to design multiple cPCR and qPCR markers that specifically detect SC and BHC in field- collected water samples in North America.

In field trials, the new species-specific markers developed in this study generally had detection rates similar to or higher than the markers currently used to detect the presence of BHC and SC DNA in environmental water samples, with similar levels of sensitivity at low concentrations of target DNA. Further, confounding or efficiency-diminishing factors (e.g., amplicons that result in gel bands of similar size to those obtained for the target species or nontarget fluorescence in qPCR trials) were not observed, indicating that these markers would be suitable as high-throughput assays to detect the presence of BHC and SC from environmen- tal water samples. Multiplexing of qPCR markers was successful in genomic DNA trials, suggesting that multiplexing may be feasible in eDNA screening, increasing throughput of the assays. However, performance of multiplexing reactions with field eDNA samples remains to be tested, and additional combinations of the various markers could be employed following further testing.

Table 3.1.6. Multiplexing of qPCR markers. Average Ct values (copy numbers) across 24 replicates for markers run individually and combined in a multiplex reaction.

Dilution:	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**
Silver Carp:				
SC-TM4	30.6 (195-424)	34.1 (10-50)	37.3 (0-12)	38.7 (0-2)
SC-TM5	30.4 (872-1498)	33.7 (60-208)	37.4 (0-30)	38.1 (0-11)
Combined:				
SC-TM4	30.7 (203-422)	34.2 (11-50)	37.9 (0-5)	38.4 (0-3)
SC-TM5	30.4 (1145-	33.9 (81-200)	37.5 (2-23)	38.4 (0-14)
Bighead Carp:				
BH-TM1	31.2 (107-244)	34.6 (8-28)	38.2 (0-4)	39.0 (0-1)
BH-TM2	31.2 (120-216)	34.8 (7-19)	37.8 (0-4)	39.3 (0-1)
Combined:				
BH-TM1	30.6 (118-263)	34.3 (8-23)	37.3 (0-5)	38.3 (0-1)
BH-TM2	31.1 (137-201)	34.6 (12-24)	38.1 (0-7)	38.7 (0-1)
Bighead and Silver Carp:				
AC-TM1	31.1 (241-358)	34.5 (16-65)	38.0 (0-12)	38.9 (0-3)
AC-TM3	29.1 (225-398)	32.3 (16-52)	36.0 (0-6)	37.7 (0-1)
Combined:				
AC-TM1	31.1 (283-536)	34.5 (22-91)	37.7 (0-16)	39.4 (0-1)
AC-TM3	29.1 (252-501)	32.4 (19-80)	35.9 (0-12)	37.6 (0-2)

In addition to potential improvements in sensitivity and throughput by the markers developed in this study, the availability of multiple new cPCR markers for eDNA screening of SC and BHC in water samples may help increase the accuracy of eDNA monitoring programs. eDNA samples are largely comprised of randomly fragmented, low-abundance DNA targets. The current program uses a single marker locus to detect the presence of SC or BHC in environmental samples, which may be sensitive to random degradation of the single marker. The use of multiple marker loci would improve overall detection rates and provide stronger evidence for the presence of BHC and SC DNA in the water. Further, the addition of qPCR technology to eDNA screening provides the

transition from simple presence/absence data provided by cPCR to the generation of data related to DNA concentration in field samples. This additional information *may* help estimate *relative* abundance or biomass of species of interest in the sampled waterway. qPCR may also reduce sample screening time by eliminating the need for gel electrophoresis and sequence verification. The new qPCR and cPCR markers developed in this study therefore represent a significant expansion of the tools available to detect the invasion of SC and BHC in North America and may improve the accuracy, resolution, and throughput of eDNA monitoring programs for these species in the future.

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 conventional PCR and with qPCR.

There was no significant difference in apparent DNA yield or quality, as measured by PCR success in dilution trials with extracted DNA, relative 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 Protocols for eDNA Sampling and Extraction

There are at least 4 different approaches currently being utilized for eDNA sampling (Ficetola 2008, Jerde et al. 2011, Lance and Carr 2012, Goldberg et al. 2011). 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 USGS and University of Idaho (Goldberg et al. 2011), 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

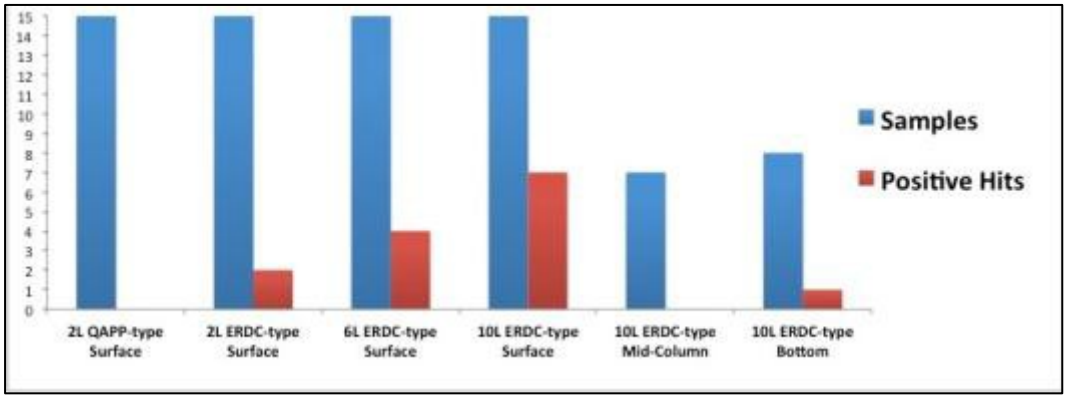
Three sampling trips were undertaken. In the first (November 2011), 15 locations in the Dresden Island Pool of the Illinois River, were sampled. At the first 8 locations, a 2-liter grab sample (i.e. QAPP protocol) from the surface, along with 2-liter, 6-liter, and 10-liter samples from the surface and one 10-liter sample from both the mid-column and near bottom zones of the river were taken (1 QAPP-type, 5 ERDC-type ERDC-type samples per location). Because of limitations on time available for the sampling effort, we decided to forgo some sample-types during the latter half of this effort -- the 10-liter ERDC-type samples from the mid-column and near-bottom were not taken at the last 8 and 7 sites, respectively. 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 4.2.1). 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.
It should be noted, however, that no water blank, or negative, controls were taken during this sampling event.

Figure 4.2.1. Alternative sampling experimental results, Trial 1.



For the second trial, there appeared to be complete inhibition within samples Figure 4.2.2). 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.

The third trial exhibited similar contamination issues with ERDC-type samples (Figure 4.2.3). We surmised that field contamination of ERDC-type samples was likely due to handling the sieve cloths on-board a sampling vessel that had

Figure 4.2.2. Alternative sampling experimental results, Trial 2.

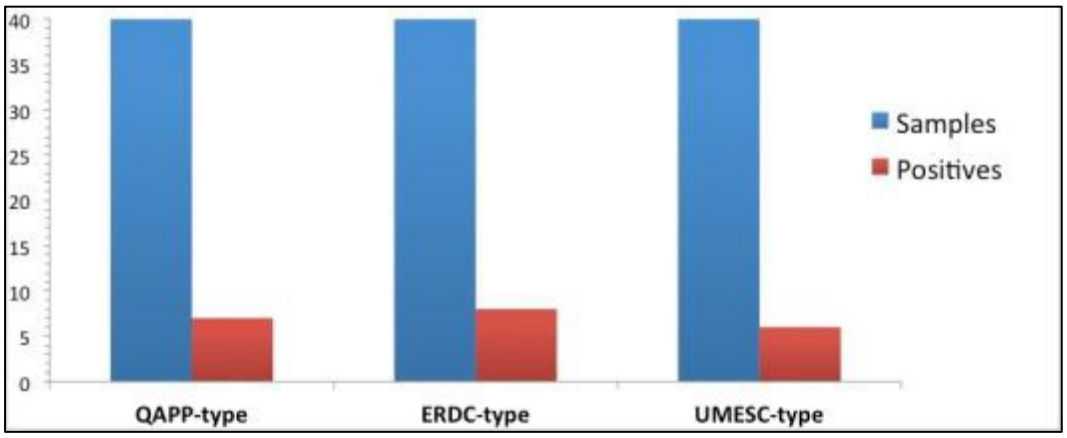
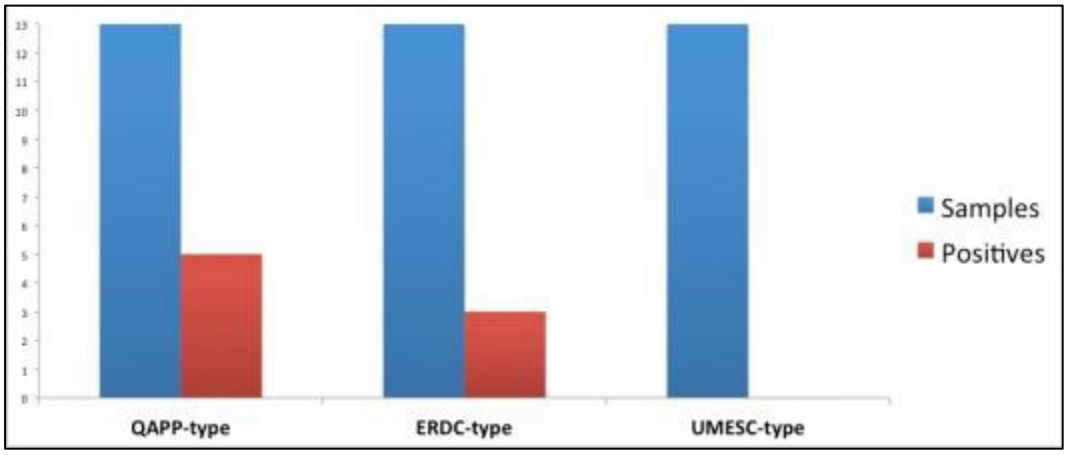


Figure 4.2.3. Alternative sampling experimental results, Trial 3.



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.

The trial showing with the most significant differences between sampling approaches was Trial 1, where the ERDC-type samples strongly outperformed the QAPP-type

samples. However, there were no negative controls taken during this trip and Trials 2 and 3 showed that ERDC-type sampling, as performed in these trials, may be relatively more prone to contamination. Also, in subsequent trials, we did not observe the same relative performance superiority of the ERDC-type samples. A final determination on the relative merit of the different sampling approaches will require additional studies. The relative value of sampling the surface vs. mid-column vs. bottom layers of a water body seems to have been answered somewhat more robustly, since all samples on the first trip would have been equally likely to have come into contact with contaminating DNA and surface samples strongly outperformed mid-column and bottom samples.

4.3 Loading Studies

The purpose of the eDNA loading studies 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. Efforts pertaining to progress on the loading studies have been documented in ECALS milestone reports. Although there may be some repetition with respect to ECALS background information here, the two loading milestone reports are presented with minimal editing for completeness.

Introduction

Since the initial detection of Asian carp moving up the Mississippi Basin, the potential for invasion of the Great Lakes by silver carp and bighead carp has been a major concern to stakeholders. To combat this problem, sampling for environ- mental DNA (eDNA) is used to monitor the waterways near Lake Michigan. This monitoring area includes the Chicago Area Waterways System (CAWS) and the Des Plaines River. By sampling waters that may be inhabited by Asian carp, the extraction and amplification of carp DNA from the collected cellular debris is possible. This technique has been successfully used in several other contexts (Ficetola et al., 2008; Foote et al., 2008) and is believed to be a highly sensitive method for species detection (Dejean et al., 2012). Compared to traditional methods for surveying aquatic invasive species (fishing, rotenone application, and electrofishing), the increased sensitivity of this method could be a valuable asset. Early detection could lead to a more rapid response to the threat of a Great Lakes invasion by Asian carp.

Currently, eDNA sampling throughout the CAWS is undertaken following the QAPP. This procedure uses conventional polymerase chain reaction (PCR) and markers developed by Jerde et al. (2011). Conventional PCR tests for the absence or presence of silver and bighead carp DNA, but does not provide any infor- mation on the actual amount of carp eDNA found in the sample. Quantification of eDNA may provide an

understanding of how eDNA behaves in the system, which will better inform us of how positive eDNA samples reflect the magnitude of the eDNA signal, and could potentially be used to distinguish between high and low densities of Asian carp. Currently, there is little information on how eDNA enters the system and how it acts in the environment. Subsequently we do not know what environmental factors influence the amount of eDNA shed, what factors influence its degradation (and thus detectability), and what factors influence its persistence and movement through the system. Our goal is to obtain information on how various factors influence the loading (or shedding) and degradation of eDNA from two Asian carp species, silver carp and bighead carp. To obtain these goals, quantitative PCR (qPCR) is used to quantify the amount of carp DNA in water samples. The following report describes results from a portion of these loading studies.

Here we describe the current qPCR assays that are being used for the loading studies. This assay differs from the current QAPP protocol used for field sampling. We also describe the sensitivity of these assays, which is vital to interpreting qPCR results. We then show results from an introductory experi- ment, describing the variability inherent in eDNA quantification. Second, we provide data from a series of lab tests that assessed the influence of fish density, temperature, spawning, and diet on eDNA shedding rates. Our third objective, testing the qPCR assay in field-like conditions (pond mesocosms), is currently underway. This experiment is designed to test the effects of fish densities and also to test the time to detection of an eDNA signal. We will report upon the third objective when that study is complete.

4.3.1 Development of qPCR Protocol for eDNA Shedding Rate Studies

Background

Quantitative PCR can be used in one of two basic methods that allow for the detection and quantification of replicated DNA. SYBR Green is a fluorescent dye that binds to pieces of double-stranded DNA formed during the polymerase chain reaction. Thus, as more strands are formed, binding of the strands by SYBR Green increases. This leads to a brighter fluorescent signal that the thermocycler detects. A more specific approach uses a TaqMan ® probe assay which incorpo- rates a fluorescently labeled probe (oligonucleotide) that binds to the amplicon of interest. As the primers and Taq polymerase amplify the target DNA, the polymerase degrades the probe and releases the fluorescent label, causing a fluorescent signal to be emitted. As more target is amplified, more fluorescence is emitted. Because the probe binds specifically to the target DNA, rather than to any double stranded DNA, only amplification of the targeted amplicon causes a fluorescent signal.

Currently we have a set of primers and TaqMan® probes designed by the USGS Upper Midwest Environmental Science Center (UMESC). We compared the sensitivity of this primer set to that of the Jerde et al. (2011) markers. Because the Jerde et al. (2011) markers do not have a TaqMan® probe, we could only compare the two primer sets directly using the SYBR Green method. We also tested the species specificity of the two primer sets. We used the UMESC primer and probe sets for all loading experiments. We describe the limit of detection (LOD) and limit of quantification (LOQ) of the UMESC markers according to our protocol. This is important for accuracy in analyzing qPCR results.

After the qPCR assay design phase, we ran a preliminary experiment which allowed us to describe the variation inherent in eDNA quantification of shedding rates under controlled conditions.

Methods

Comparisons between the Jerde et al. (2011) and UMESC markers were made using SYBR Green master mix and a thermal gradient qPCR. Our DNA template was tissue extracted genomic DNA from each species. Concentrations of original extractions were quantified using a spectrophotometer, and then subsequently diluted to a medium concentration and low concentrations. We also checked for cross reactivity of the primers by running the silver carp extraction with bighead carp primer/ probe sets and the bighead carp tissue extraction with silver carp primer/ probe sets. Results from the thermal gradient qPCR are reported in threshold cycles. Final optimization of annealing temperatures and primer/ probe concentrations for each assay (silver carp and bighead carp) was achieved using a series of thermal gradient reactions with varied reagent concentrations. Table 4.3.4 lists the primer/probe sequences, qPCR protocols and reagents used.

For all other qPCR assays, quantification of samples was inferred using a standard curve. The standard curve was made with a set of serial dilutions of a plasmid that includes the target amplicon. All standards and samples were run in duplicate (preliminary studies) or triplicate on each sample plate. Up to eight different plates of samples were run for each experiment. Average efficiencies from each experiment ranged from 88.5% to 96.6%. The limits of quantification and detection (LOQ and LOD) were determined for each assay by running the standard curve dilution series with eight replicates. The LOQ was determined as the lowest standard dilution at which all 8 replicates amplified. The LOD was assessed as the standard dilution that was 10-fold below the LOQ.

For the preliminary study, we placed single juvenile silver carp in 40 L tanks with flowing water. Three different flow rates (1 L/hr, 2 L/hr, and 3 L/hr) were tested

to determine the optimal rate for quantifiable eDNA detection. Fish were left in the tanks for seven weeks and sampled nearly every day. All samples were run in duplicate with our qPCR assay.

Results

We found the UMESC primers to be more sensitive than the Jerde et al. (2011) markers. As shown in Table 4.3.1, the UMESC primers amplified genomic DNA extracts more efficiently. At the low concentrations of genomic DNA most relevant to eDNA monitoring, the UMESC silver carp primers amplified 3 threshold cycles earlier than the Jerde et al. (2011) primers. Furthermore, the UMESC bighead carp primers amplified 11 threshold cycles earlier than the Jerde et al. (2011) markers. This is equal to a 10-fold (for silver carp) and nearly 10,000-fold (for bighead carp) difference in eDNA detection sensitivity. Each 3 threshold cycle (Ct) difference corresponds to a 10-fold difference in initial target DNA concentration. For different primer sets testing the same DNA sample, differences in Ct reflect differences in amplification efficiency, resulting in different sensitivity. However, we also found the UMESC markers to more readily cross-amplify with the other non- target carp species compared to the UND markers. Our limit of quantification (LOQ) was our 10^2 standard (~200 copies/5 µl reaction), thus we can quantify ~40 copies/µl. Our limit of detection (LOD) for both silver carp and bighead assays was our 10^1 standard (~20 copies/5 µl reaction). Therefore, we can detect around 4 copies of DNA/µl. These numbers vary slightly depending on the concentration of the plasmid stock. For further analyses, any samples with detectable eDNA at concentrations below the LOQ were assigned a quantity of half the LOQ.

For the preliminary study, a scatter plot (Figure 4.3.1) shows the variability among samples of the same tank. There was high variability throughout the seven week period, with the first week having the highest variation. The low flow rate (1 L/hr) tank had the highest amount of detectable DNA. After adjusting the amount of DNA detected by flow rate, however, the shedding rates of DNA in all three tanks were similar (Table 4.3.2).

Table 4.3.1. Comparison of Jerde et al. (2011) and UMESC qPCR primer sensitivity and cross-reactivity using SYBR Green with a thermal gradient for the annealing temperature and medium or low concentrations of template genomic DNA.

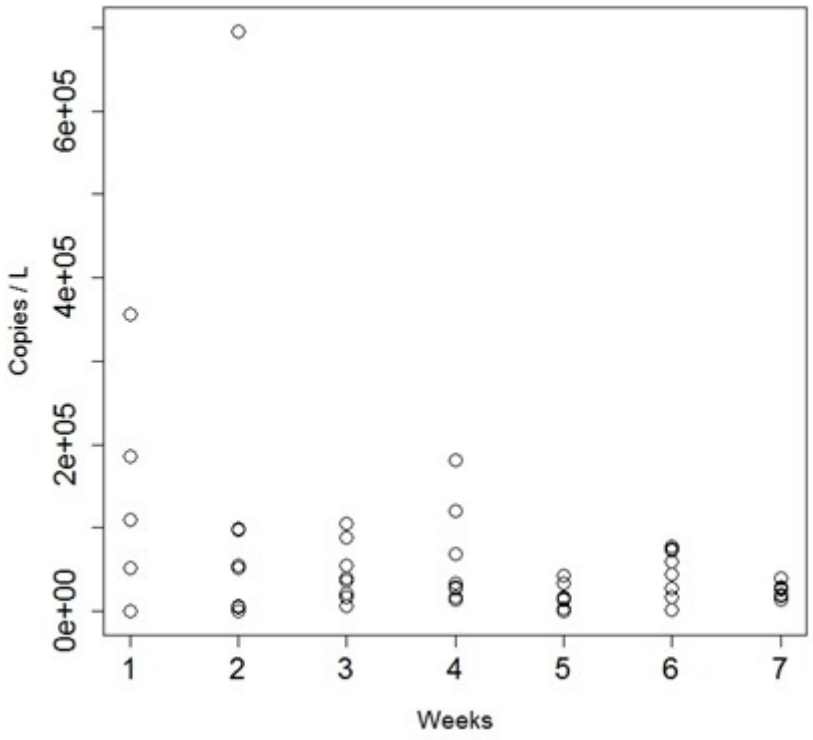
	Medium SC	**Low SC**	**Medium BH**	**Low BH**
Primers	1.1 ng/reaction	0.11 ng/reaction	1.2 ng/reaction	0.12 ng/reaction
Jerde SC	23.36 cycles @ 53.9 C	30.32 cycles @ 53.9 C	36.38 cycles @ 53.9 C	N/A
Jerde BH	> 40 cycles @ 50.0 C	N/A	33.05 cycles @ 50.0 C	> 40 cycles @ 50.0 C
UMESC SC	23.75 cycles @ 58.0 C	27.27 cycles @ 58.0 C	25.29 cycles @ 58.0 C	N/A
UMESC BH	28.03 cycles @ 58.0 C	N/A	22.29 cycles @ 58.0 C	27.17 cycles @ 58.0 C

The Ct (threshold cycle) at the optimum annealing temperature for each combination of primer and template DNA is shown. The optimum annealing temperature was deter- mined as the temperature at which the primers are most sensitive (Ct is lowest). SC- Sil- ver Carp; BH- Bighead Carp

Table 4.3.2. Amounts of eDNA (copies of eDNA/L) and shedding rates (copies of eDNA/hr) for fish kept at three different flow through rates.

	1L/hr	**2L/hr**	**3L/hr**
Average Copies of eDNA/L	61,000	33,000	18,000
Average Copies of eDNA/hr	61,000	66,000	54,000
Standard Deviation	+/- 50,000	+/- 53,000	+/- 92,000

Figure 4.3.1. Scatter plot from preliminary study showing variation of eDNA sample quantification (copies of eDNA/L versus sampling week). Fish were added to the tank at the start of week 1.



Discussion

We showed that qPCR can quantify eDNA from water samples; however, these assays must be optimized (best annealing temperature, primer concentrations, etc.) to obtain the highest sensitivity for detecting the target DNA. The UMESC markers used in our studies are not species-specific; however, due to the small genetic difference between silver and bighead carp, species-specific markers may not be obtainable. Finding genus-specific markers that do not cross amplify with Asian carps other than bigheaded carps, may be adequate for field collections. Sequencing of samples can then allow for identification of species, if required.

We found that quantification of eDNA samples can be highly variable even when sampling the same individual under controlled conditions. Environmental DNA is unlikely to be homogenous and probably has a clumped distribution. Masses of tissue, cells or fecal debris that contain high amounts of DNA are not evenly dispersed in the

environment. Nevertheless, our preliminary study showed that silver and bighead carp appear to shed at similar rates under the same condi- tions. The preliminary study also revealed that averaging quantities from multiple samples at one site may provide a more accurate estimate of eDNA quantity. Averaging the results of many samples should minimize the effects of this inherent sampling variability, and reduce the effect of outliers that occur simply due to the clumped nature of eDNA. We hypothesize that the high points in eDNA shedding rates observed during weeks 1 and 2 in the preliminary study (Figure 4.3.1) could be due to the fish being stressed by new surroundings or due to fish handling during introduction. Thus, for subsequent experiments, we used samples from only the second to fourth weeks. Even with early samples removed, quantification variability remained high. Such high variability may not allow for the discrimination of small effect sizes in shedding rates should they exist. Thus, future studies should address detection of larger effects.

4.3.2 Quantification of eDNA Shedding Rates: Fish Density, Temperature, and Spawning

Background

In order to understand how eDNA behaves in the environment, we must understand how it enters the system. We addressed three hypotheses that could influence the shedding rate of eDNA by these fish. It is assumed that the amount of DNA shed will increase linearly as the number of fish increase. If this is the case, then it might be possible to provide an estimate of fish density from quantifying an eDNA sample. We hypothesized that as the number of fish in a tank increased so would the shedding rate (amount of DNA in a sample).

We tested the influence of temperature on shedding rate. We hypothesized that fish in warmer water temperatures may be more active and thus shed more DNA, so we expected to observe higher eDNA shedding rates from fish kept in warmer temperatures.

Spawning events result in large amounts of gametes being released into the water. The eDNA signal from such events may result in a distinct signature. We quantified the amount of eDNA in water samples given a known amount of sperm that had been added to the water, to see if spawning events can indeed lead to a strong eDNA signal. We also described how this eDNA signal degrades over time in a closed system (no flow).

Methods

Experimental Set Up

Juvenile fish (60-100mm) were placed in 40 L glass aquaria and sub-adult fish (100-300mm) were housed in 379 L plastic round tanks. The small tanks were set at a flow rate of 2 L/hr and large tanks at 19 L/hr. For density assays, treatments included 1, 3, or 6 fish (with 4 replicates for each treatment). Tests were run for both age classes of each species. For the temperature assay, treatments included Low (19°C), Medium (25°C), and High (31°C). One sub-adult fish was kept in each large tank. There were three replicates per temperature treatment. For the sperm degradation study, milt from bighead carp and silver carp was collected at separate times and from multiple males of each species. This milt was then mixed. For each study (bighead and silver) half of the sperm was placed on dry ice and frozen, and the other half was kept on wet ice ("fresh"); 500 µl of sperm (either fresh or frozen) was then added to each of three 40 L glass aquaria. Environmental DNA samples were then collected every day for 21 days after the addition of sperm.

qPCR

Sample Processing: All samples (50 ml) were taken in duplicate using either a clean serological pipette for experiments run in the 40 L aquaria, or using a siphon to sample from the 379 L plastic aquaria. All samples were taken below the surface but not from the bottom. Samples were then centrifuged for 30 minutes at 5000 RCF at 4°C. Afterwards, the water was decanted off, and samples were left to dry for at least 10 minutes before adding 250 µl of the extraction TDS0 buffer (AutoGen Inc., Holliston, MA). Samples were then frozen until extracted. Samples were digested using Proteinase K (AutoGen Inc. Holliston, MA) and left overnight in 55°C water bath. Samples were extracted with an AutoGen (AutoGen Inc. Holliston, MA) automated robot, using a phenol chloroform extraction method.

qPCR Assay: Samples from the second to fourth experimental week were then run using the appropriate species' primer/ probe set. Samples were run in triplicate and each plate included a standard curve. Quantifications of eDNA were converted from copies per reaction to copies per liter (eDNA amount) or copies per hour (eDNA shedding rate).

Analysis

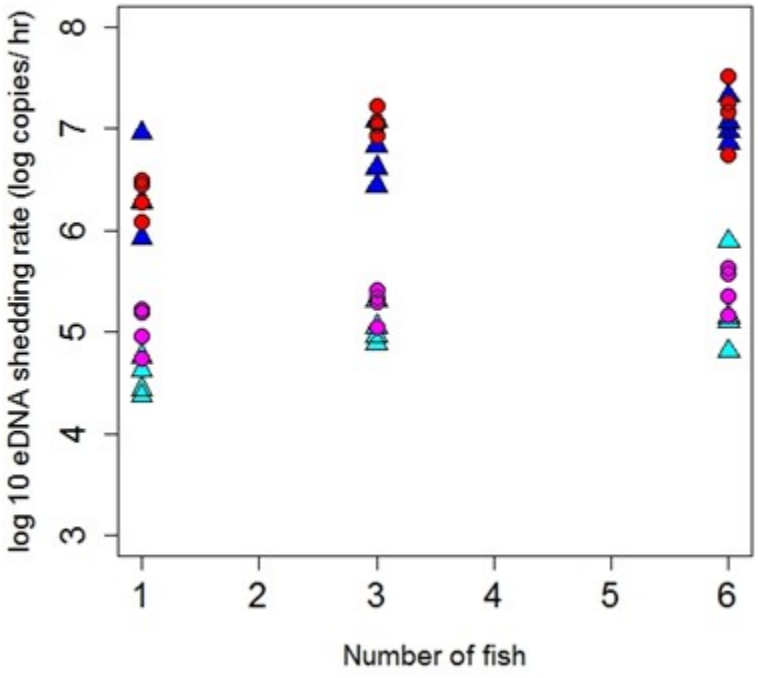
A total of eight samples from each tank was collected and averaged to obtain the average eDNA shedding rate for that tank. Box-plots of the eight samples per tank were used to identify extreme outliers in the dataset. Outliers were identified as points being 1.5 times the inter-quartile range of the data. Extreme outliers were classified as those that cause a 2-fold or higher change in the average. Only the extreme outliers were removed from data sets; the averages for each treatment were calculated. Data (average eDNA shedding rates) were log transformed to fit the assumption of normality. Linear regression was then used to look for any correlation between eDNA shedding rate and treatment factors. ANOVAs and subsequent pairwise tests with Bonferroni corrections were also used to look for statistically significant treatment differences. Statistical significance was defined at $p = 0.05$.

Results

Fish Density. All six density experiments showed a significant correlation between eDNA shedding rate and number of fish (Figure 4.3.2; Table 4.3.3). Running a one-way ANOVA using fish density as a factor, all analyses showed significant or near significant treatment differences. Post-hoc pairwise compari- sons using the Bonferroni correction showed high versus low density to be significant in three of the experiments and medium versus low density was also significant in the Bighead sub-adult test (Table 4.3.3). We also looked at eDNA shedding rates and total length or total weight of all fish in each tank (Figure 4.3.3). There was not a strong linear relationship between eDNA shedding rate and total length of fish; however there was a very strong relationship between eDNA shedding rate and total mass of fish ($F=468.4$, DF (1,46), $p < 0.01$, $R2 = 0.91$, slope 0.94, intercept 4.2, Figure 4.3.3).

It appears that there is a difference between age class and eDNA shedding rate, but no difference between species within the same age class. We will apply further statistical tests to assess this.

Figure 4.3.2. Scatterplot of fish density (number of fish per tank) and eDNA shedding rates for silver carp and bighead carp.



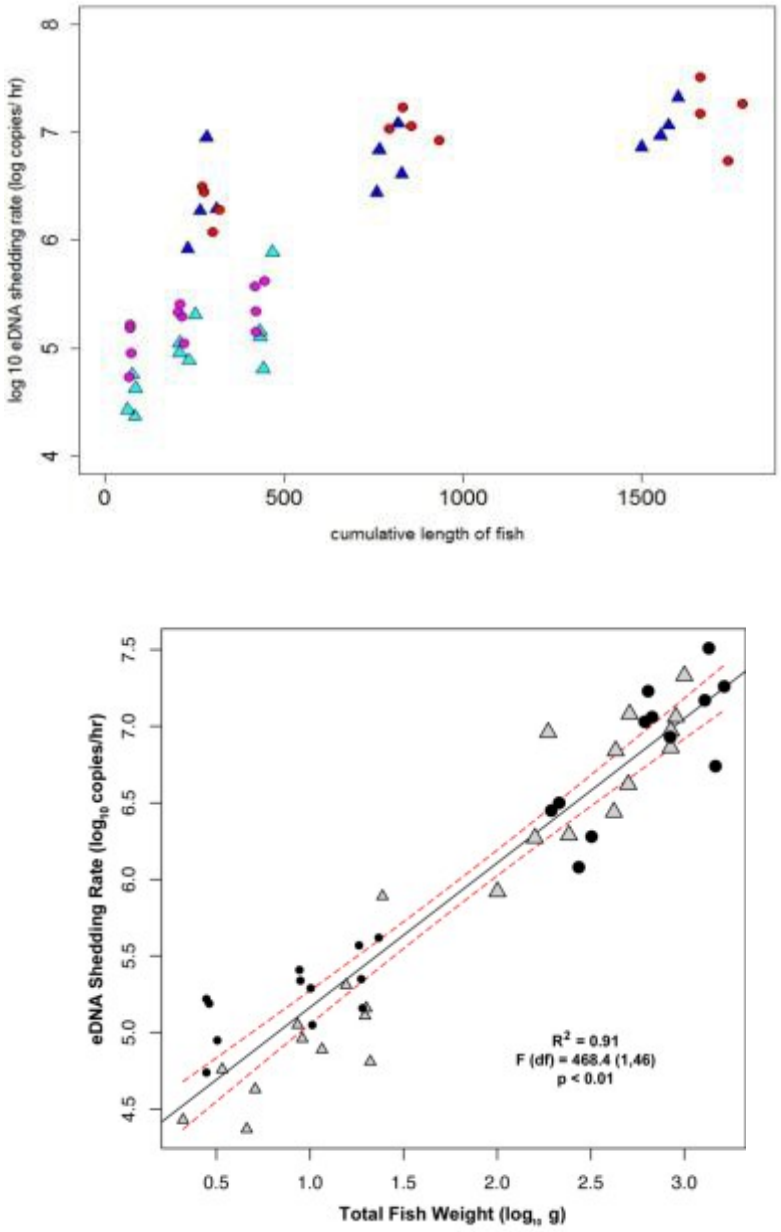
Dark Blue Triangles – Silver Carp sub-adults; Light Blue Triangles – Silver Carp juveniles; Red Circles- Bighead Carp sub-adults; Pink circles – Bighead Carp juveniles

Table 4.3.3. Regression and ANOVA statistics for all temperature and density loading studies.

		Temp SC Sub-Adult	**Temp BH Sub-Adult**	**Density SC Juvenile**	**Density SC Sub-Adult**	**Density BH Juvenile**	**Density BH Sub-Adult**
Regression	R^2	0.07	0.09	0.48	0.5	0.44	0.59
	F (dof)	0.49 (1,7)	0.69 (1,7)	9.17 (1,10)	10.07 (1,10)	7.94 (1,10)	14.37 (1,10)
	p	0.51	0.43	0.01*	0.01*	0.02*	<0.01*
ANOVA	F (dof)	5.7 (2,6)	0.87 (2, 6)	5.57 (2,9)	4.83 (2,9)	4.02 (2,9)	16.33 (2,9)
	p	0.04*	0.46	0.03*	0.04*	0.06	<0.01*
Post-hoc pairwise comparisions	p	Med. vs. Low 0.05*	High vs. Low 0.03*	High vs. Low 0.04*	High vs. Low 0.06	High vs. Low <0.01*	Med. vs. Low <0.01*

Significant post-hoc pairwise comparisons with Bonferroni corrections are shown with high, medium and low representative of the temper- ature or density treatment.* statistically significant ($p < 0.05$)

Figure 4.3.3. Shedding rates of eDNA for two age classes of two species.



Top panel shows mean eDNA shedding rates versus total length of fish per tank. Bottom panel shows mean eDNA shedding rate versus total weight of fish per tank. Top panel: Dark blue triangles – Silver Carp sub-adults; Light blue triangles – Silver Carp juveniles; Red circles- Bighead Carp sub-adults; Pink circles – Bighead Carp juveniles. Bottom panel: Bighead carp sub- adults – large, black circles; bighead carp juveniles –small, black circles; silver carp sub-adults – large, grey triangles; silver carp juveniles – small, grey triangles. Dashed line indicates 95 % CI.

Temperature. Shedding rate did not correlate with temperature for either carp species (Figure 4.3.4), as linear regressions were not significant. Using treatment type (low, medium or high temperature) as a factor, an ANOVA and post-hoc pairwise tests found a significant difference between the Silver Carp eDNA shedding rates of the low and medium temperature treatments (Table 4.3.3). Silver Carp in the medium temperature treatment shed less eDNA than those fish in the low temperature treatment. However, there was no significant difference between either the low or medium treatment and the high temperature treat- ment.

Sperm Degradation. For both Silver Carp and Bighead Carp assays eDNA detection was highest on the first day after addition of sperm. By the fourth day after addition of sperm to the tanks, over 99% of the original amount was lost (not detected; Figure 4.3.5). However, even up to the 21st day, some eDNA was detectable.

Discussion

Fish density was correlated to eDNA shedding rate, with the largest differences between the low and high density treatments. We believe that the small differences among treatments did not allow for a more precise discrimination among eDNA shedding rates, and that testing the effect of larger differences will lead to higher correlations.

We found no effect of water temperature on shedding rate; thus no support for our hypothesis that fish in warmer water are more active and shed more eDNA. This is similar to the findings of Takahara et al. (2012) in a similar study using common carp. However, Takahara et al. (2012) predicted that fish behavior may play a role in seasonal eDNA detection. In field sampling, they detected more eDNA in warmer stretches of water than cooler stretches, but they suggested that carp prefer to congregate in theses warmer locations, thus resulting in stronger eDNA signal in such locations.

Finally, the sperm studies found that peak loading (amount of eDNA) was detected one to two days after the initial addition of sperm. After 4 days, 99% of the eDNA quantified from the first day was undetectable. Some eDNA (< 1%), however, was still detectable up to the 21st day of the experiment. Thus spawning activity may be detectable, if samples within a small area show high eDNA quantification but much lower signal in the surrounding area, and if that signal drops rapidly over the next few days. However, this possibility should be tested in the field against other measures of spawning, such as egg counts, and other conditions that could result in high eDNA, such as aggregations of fish feeding at a concentrated food source. The study also indicated that eDNA is detectable for at least 21 days (though at low levels) even after the source of DNA is gone.

Figure 4.3.4. Scatterplot for the regression of eDNA shedding rate and temperature for both silver carp (colored triangles) and bighead carp (colored circles). Color is indicative of the treatment type (low, medium, high temperature), but the regression was run using average tank temperature as a continuous variable.

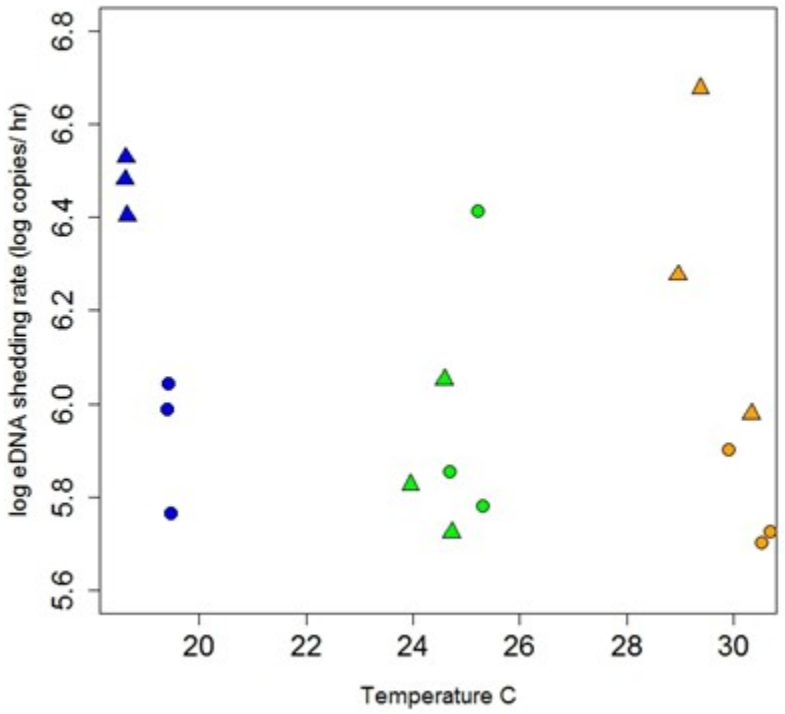
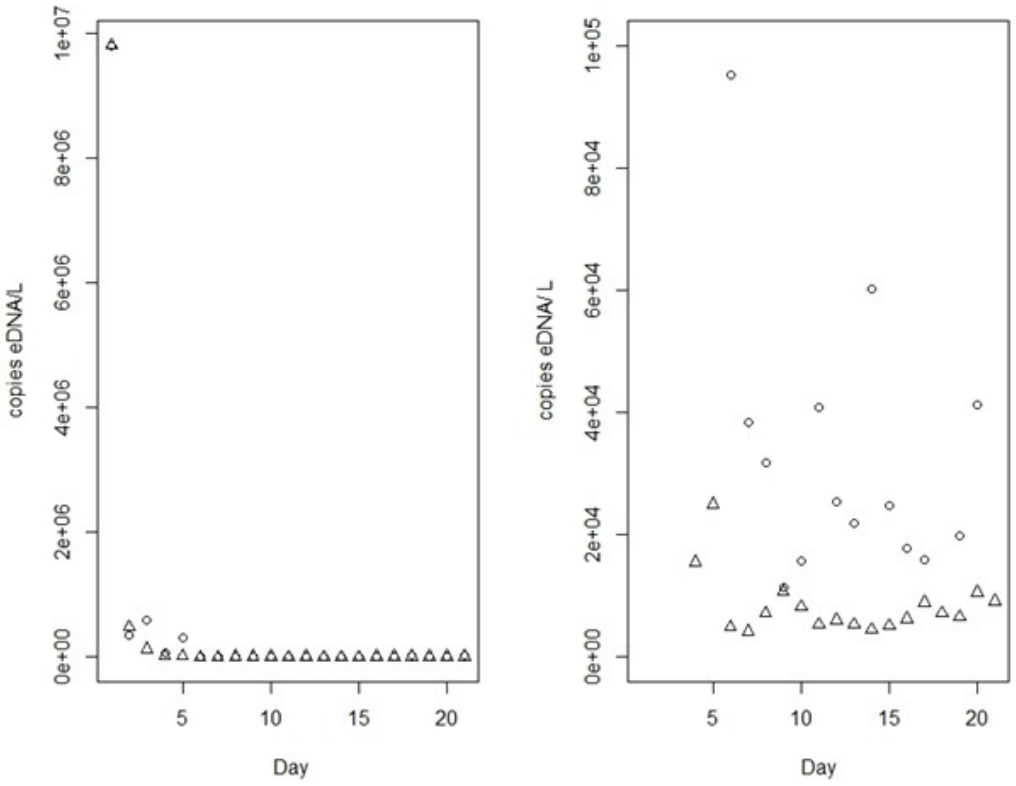


Figure 4.3.5. Results of sperm loading and degradation studies.



The plot on the right is a magnified view of samples from day 4 through day 21. No averaged sample reached 0. Circles – bighead carp sperm samples; Triangles – silver carp sperm samples.

Summary

Water samples can be quantified for carp eDNA using qPCR. The sensitivity of the assay will depend on time-consuming but necessary optimization of the analysis (temperature, reagent amounts). The eDNA signal can be highly variable, likely reflecting clumped eDNA distribution. We found no correlation between water temperature and eDNA shedding rates. We observed a correlation between eDNA loading and fish density. Inhibition of the polymerase chain reaction by compounds in the sample that are co-extracted with the DNA will lead to false negatives. Positive internal controls can be used to identify inhibited samples, and thus avoid false negatives.

4.3.3 Quantification of eDNA Shedding Rates: Diet

Background

The source of the shed cellular debris that contains the eDNA is currently unknown. It is believed to either come mostly from the exterior skin or scale cells of the fish or from cells lining the gut and shed through excrement. We hypothe- sized that if the gut lining is the source of most shed eDNA, fish fed more food would also shed more eDNA. For this experiment, we compared the eDNA shedding rates of fish that were not fed, to fish that were fed with different amounts of green algae, and to fish fed with brine shrimp.

Methods

Experimental Set Up

Juvenile fish (60-100mm) were placed in 40 L glass aquaria and sub-adult fish (100-300mm) were housed in 379 L plastic, round tanks. The small tanks were set at a flow rate of 2 L/hr and large tanks at 19 L/hr. For the diet study, four experiments were run: silver sub-adults, silver juveniles, bighead sub-adults, and bighead juveniles. Each experiment had four treatments: no food, low feeding rate of algae (soft food), high feeding rate of algae (soft food), and low feeding rate of brine shrimp (rough crustacean food). No high feeding rate of brine shrimp diet was used due to space limitations. Each treatment had three replicates. Three fish were placed in each tank. Daily feeding amounts were calculated as a percent of the average fish body mass. Feeding rate percentages differed between the sub-adult and juvenile tests (see x-axis Figure 4.3.6). Fish were trained on their diet type (algae or shrimp) before the experiment began. Studies were run from October 2012 through August 2013. Each study ran approximately four weeks.

qPCR

Sample Processing: Water samples (50 ml) were taken every other day for 2.5 weeks in duplicate using either a clean serological pipette for experiments run in the 40 L aquaria, or using a siphon to sample from the 379 L plastic aquaria. All samples were taken below the surface but not from the bottom. Samples were then centrifuged for 30 minutes at 5000 RCF at 4°C. Afterwards, the water was decanted off, and samples were left to dry for at least 10 minutes before adding 250 µl of the extraction TDS0 buffer (AutoGen Inc., Holliston, MA). Samples were then frozen until extracted. Samples were digested using Proteinase K (AutoGen Inc. Holliston, MA) and left overnight in 55°C water bath. Samples were extracted with an AutoGen245 (AutoGen Inc. Holliston, MA) automated robot, using a phenol chloroform extraction method.

qPCR Assay: Samples from the second to fourth experimental week were then run using the appropriate species' primer/ probe set designed by USGS Upper Midwest Environmental Science Center (UMESC) (Table 4.3.4). Samples were run in triplicate and each plate included a standard curve. Quantifications of eDNA were converted from copies per reaction to copies per liter (eDNA amount) or copies per hour (eDNA shedding rate).

Figure 4.3.6. Box plots of treatment (diet) averages for eDNA shedding rates in four experiments. Percent weight gain or loss and average starting weights are also shown below each plot.

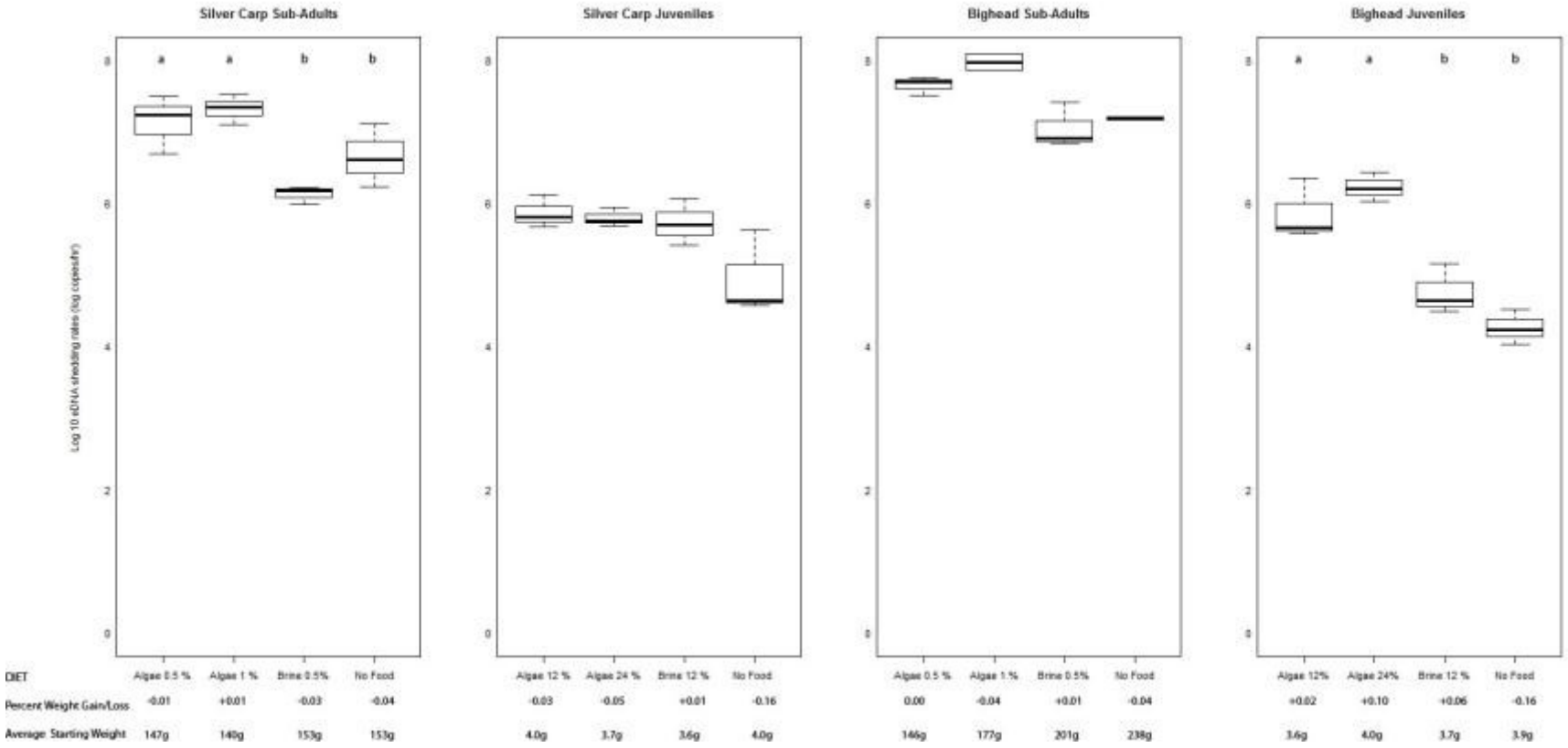


Table 4.3.4. Primer set and probes used for the qPCR analyses. UMESC primers from D-loop region.

Species	Forward	Reverse	Probe	Annealing Temperature* (deg C)	Amplicon Length (bp)
Silver carp	GGTGGCGCAG AATGAACTA	TCACATCATTT AACCAGATGCC	CCATGTCCGTGA GATTCCAAGCC	58.0	108
Bighead carp	GGTGGCGCAA AATGAACTAT	GCAAGGTGAA AGGAAACCAA	CCCCACATGCCG AGCATTCT	58.0	190

Analysis

Average shedding rates determined from previous experiments (effects of temperature and biomass) were calculated from 8 subsamples per tank over the 2.5 week period. However, due to equipment complications during the diet experiments, the number of subsamples per experiment varied. Averaged shedding rates for each tank were calculat- ed from: 7 subsamples for bighead sub-adults; 6 subsamples for silver sub-adults; 5 sub- samples for bighead juveniles and 3 subsamples for silver juveniles. In the bighead sub- adult test, fish mortality led to the removal of one tank (replicate) for both the unfed treatment and the high feeding rate of algae treatment.

Box plots of the subsamples per tank were used to identify extreme outliers in the da- taset. Outliers were defined as points 1.5 times the inter-quartile range of the data. Ex- treme outliers were classified as those that cause a 2-fold or higher change in the mean. Extreme outliers were removed from data sets and the means for each treatment were calculated. Data (average eDNA shedding rates per tank) were log transformed to fit the assumption of normality. ANOVAs and subsequent pairwise tests with Bonferroni cor- rections were used to look for statistically significant treatment differences. Statistical significance was defined at $p = 0.05$.

We also looked at the average percentage of body weight gained or lost in each treat- ment. Figure 4.3.6 shows these weight gain/loss percentages above the average weights in the box plot.

Results

Preliminary analyses revealed that the polymerase chain reaction was inhibited in samples from the tanks with the algae-fed juvenile fish. No amplification was observed in these samples, even after spiking the samples with a known amount of DNA before running the reaction. Samples from the tanks of unfed fish and brine shrimp-fed fish were not inhibited. The algae-fed sub-adults had a lower percentage of food added to their tanks, and subsequently, we detected no inhibition in these samples. We found that a 1:10 dilution of the inhibited samples (all samples from the algae-fed juvenile fish) allowed for recovery of the polymerase chain reaction and DNA quantification. The quantification from these diluted samples was then multiplied by 10 to correct for the dilution factor.

As shown in Figure 4.3.6, unfed fish still shed DNA into the water; however, fed fish generally had higher eDNA shedding rates, particularly the fish fed an algae diet. The difference between fed and unfed fish was approximately a 10-fold increase or higher in average DNA shedding rates among silver sub-adults and bighead juveniles. Similarly, bighead sub-adults and silver juveniles had shedding rate increases between non-fed and algae-fed treatments, but not to the same degree (Table 4.3.5). Brine-fed fish gener- ally had shedding rates similar to the unfed fish, except for silver juveniles.

Table 4.3.5. Average log 10 eDNA shedding rates of each treatment (standard deviation in parentheses).

	Silver Carp Sub-Adult	**Silver Carp Juvenile**	**Bighead Carp Sub-Adult**	**Bighead Carp Juvenile**
High Algae	7.13 (0.20)	5.80 (0.14)	7.98 (0.16)	6.23 (0.21)
Low Algae	7.14 (0.41)	5.87 (0.23)	7.78 (0.32)	5.87 (0.43)
Low Brine Shrimp	6.03 (0.30)	5.73 (0.32)	7.14 (0.29)	4.77 (0.35)
No Food	6.10 (0.19)	4.96 (0.59)	7.19 (0.02)	4.27 (0.25)

We found statistically significant differences among treatments in all four experiments ($p \leq 0.05$; Table 4.3.6). Pairwise t-tests indicate that for silver sub-adults and bighead juveniles, algae-fed groups were significantly different from brine shrimp-fed and unfed groups. For the sub-adult bighead carp no pairwise differences were significant; however, results followed a similar trend to results from the bighead juveniles and silver sub-adult experiments in that the brine shrimp-fed or unfed treatments shed less eDNA relative fish from the algae-fed treatments. Finally, although the ANOVA results showed significant ($p = 0.05$) differences for the juvenile silver carp experiment, none of the post-hoc pairwise comparisons were significantly different at the $p = 0.05$ level. Unlike the previous three experiments the brine shrimp-fed treatment and the algae fed-treatments were not different from one another ($p = 1.00$). Unfed silver carp juveniles shed less DNA that fed fish, however differences were not statistically significant.

Table 4.3.6. ANOVA and post-hoc pairwise comparison (with Bonferonni correction) statistics for each of the four diet experiments.

	Silver Carp Sub-Adult	**Silver Carp Juvenile**	**Bighead Carp Sub-Adult**	**Bighead Carp Juvenile**
# subsamples	6	3	7	5
# treatments	4	4	4	4
ANOVA				
F (dof)	13.83 (3,8)	4.18 (3,8)	6.28 (3,8)	24.74 (3,8)
P	< 0.01*	0.05*	0.03*	< 0.01*
Post-Hoc Pairwise Comparisons				
High Algae vs. Low Algae	1.00	1.00	1.00	1.00
High Algae vs. Low Brine Shrimp	0.01*	1.00	0.08	< 0.01*
High Algae vs. No Food	0.01*	0.13	0.14	< 0.01*
Low Algae vs. Low Brine Shrimp	0.01*	1.00	0.14	0.02*
Low Algae vs. No Food	0.01*	0.09	0.28	< 0.01*
Low Brine Shrimp vs. No Food	1.00	0.18	1.00	0.55

Three replicates per treatment; except for the bighead carp sub-adult study which had 3 replicates for the low algae and low brine shrimp treatments but only 2 replicates for both the no food and the high algae treatments. Post-hoc pairwise comparisons are Bonferroni-corrected. * Significant (p = 0.05).

Discussion

Overall, fish fed the soft, algae diets shed more DNA than unfed fish. In general, fed fish shed about one order of magnitude more DNA than non-fed fish (Figure 4.3.6). We accept our hypothesis that gut cells shed via feces is a major source of shed DNA. Non-fed fish shed detectable amounts; however, feeding leads to higher shedding rates, and statistically higher rates in two of the experiments (silver sub-adults and juvenile bigheads).

Could shedding rate differences be due to size of the fish rather than the actual differences in diet? As previously shown, greater biomass (more fish or larger fish) leads to a greater amount of detectable DNA (previous July Interim report; Takahara et al. 2012). We tried to use fish of similar length and weight in each treatment; however, differences did exist. If fish in the non-fed and brine shrimp-fed treatments were smaller in size, they would be expected to shed less due to this size difference. However, Figure 4.3.6 shows that in the sub-adult experiments, fish in the unfed and brine shrimp-fed treat- ments had higher average weights than either of the algae-fed treatments, yet shed less than the smaller fish from the algae-fed treatments. This suggests that the increase in shed DNA was likely attributed to an increase in excrement and sloughed off cells from the gut. Similarly, in the juvenile studies, the average initial weight of the low algae and no food treatments were similar, and yet the algae-fed treatments still had higher shed- ding rates. Size differences among treatments may also have contributed to a lack of statistical significance in the bighead sub-adult experiment. Although the algae-fed fish shed at higher rates, there was not a significant difference between the algae treatments and the larger unfed and brine-shrimp fed fish. The larger average size of the unfed and brine shrimp fed fish may have resulted in higher shedding rates due to greater body mass, masking effects of diet. In fact, size difference of fish among treatments is greatest in the bighead sub-adult experiment relative to the other three experiments.

We hypothesized that rough crustacean food may lead to more sloughed off gut cells and thus higher shedding rates, as the crustacean exoskeletons would be more abrasive on the digestive tract compared to the softer algae diet. We do not have evidence to support this; and in fact, the brine shrimp-fed treatments generally had lower average shedding rates more similar to the non-fed treatments. It is possible that the brine shrimp food was less available to the fish than the algae food, leading to less excretion. Unlike the al- gae food which stayed in the water column until it was eaten, the brine shrimp only stayed in the water column for a couple of hours before dying and dropping to the bot- tom. Although amount of feces was not quantified, the unfed tanks were observed to have little to no feces, and the tanks fed brine shrimp had some feces but less than the algae diet tanks. The lower shedding rates generally observed in the brine shrimp-fed treatments is likely a result of lower feeding rates due to limited availability of the crustacean food.

Alternatively, silver carp may have a reduced gut length when on a zooplankton diet relative to when eating less nutritious phytoplankton (algae), which requires more digestion and thus a longer gut. Such environmentally induced phenotypic plasticity in gut length has been reported in perch (Olsson et al., 2007) and in silver carp (Ke et al., 2008). Because we did not measure gut length after the experiment, we cannot draw conclusions about this potential factor from this study.

Summary

Fish do shed DNA at higher rates when fed, likely due to cells sloughed off in the excrement. Non-fed fish still shed detectable amounts of DNA but at approximately 10-fold lower rates compared to the fed fish (especially those fed algae). Non-fed silver carp juveniles and bighead sub-adults shed lower amounts of eDNA relative to algae-fed fish, but differences were not statistically significant. Lack of significance may be due to the fewer replicates for each treatments that we had in the silver juvenile experiment (n = 3). For the bighead sub-adults, shedding rates trended similarly to the bighead juveniles and sub-adult silvers, but did not show significant pairwise differences in shedding rates. This may be due in part to the unequal average size of the fish among treatments, with unfed and brine shrimp fed fish being larger than the algae-fed fish. The lower shedding rates found in most of the brine shrimp-fed treatments is likely due to the lim- ited availability of the food to the fish during these experiments; however, the potential for gut length change dependent on diet type may also have an influence.

4.4 Degradation Studies

Efforts pertaining to progress on the degradation studies through December 2013 have been documented in the 2013 ECALS milestone report. Although there may be some repetition with respect to ECALS background information here, the entire degradation studies milestone report is presented in its entirety, with minimal editing, for completeness.

Introduction

Invasive aquatic nuisance species pose a major threat to aquatic ecosystems worldwide. Invasive Asian carp species, 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), and possibly Lake Michigan and the Great Lakes ecosystem, electrical barriers have 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 river mile (RM) 278 in Dresden Island Pool, 18 miles downstream from the barrier and 55 miles from Lake Michigan. This front has not progressed upstream since 2006.

Should a self-sustaining Asian carp population become established in the Great Lakes, populations of native fishes and many threatened or endangered plant and animal species could be negatively affected. 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 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 because it can detect the presence of Asian carp DNA in water when fish populations are at very low abundance (though other vectors, such as piscivorous birds may deposit Asian carp DNA into a system). However, the behavior of Asian carp eDNA once it is released into the water, in particular the rate at which it degrades, is largely unknown. Such data, and information on how different environmental factors influence degradation, could significantly augment our ability to use eDNA data in management decisions. To date, though, there have only been a few published reports describing loss

of eDNA “signal” or decreasing eDNA concentrations over time (Dejean et al. 2011, Thomsen et al. 2011, Thomsen et al. 2012; Figures 4.4.1, 4.4.2, and 4.4.3). There are no published data on degradation rates in Asian carp eDNA. Studies of how different environmental factors influence eDNA degradation in aquatic systems also appear to be lacking.

This report describes efforts to better understand Asian carp eDNA degradation. In November 2011 an expert panel was convened in Chicago for conceptual discussions and identification of research priorities for the eDNA Calibration Study funded by the Great Lakes Restoration Initiative (http://www.asiancarp.us/ecals.htm). As part of those discussions, several factors that were identified as potential environmental influences on the degradation of eDNA in an aquatic system. Research subtasks were designed to ad- dress the following as factors influencing eDNA degradation: water temperature, light exposure, water pH, microbial loads, water energy (turbulence), total organic content of water, and dissolved oxygen in the water.

Study design was focused on observing comparative degrees of influence of different factors on degradation. Trials were run in simplified matrices (not complex environmental matrices, like rivers or lakes) and, while general patterns may be robust, the actual rates of degradation that were observed may be very different from rates in the field. Also, our trials utilized a single DNA marker and observed rates of degradation or “signal loss” may vary based on marker features (e.g. marker length or target gene).

Figure 4.4.1. DNA degradation rates following removal of a) frog tadpoles from beakers and b) sturgeon from naturalistic outdoor mesocosms. “Detectability” is the proportion of conventional PCRs with positive results (bands) on an agarose gel at each time point. From Dejean et al. 2011.

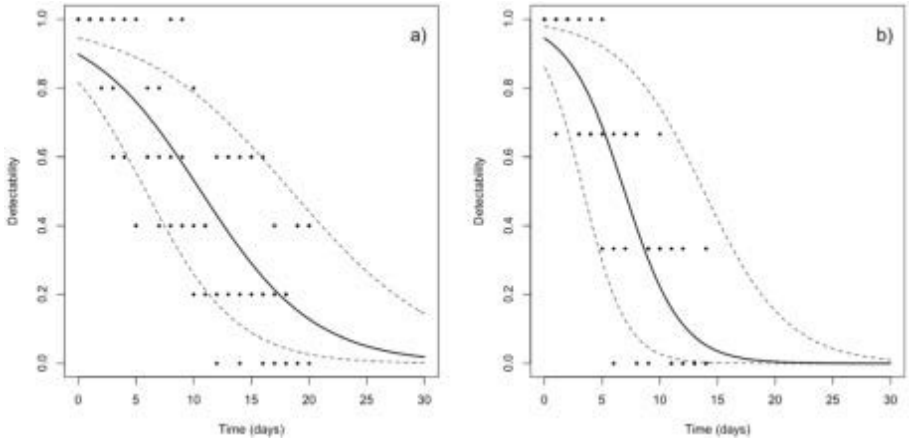


Figure 4.4.2. DNA degradation rates following removal (on Day 64) of larval toads and (left) and newts (right) from outdoor mesocosms. From Thomsen et al. 2011.

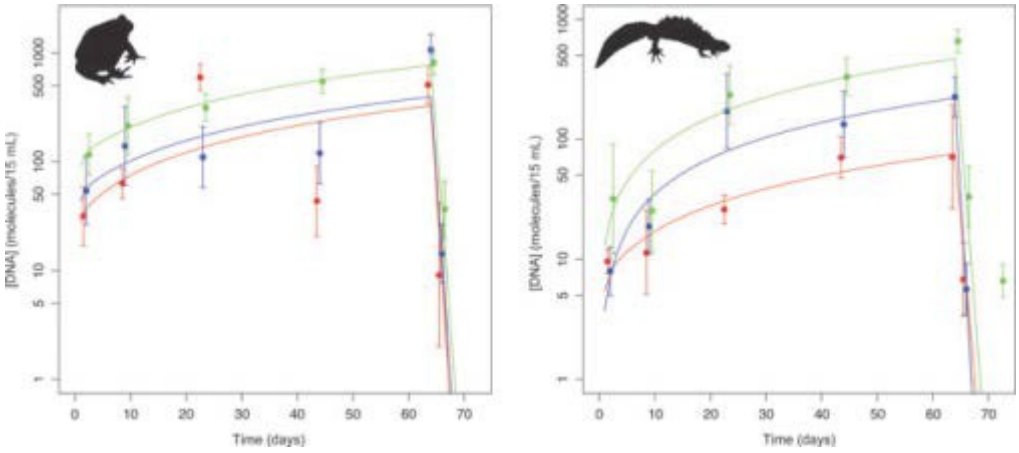
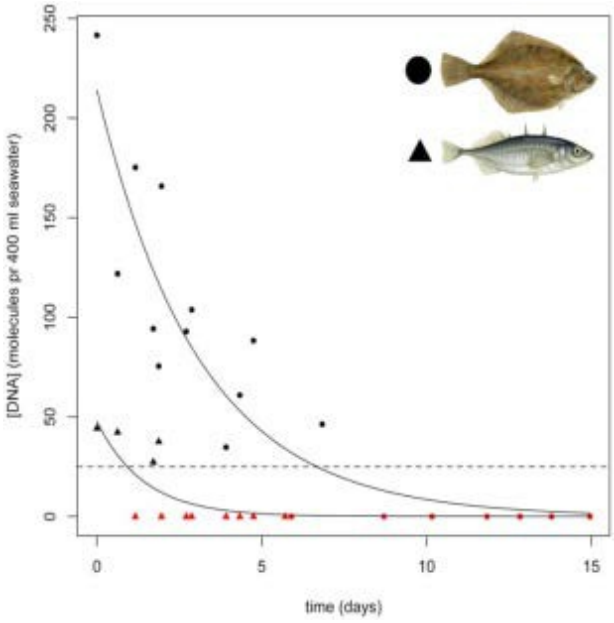


Figure 4.4.3. Temporal change in estimated eDNA abundance in large water sample. Aliquots from larger sample removed and assayed with quantitative PCR (qPCR) for two species of marine fish (*Platichthys flesus* and *Gasterosteus aculeatus*) at several intervals over 15 days. Red symbols are time points with no eDNA detections and the dashed line represents calculated detection threshold for qPCR assay. Solid lines represent exponential decay model fits. From Thomsen et al. 2012.



Methods

Characterize Baseline eDNA Degradation

Prior to executing subtasks where the influence of various factors in eDNA degradation would be assessed, we performed an experiment to observe degradation under "standard" conditions in order to test the initial experimental set up and provide a baseline observation of eDNA degradation within such a set-up. Degradation, for the purposes of this study, refers to the diminishing abundance of target DNA (i.e. marker) over time. The fate of the degraded target DNA and degradation products, or the molecular-level processes responsible for degradation of the target DNA were beyond the scope of our efforts.

For the initial set of trials we identified a diluted mixture of slime, feces, etc. collected from filtration units attached to tanks holding Asian carp as an optimal genetic material for degradation studies. We refer to the final material as "Asian carp slurry" (Figure 4.4.4). This material represents the types of materials that Asian carp eDNA are expected to originate from (Ficetola et al. 2008), but is highly variable in DNA content. Other studies (Dejean et al. 2011, Thomsen et al. 2011) have tracked degradation by housing target organisms in vessels or mesocosms, removing the organisms, and then tracking the change in eDNA detection or abundance (i.e. molecule copy number) over time. When we, in similar fashion, explored the option of tracking degradation using water removed from carp holding tanks at ERDC, we found that eDNA concentrations following extraction and purification were lower than our targeted range (several hundred to 1000+ copies per microliter (µl)).

Throughout all the degradation studies, carp slurry was prepared by adding 3 g wet filtrate from carp tanks to 50 ml of deionized, purified water. The final concentration in the slurry was therefore 60 mg/ml filtrate in water. All experiments to date have been conducted in 15 ml polypropylene screw-top centrifuge tubes, with 2 ml of fresh 60 mg/ml slurry further diluted into 12 ml purified water (14 ml total solution). At each sampling time point, 8 replicates plus one blank sample for each treatment were randomly collected and centrifuged at 4° C at 4000 rpm for 15 min., following which the supernatant was careful drained (and discarded) without disturbing the collected material (i.e. pellet) at the bottom of the tube. Samples (pellets) were stored at -20° C until DNA extraction.

For characterizing baseline degradation of eDNA in the slurry, 80 of the 15 ml tubes were prepared as described above and then placed on orbital shakers and shaken at 66 rpm. Tubes were kept at 22° C and in the dark for the duration of the trial. An additional 10 tubes, to be used as negative controls (or blanks), were filled with 14 ml of purified water (no slurry), sealed, and placed on attachable shaker racks. At each sampling point

– days 0, 1, 2, 3, 5, 7, 10, 14, 21, and 28, eight slurry samples and one blank sample were randomly selected for processing and analysis. DNA from each sample (and control) was extracted using a modified cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1987). Eluted DNA from each sample extraction, along with extract from neg- ative controls, was then subjected to 3 replicate qPCRs using primers and a probe devel- oped by the eCALS team for bighead carp (Table 4.4.1), a commercial qPCR kit reagent mix (Table 4.4.2), and a standard qPCR thermal-cycle program (Figure 4.4.5).

Figure 4.4.4. Collection of waste material (filtrate) from bighead carp tanks (not pictured) and samples (tubes) on tube racks.

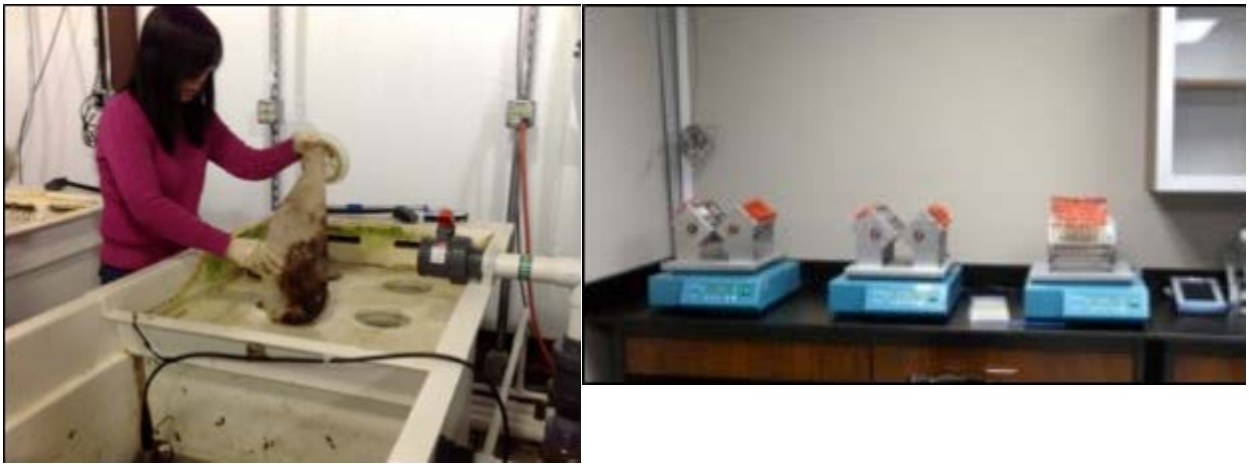


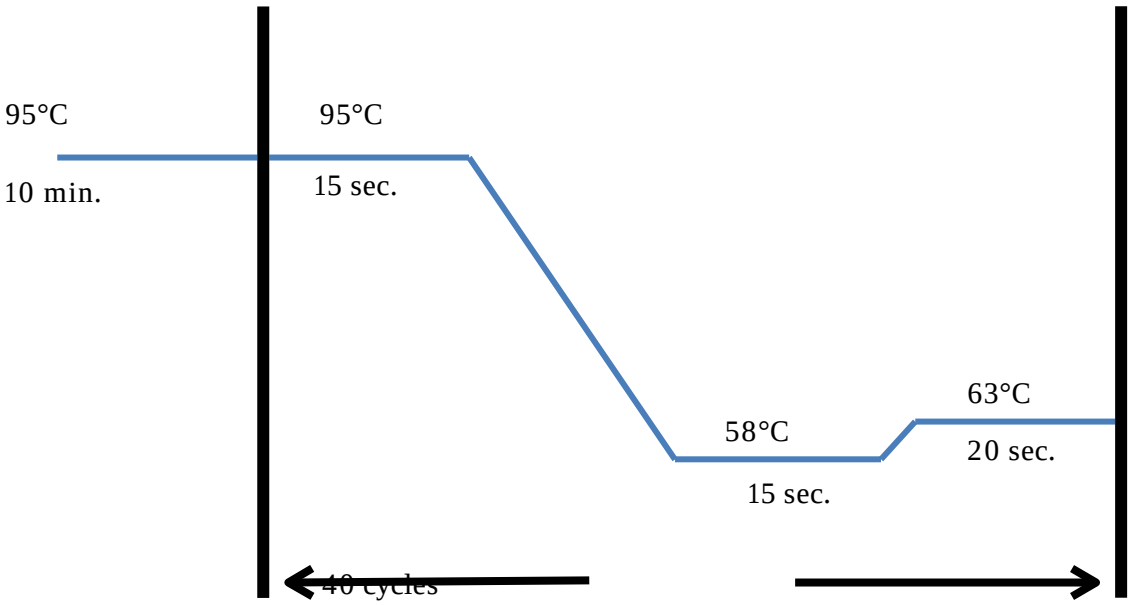
Table 4.4.1. Primers and probe used for qPCR analysis of samples from degradation tasks.

Primer/Probe	ID	Sequence (5'-3')
Forward primer	UMESC-BH-F	GGTGGCGCAAAATGAACTAT
Probe	UMESC-BH-P	FAM-CCCCACATGCCGAGCATTCT-TAMRA
Reverse primer	UMESC-BH-R	GCAAGGTGAAAGGAAACCAA

Table 4.4.2. Taqman qPCR Master Mix (20 µL reaction).

Reagent	Quantity (µL)
2X TaqMan Environmental Master Mix	10
Forward Primer (10 µM)	1
Reverse Primer (10 µM)	1
Probe (2.5 µM)	1
Water	6
DNA Template	1

Figure 4.4.5. Thermal-cycle program utilized for qPCR assays of target DNA abundance in degradation trials.



Characterize Influence of Temperature on eDNA Degradation

Four temperatures levels (4° C, 12° C, 20° C and 30° C) were tested in this trial. Temperature levels were selected to represent the range of water temperatures for the CAWS from 2011 as described in a water quality dataset (Chicago Area Waterways Ambient Water Quality Monitoring Program) made available online by the Metropolitan Water Reclamation District of Greater Chicago (www.mwrd.org). Water temperatures were maintained by placing tubes in temperature-controlled rooms or chambers and allowing

sample temperatures to equilibrate with room temperatures. For each treatment class, 64 tubes were filled with 14ml of fresh slurry mixture (as described above) and 8 with purified water (as described above). Tubes were placed on orbital shakers and shaken at 66 rpm in the dark for 14 days in one of four temperature-controlled rooms. Random subsets of 8 samples and one control blank were removed at each sampling point (days 0, 1, 2, 3, 5, 7, 10, 14) from each temperature class. Following centrifugation and supernatant removal (as described above), samples were stored at -20^{o}C until DNA extraction. Samples were extracted using the CTAB protocol and then assayed with qPCR as described above.

Differences between treatment classes (temperatures) in estimated Asian carp eDNA abundances were analyzed with 2-way analysis of variance with treatment, day, and treatment x day interactions as factors, and with pairwise Tukey Honest Significant Difference calculations (R version 2.15.0; R Development Core Team 2012). This same basic analytical approach was applied in all trials.

Characterize Influence of pH on eDNA Degradation

Four pH classes were selected (6.5, 7, 7.5 and 8.0) for the trial, based on pH ranges observed in the 2011 CAWS water quality data. Fresh slurry was prepared (as described above) for each pH class and pH levels adjusted to target levels using 1M NaOH (unadjusted pH of slurry ≈ 6.5). Five additional tubes were set up for each pH class to serve as pH controls. On a daily basis, pH level was measured in these controls and adjusted with 1M NaOH to return the target level. All remaining slurry samples in each pH class were then amended with the average amount of NaOH required to return their associated controls to the target level. Tubes (n = 64 per pH class) were placed on orbital shakers and shaken at 66 rpm in the dark for 14 days. Random subsets of 8 samples and one control blank were removed at each sampling point (days 0, 1, 2, 3, 5, 7, 10, 14) from each pH class. Following centrifugation and supernatant removal (as described above), samples were stored at -20^{o}C until DNA extraction. Samples were extracted using the CTAB protocol and then assayed with qPCR as described above.

Characterize Influence of Microbial Loads on eDNA Degradation

First Microbial Trial

Prior to executing this subtask we verified that large numbers of viable bacteria were associated with the slurry. In order to obtain a near-zero or very low microbial load class for this trial, we applied a mixed antibiotic solution (100 mg/ml ampicillin and 100 mg/ml kanamycin) to slurry samples (2ml slurry + 12 ml purified water). In an earlier

trial this antibiotic treatment killed a large majority of the microbes in the slurry. In order to augment the microbial loads for high microbial load classes, we added 2 ml of slurry to 15 ml tubes containing either a) 6 ml pond water + 6 ml purified water or b) 12 ml pond water. Pond water was collected from a nearby mesotrophic pond at ERDC that is frequented by turtles, waterfowl, and wading birds among other taxa. In addition to the three microbial load classes described above, a nontreated class (2 ml slurry + 12 ml purified water) was included in the trial.

Tubes (n = 64 per microbial load class) were placed on orbital shakers and shaken at 66 rpm in the dark for 14 days. Random subsets of 8 samples and one control blank were removed at each sampling point (days 0, 1, 2, 3, 5, 7, 10, 14) from each class. Following centrifugation and supernatant removal (as described above), samples were stored at -20^{o} C until DNA extraction. Samples were extracted using the CTAB protocol and then assayed with qPCR as described above. Additionally, at each time point, 3 samples were removed from each class and assessed for microbial load. Microbial load was assessed by removing 50 µl aliquots from each sample tube, plating serial dilutions (10^{0} to 10^{-4}) of these aliquots onto tryptic soy agar (TSA) media and counting colonies after a 24 hour incubation period at 30°C. Colony counts were recorded as colony forming units (CFU) and the average CFU/ml of sample was calculated for each time point.

Second Microbial Trial

Because the different pond water treatments appeared to differ little in their impact on degradation, a second microbial load trial was run. In this second trial, antibiotic solutions were made by adding 1 g of ampicillin and 1 g of kanamycin to 10 ml of deionized water, then filtering the solution through a 0.22 micron syringe filter. Diluted solutions were then made by adding 10 or 20 ml of the antibiotic solution to 1 L of water, generating 10x and 20x antibiotic concentrations, respectively. Treatment setups for this trial were 1) 2 ml slurry + 12 ml water, 2) 2 ml slurry + 12 ml pond water, 3) 2 ml slurry + 12 ml water with 10x antibiotic solution, and 4) 2 ml slurry + 12 ml water with 20x antibiotic solution. Tubes (n = 64 per microbial load class) were place on orbital shakers and shaken at 66 rpm at 22 °C in the dark for 14 days. Random subsets of eight samples and one control blank were removed at each sampling point (days 0, 1, 2, 3, 5, 7, 10, 14) from each class. Additionally, at each time point, three samples were removed from each class and assessed for microbial load by removing 50 µl aliquots from each sample tube, plating serial dilutions (10^{0} to 10^{-4}) of these aliquots onto tryptic soy agar (TSA) media and counting colonies after a 24 hour incubation period at 30°C. Colony counts were recorded as colony forming units (CFU) and the average CFU/ml of sample was calculated for each time point.

Characterize Influence of Water Energy (Turbulence) on eDNA Degradation

We designed this trial with four different water energy classes (0, 66 rpm, 132 rpm and 200 rpm) based on the range of shaking speeds that could be achieved with our orbital shakers. Tubes (n = 64 per microbial load class) for each treatment level were placed on four identical orbital shakers and shaken at 22 °C in the dark for 14 days at speeds identified above. Random subsets of 8 samples and one control blank were removed at each sampling point (days 0, 1, 2, 3, 5, 7, 10, 14) from each class. Following centrifugation and supernatant removal (as described above), samples were stored at -20° C until DNA extraction. Samples were extracted using the CTAB protocol and then assayed with qPCR as described above.

Characterize Influence of Light Exposure on eDNA Degradation

Because of the need for equivalent exposure of all samples to limited light sources and the potential for polypropylene tube walls to block some wavelengths of light, the experiment was carried out in four 15 cm W x 30 cm L x 20 cm H open-top aquarium tanks (Figure 4.4.6). Tanks were filled with 3 liters of water and 500ml of eDNA slurry with an exposed surface area of 14 cm x 29 cm Beside each tank was a 250 ml sealed bottle of water that served as a negative control. The tanks were enclosed in individual 66 cm W x 66 cm L x 86 cm H dark chambers constructed with PVC pipe and black plastic wrap to block extraneous light. Four durations of light exposure (9, 11, 13, and 15 hours per 24 hours) were used as treatments in this trial to mimic seasonal variation in daylight hours near the CAWS. Inside each chamber one 30-watt, 80% lumens full-spectrum fluorescent bulb was suspended 58.5 cm above the water surface and set to a timer to control light exposure duration. Tanks rested on timer-controlled stir plates, which were programmed to continually cycle between 90 minutes resting and 30 minutes stirring. The room temperature was maintained at 22 °C.

Characterize Combined Influence of Temperature, pH, and Microbial Loads on eDNA Degradation

Temperature, pH, and microbial loads all appeared to affect the amount of DNA persisting at different time points and in consistent patterns during the preceding trials. In order to characterize how a combination of these factors might influence degradation rates we ran a long-term trial with three treatment classes: 1) the slow degradation class was treated with low temperature, low pH, and low microbial loads, which we expected to result in relatively slow degradation, 2) the baseline degradation class was treated with room temperature, unadjusted pH, and unadjusted microbial loads, which we expected to exhibit degradation similar to the baseline we originally observed, and 3) the rapid

degradation class was treated with a high temperature, high pH, and high microbial loads, which we expected to result in relatively rapid degradation.

Figure 4.4.6. Experimental setup for light exposure trial.

A fresh stock of slurry was prepared (as above) and 350 ml was aliquoted into 2100 ml of either sterile water (slow and baseline degradation classes) or pond water (rapid degradation class; same pond as used in earlier microbial load trials). The slow degradation class solution were treated with 49 ml of a 1000X mixed antibiotic solution (100 mg/ml ampicillin and 50 mg/ml kanamycin)) solution (= 20X antibiotic treatment from the second microbial load trial). The pH of the slow degradation solution was adjusted to 6.5 and the rapid degradation solution to 8.0 using 1 N HCl and I N NaOH solutions. For each treatment class, a total of 141 15-ml screw-top polypropylene tubes were filled with 14 ul of solution from prepared for this trial and capped. A total of 51 15-ml tubes were filled with 14 ml of water to serve as negative control (or water blank) tubes. Five tubes within each treatment class were set aside as "pH indicator" samples. Eight tubes from each treatment class (n = 24) were randomly selected as Day 0 samples. The remaining 133 tubes from the slow degradation class were then placed in a 4^{o} C chamber and the 133 rapid degradation tubes in a 30^{o} C chamber. The remaining 131 tubes for the baseline degradation class were maintained at room temperature (22^{o} C). Because daily pH checks and adjustments were not feasible, the pH indicator tubes were used as models for maintaining pH levels for the slow and rapid treatment classes and for tracking pH levels in the baseline class. The pHs of indicator samples were measured daily and

adjusted back to the target level. The average volume of HCl or NaOH required to bring indicator samples down or up to target levels in each class was then added to all remaining samples in that class. This process occurred every day until pH was observed to stabilize over several days, after which pH checks were made every 2 days. The pH levels of the baseline class indicator samples and other samples were not adjusted, only tracked. On each of the 17 sampling dates, three tubes were randomly selected from among the eight sample tubes randomly selected for removal. Before DNA extraction, 50 µl aliquots were removed from each of the three tubes and used to assess microbial loads. We plat- ed serial dilutions (10^0 to 10^{-4}) of these aliquots onto tryptic soy agar (TSA) media and counted colonies after a 24 hour incubation period at 30°C.

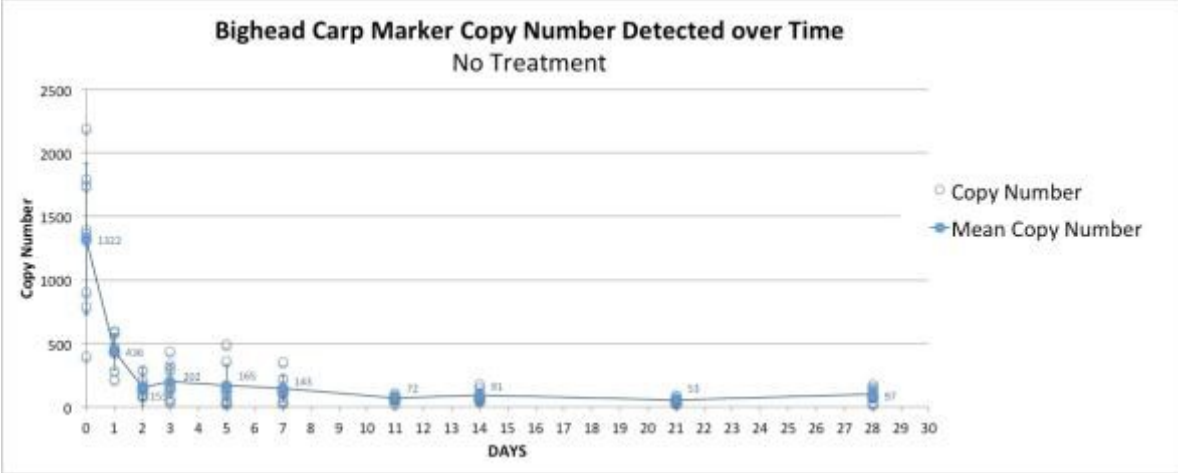
Random subsets of 8 samples and one control blank were removed at each sampling point (days 0, 1, 2, 3, 5, 7, 10, 14, 21, 28, 35, 42, 49, 56, 63, 77, and 91) from each treatment class. Following centrifugation and supernatant removal (as described above), samples were stored at -20°C until DNA extraction. Samples were extracted using the CTAB protocol and then assayed with qPCR. Instead of using the qPCR marker UMESC-BH, we used a qPCR marker developed by ERDC, BHTM-1 (Ch. III), because BHTM-1 appeared to provide potentially more accurate DNA estimates than UMESC-BH.

Results

Baseline eDNA Degradation

DNA degraded quickly under baseline conditions (Figure 4.4.7), with about a 70% reduction after one day and a 90% reduction after 2 days. Of interest is the persistence of a small portion (around 4-7%) of Asian carp eDNA through the end of the 28-day trial. Also of interest is the appearance of a slight increase in the amount of eDNA from Day 2 to Day 3, from Day 11 to Day 14, and from Day 21 to Day 28. Some of this discrepancy, especially earlier in the trial, could be a function of the large variances around the mean copy numbers. However, the Asian carp slurry used in these trials likely has a number of co-occurring PCR inhibitors, including those typically associated with fecal material (bile salts and complex polysaccharides). Unexpected patterns in the degradation curve could be a result of the breakdown of PCR inhibitors over time, allowing for different PCR efficiencies and yields for different time points, and the appearance of increasing DNA abundances (but really just more effective PCR). The role of PCR inhibitors in our trials is currently being examined.

Figure 4.4.7. Degradation of DNA from Asian carp slurry over 28 day span. The slurry was comprised of mixed filtrate (slime, feces, food, etc.) from tanks holding bighead carp.



Characterize Influence of Temperature on eDNA Degradation

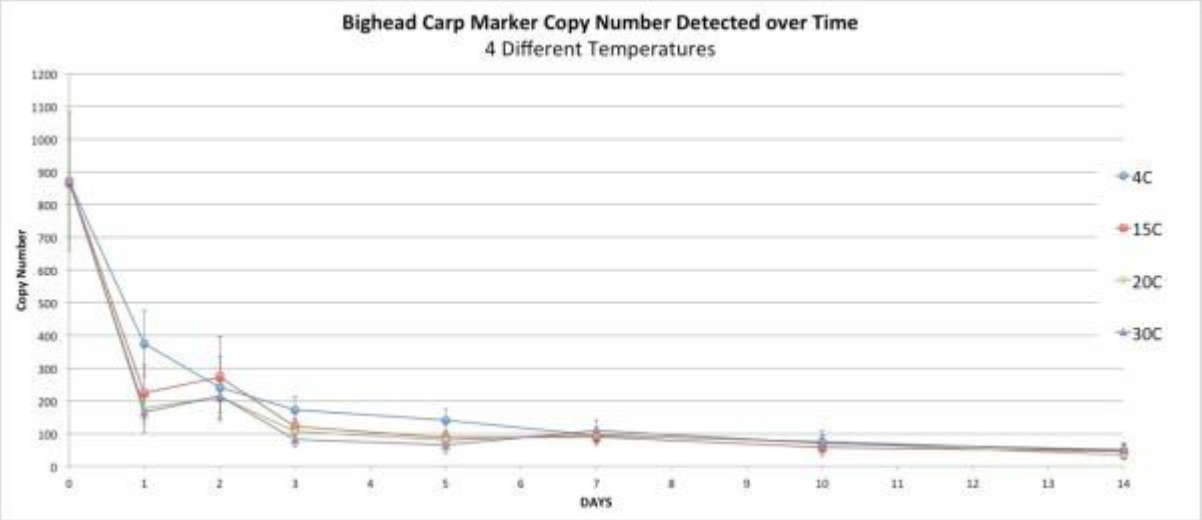
DNA degraded quickly under all four temperature treatments (Figure 4.4.8), with about a 70% reduction after one day, and nearly 90% reduction after three days. Degradation was significantly different ($p < 0.05$) across temperatures (Table 4.4.3), with a strong trend for slower degradation (more DNA across time) in the 4° C class and more rapid degradation in the 30° C class.

An apparent increase in DNA abundance on Day 2, likely due to changing concentra- tions of PCR inhibitors (not actual DNA amounts), partly obscures the general pattern of declining DNA abundance over time. If this is the case, the lack of any upward spikes in apparent DNA abundance in the 4° C class seems to indicate that inhibitors are not de- grading at this temperature. Differences in DNA abundance between this temperature class, in which PCR is inhibited, and the other 3 classes (15, 20, and 30° C), which may be inhibited to a much lesser degree, may therefore be artificially small -- the amount of DNA in the 4° C trials may actually be greater than what we observed, while, after Day 2, the amount of DNA estimated in the other classes may be closer to reality. Further tests of inhibition in these samples and chemical analysis of similar samples should shed some light on that hypothesis.

In any case, the rapid initial decline in intact DNA is consistent with no-treatment trial results. Likewise, the pattern of a small portion of eDNA persisting over long periods,

which occurred across the range of temperatures we tested, is also consistent with no-treatment trial results.

Figure 4.4.8. Degradation of DNA from Asian carp slurry over 14 day span at 4 different water temperatures. DNA abundance for the 4° C (4C) treatment class at Day 1 was significantly greater than any other temperature class, while DNA abundances for the 4° C treatment class were significantly greater than DNA in the 30° C (30C) treatment class on Day 3 and Day 5.



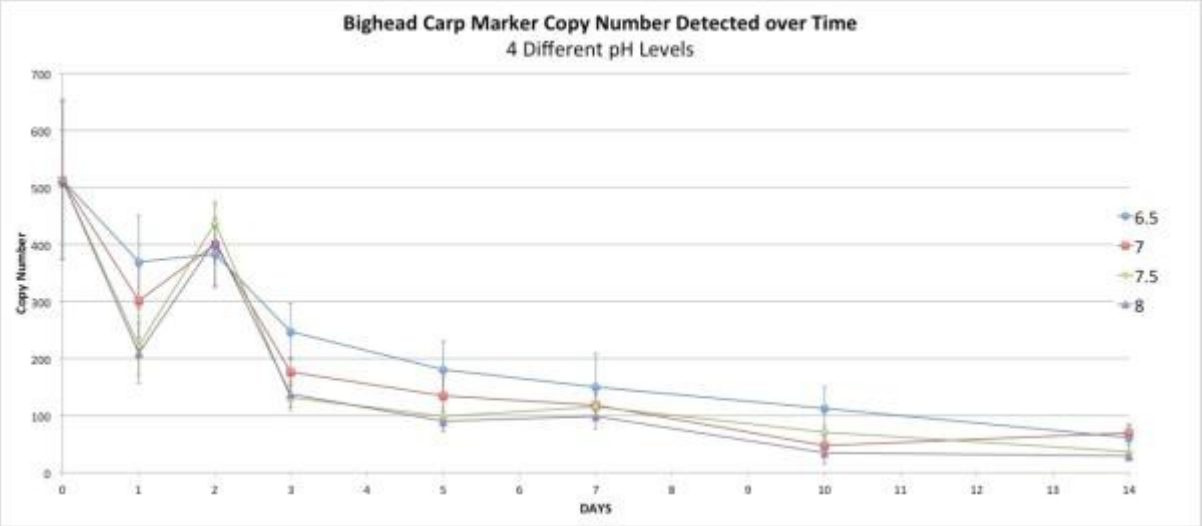
Characterize Influence of pH on eDNA Degradation

DNA degraded less rapidly in these trials (Figure 4.4.9) than in the trials described above, assumedly due to differences in contents or attributes of the slurry. The pH = 6.5 treatment class required little or no amendment to achieve the target pH, while the other classes frequently required daily amendments with NaOH. Each treatment showed the same basic pattern of degradation, with DNA losses at Day 1 ranged from 28% (6.5 class) to 59% (8.0 class), and overall losses of 88% (6.5 class) to 94% (8.0 class) on Day 14. All treatments evidenced a considerable spike in apparent DNA abundance at Day 2, Overall, pH significantly influenced degradation ($p = 0.004$), and in particular, the pH class 6.5 exhibited significantly slower degradation than the pH classes 7.5 ($p = 0.024$) and 8.0 ($p = 0.003$).

Table 4.4.3. List of temperature classes with significantly different ($p < 0.05$) mean estimates of DNA abundance across 14 days of degradation. Significant differences between means determined by Tukey's Honest Significant Difference Test.

Day	Higher Mean Estimate	Lower Mean Estimate
1	4° C	12° C
	4° C	30° C
3	4° C	12° C
	4° C	20° C
	4° C	30° C
	12° C	30° C
5	4° C	30° C
	12° C	30° C
10	4° C	30° C
	4° C	20° C
	12° C	20° C
	12° C	30° C
14	4° C	20° C
	4° C	30° C

Figure 4.4.9. Degradation of DNA from Asian carp slurry over 14 day span at 4 different pH levels. DNA abundance for the pH = 6.5 treatment tended to degrade at a slower rate than the other treatment classes.



Characterize Influence of Microbial Loads on eDNA Degradation

DNA degraded very rapidly in the first trial (Figure 4.4.10) with apparent 81-96% reductions by Day 1. However, these Day 1 estimates are clearly affected by PCR inhibition, as there are apparent eDNA recoveries on subsequent days, including the second highest estimates (after Day 0) much later at Day 10. The actual degree of degradation was, at most 82-90% (Day 10 values), but almost certainly much less. Microbial loads were not a significant factor in apparent degradation results ($p = 0.655$).

The ampicillin-kanamycin treatment class had a much smaller estimated microbial load relative to the other treatment classes (Figure 4.4.11). The addition of pond water, in contrast, had only a small impact on microbial loading relative to the observed indigenous load in slurry. A typical challenge with estimating microbial loads is that some microbial constituents grow better than others in different media and this can bias observations on relative microbial abundances and diversities. Here, the relative contributions of slurry and pond water to microbial loads may or may not be influenced by such biases, but as we utilized the same starting materials across treatments, we as- sume inter-treatment biases to be minimal.

In Trial 2, initial degradation appears to be similarly rapid, at least for the pond-water and untreated samples (Figure 4.4.12). The 10X and 20X antibiotic treated samples show a similar decline, but an apparent recovery to near starting levels of DNA on Day 7

indicates that PCR inhibitors likely exerted a strong downward bias in DNA estimates during those periods. The pond water and untreated samples never exhibited a spike in DNA estimates on the same scale and our assumption is that the general trend seen for these samples is accurate. Over the course of 14 days, 84-92% of the total DNA appeared to have been degraded in the pond water and no treatment samples, whereas only about 62% and 36% of the DNA appeared to be degraded in the 10X and 20X antibiotic treated samples, respectively. The general trends for microbial counts in Trial 2 (Figure 4.4.13) seem to largely track the DNA trends, with the pond water and no treatment samples typically showing the highest counts for most of the trial (and highest total DNA degradation), while the 10X and 20X antibiotic treated samples exhibited, with the exception of the 1oX samples on Day 14, the lowest cell counts (and relatively less total DNA degradation). Notably, the 20X samples had significantly lower bacterial estimates and significantly less total degradation.

Figure 4.4.10. Degradation of DNA from Asian carp slurry over 14 day span at 4 different microbial load levels. Trial 1 with two different pond water treatments.

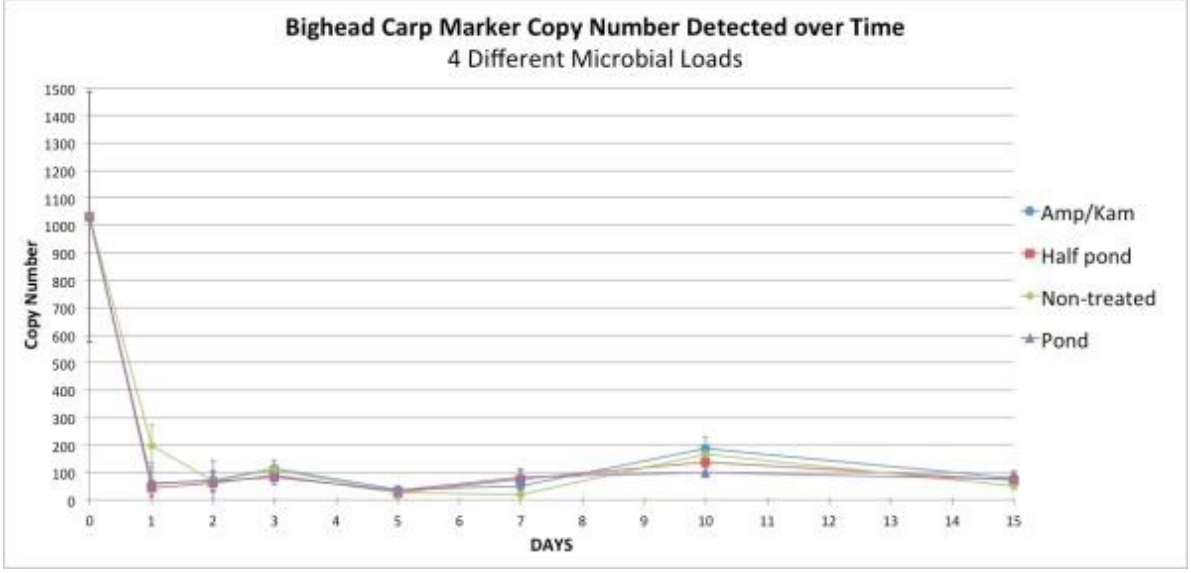


Figure 4.4.11. Differences in microbial loads (colony forming units) across treatments and days. Trial 1 with two different pond water treatments.

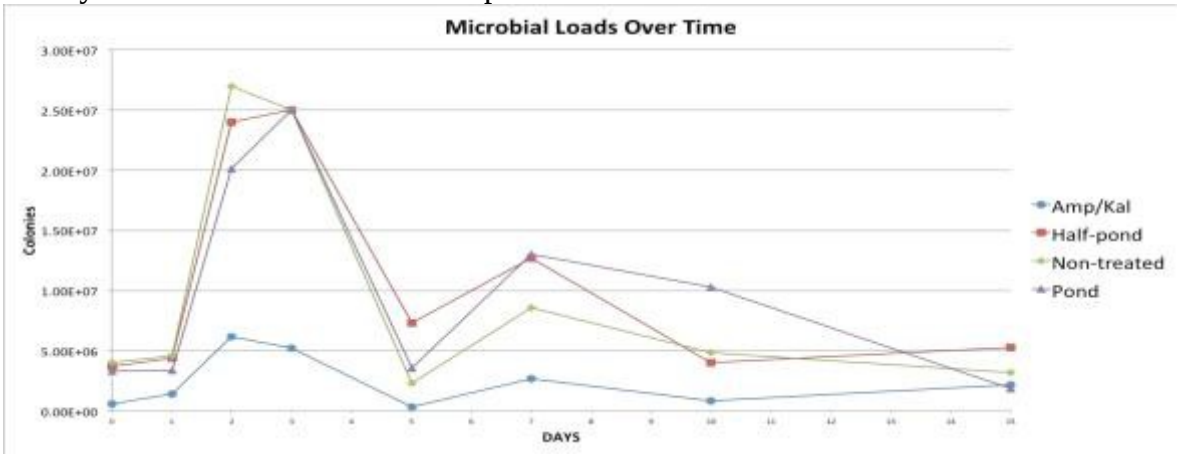


Figure 4.4.12. Trial 2, degradation of DNA from Asian carp slurry over 14 day span at 4 different microbial load levels.

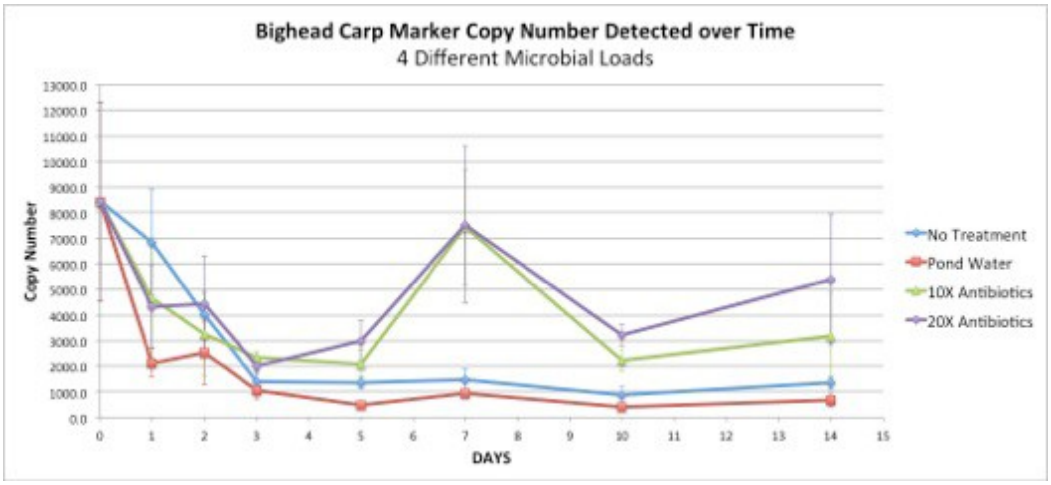
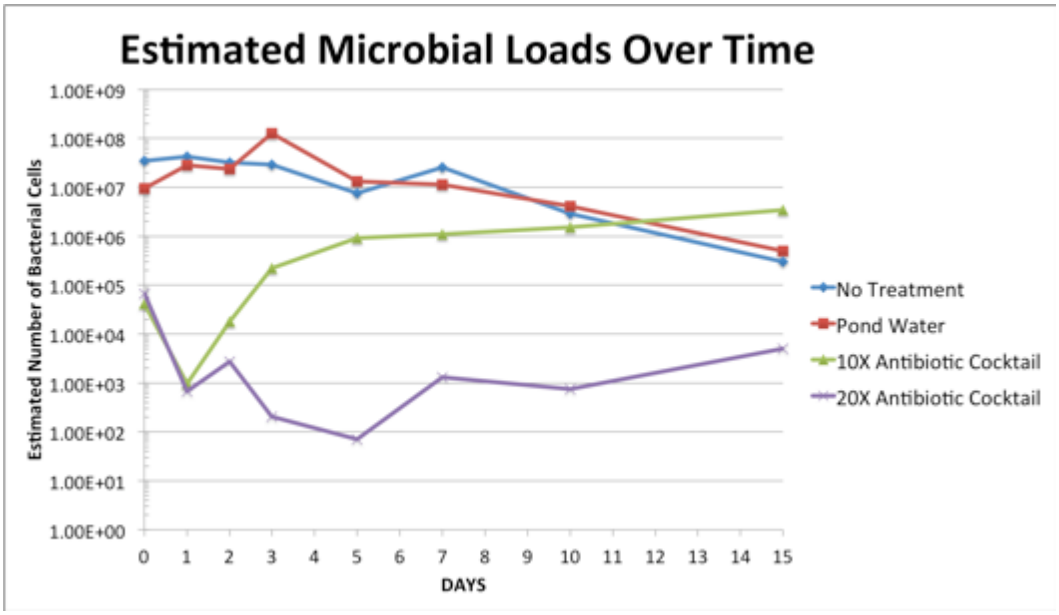


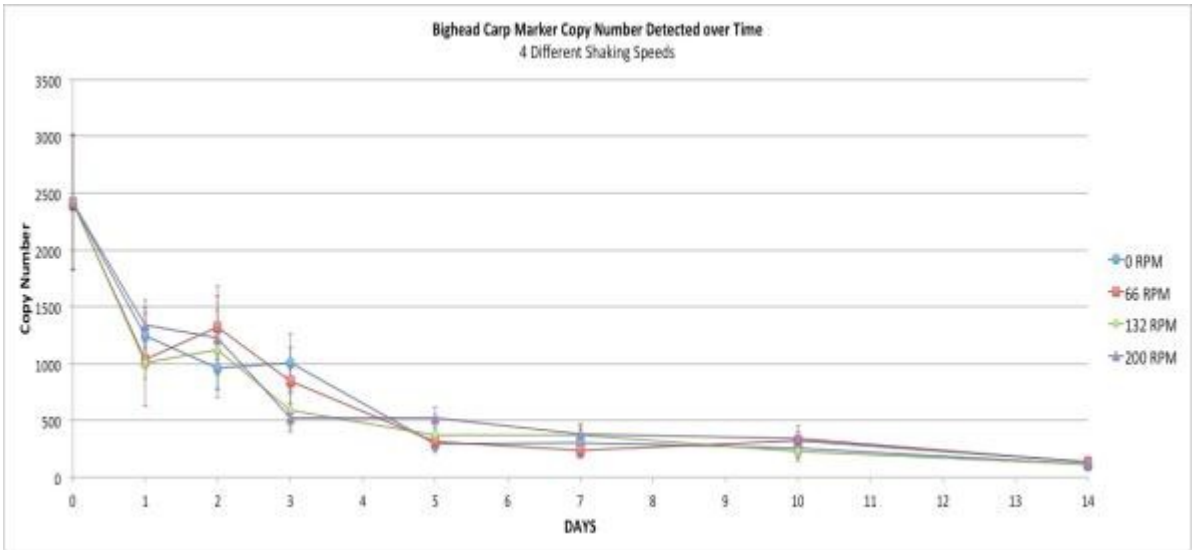
Figure 4.4.13. Trial 2, differences in microbial loads (colony forming units) across treatments and days.



Characterize Influence of Water Energy (Turbulence) on eDNA Degradation

DNA degraded less rapidly in this trial (Figure 4.4.14) than in some other trials, again, likely due to differences in contents or attributes of the slurry. By Day 1, 44-58% of DNA had degraded. By Day 14, 94-95% of DNA had degraded, but the apparent increase in DNA at Day 2 would indicate that, as with other trials, the Day 1 estimates were likely affected by PCR inhibition and actual DNA degradation losses were lower. By Day 14, 94-95% of DNA had been lost to degradation. The rate at which samples were shaken did not significantly impact the rate of degradation ($p = 0.958$).

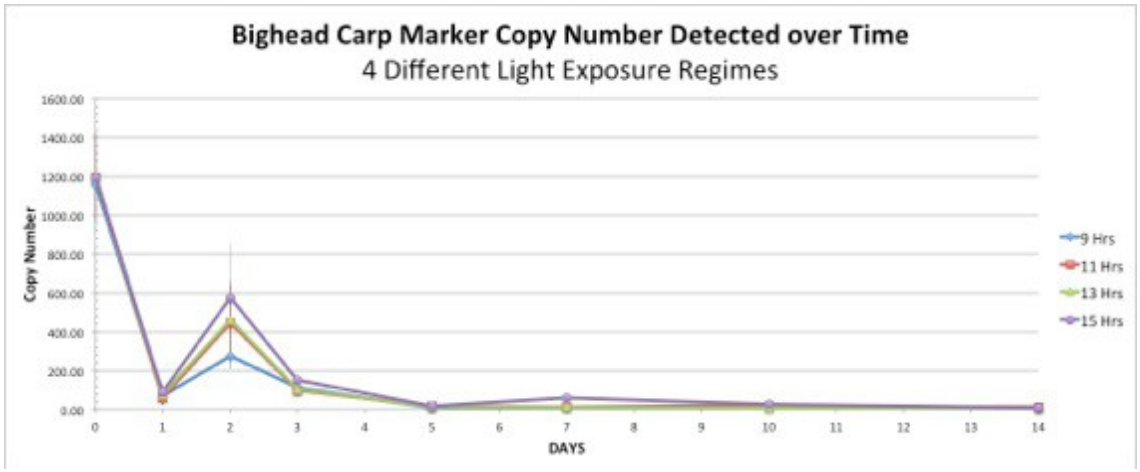
Figure 4.4.14. Degradation of DNA from Asian carp slurry over 14 day span at 4 shaking speeds on orbital shakers.



Characterize the Influence of Light Exposure on eDNA Degradation

We observed no significant difference in degradation of eDNA in samples experiencing light exposures ranging from 9hrs/day to 15 hrs/day for 14 days (for a 210 total hour exposure; Fig. 4.4.15). The general pattern of DNA degradation was similar to that in other trials with rapid initial degradation followed by slower rates and DNA persisting beyond the end of the experiment. The lack of any observed effect of light exposure on DNA degradation may have been due the relatively very low energy incidence of light from the full spectrum bulb relative to the energy incidence of sunlight at ground level (6-7 orders of magnitude less energy based on visible light measurements). Measures of relative energies in the UV range are forthcoming.

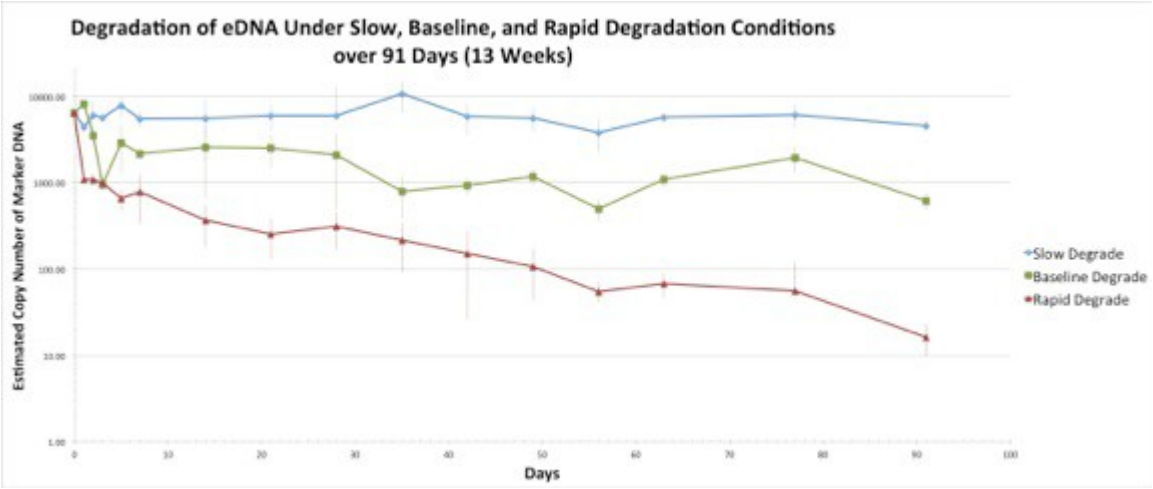
Figure 4.4.15. Degradation of DNA from Asian carp slurry over 14 day span with different length exposures to full spectrum light.



Characterize Combined Influence of Temperature, pH, and Microbial Loads on eDNA Degradation

As expected, DNA degraded more quickly under the rapid degradation treatment than in the untreated samples and under the slow degradation treatment (Fig. 4.4.16). After a total of 91 days, only about 30% of the DNA was lost under the slow degradation experiment, whereas approximately 90% of the DNA was lost in the untreated samples, and over 99% was lost under the rapid degradation treatment. Because of the variability in DNA estimates from sampling point to sampling point within each treatment, points where shifts in degradation rates occurred were identified based on identifying days where DNA estimates appeared to represent central tendencies in the data. Based on this approach, the rapid degradation samples exhibited a steep decline within a day of the start of the treatment (Day 0 to Day 1 ≈ 90% DNA loss), while the untreated samples exhibited a rapid decline over about 5 days (Day 0 to Day 5 ≈ 66% DNA loss). The slow degradation samples appeared to only undergo an even lower degree of degradation (Day 0 to Day 3 ≈ 5%). After initial declines, degradation rates slowed considerably, with the rapid treatment samples losing approximately 16% more of their DNA over 90 days (~ 5-6% of remaining DNA lost per day), the untreated samples losing an addition- al 24% of their DNA over 86 days (~1-2% of remaining DNA lost per day), and slow deg- radation samples losing an additional 24% of their DNA over 88 days (~0.3-0.4% of remaining DNA lost per day). Even after 91 days, all trials exhibited some remaining DNA.

Figure 4.4.16. Degradation of DNA from Asian carp slurry over 91 day span with three different treatments: 1) slow degrade – low pH, low temperature, low microbial loads, 2) baseline degrade – no pH adjustment, room temperature, no microbial manipulation, and 3) rapid degrade – high pH, high temperature, high microbial loads.



Discussion

Degradation rates in our trials typically exhibited similar patterns to those observed in Thomsen et al. (2011) where eDNA abundance declined rapidly over the course of only a small number of days. Likewise, if we use the conventional PCR markers currently employed for Asian carp eDNA monitoring (Jerde et al. 2011) that require on the order of 10-200 copies of target DNA per microliter of extracted DNA solution for successful detection (Jerde et al. 2012), we would have observed a gradual decrease in detections over the course of a number of days, as seen in Dejean et al. 2011. However, in our trials we also observed a recalcitrant portion of DNA, often as much as 10%, that persisted beyond the length of our trials. In by far the longest running experimental trial of degradation of eDNA–types samples (the longest trial to date was Dejean et al. 2011, where they tracked eDNA detections over 1 month), our combined factors trial ran for 91 days (13 weeks). Even over this much longer period, a recalcitrant portion of DNA persisted beyond the length of the trial. The portion of DNA that is recalcitrant is clearly affected by environmental factors. The capacity of a slowly degrading fraction of eDNA to accumulate over time or to be protected from degradation through binding with other substrates is of particular importance to developing both conceptual or predictive models of eDNA behavior.

Within the scope of our study design, water temperature, pH, and microbial loads appeared to significantly influence degradation rates. Warmer water, higher pH (within the range of 6.5 to 8.0), and greater microbial abundance were associated with higher

degradation rates. These factors are likely interdependent, as the slurry we used appeared to contain significant endogenous microbial activity (added microbial biomass from a local pond did not significantly increase degradation, but antibiotics significantly lessened degradation) and microbial activity is significantly affected by water temperatures. The levels of pH likely also affect microbial impacts on eDNA, for instance by affecting the molecular interactions between extracellular enzymes (e.g. exonucleases) and target substrates (e.g. nucleic acids, cell membranes). For example, microbes are believed to rapidly degrade free-floating DNA in environmental waters and factors that impact this activity could significantly influence the amount of eDNA available for detection.

DNA is generally stable at moderate pH, though in our case we appear to see an increase in degradation at higher pH. Lower pHs may increase the binding affinity of DNA molecules to other environmental molecules or particles, such clays, humic acids, or fulvic acids (reviewed in Levy-Booth et al. 2007). Particle-bound DNA is protected from nucleases and degraded more slowly than unprotected DNA (Ogram et al. 1998, reviewed in Trevors 1996).

We assume that all or nearly all of the DNA in our samples is "extractable" using the well-vetted, widely-used CTAB protocol we employ, and that it is highly unlikely that the spikes of DNA we observed in Days 2 or 3 (and Days 7 or 10 in some cases) could be explained by "troves" of DNA that only became extractable and detectable after a few days. A more likely scenario is that changing concentrations of PCR inhibitors in the samples result in changing PCR efficiencies, and that the spikes have little to do with the actual changes in target DNA abundance. In such a scenario, as inhibitor concentrations decrease over time, qPCR efficiency increases, and estimates of DNA abundance increase in magnitude (and become more accurate). In some cases, the improved qPCR efficiency would result in DNA estimates greater than those from preceding, more inhibited sampling points. The potential role of inhibition in causing this pattern is currently being studied.

There are many different classes of substances that can interfere with PCR (Bessetti 2007), including some, like bile salts and complex polysaccharides, that are associated with fecal material (which comprises the bulk of our slurry). Ideally, these inhibitory compounds would be eliminated when DNA is extracted from samples and then isolated into a purified solution. However, extraction protocols commonly achieve less than 100% purification of DNA and compounds other than nucleic acids (e.g. inhibitory compounds) are often found in final DNA solutions. In a related scenario, extremely high levels of DNA and other nucleic acids can be inhibitory to PCR (or qPCR) – a sort of self-inhibition. In such a case, as DNA in solution degraded over time, qPCR from later sam-

pling points would become increasingly less inhibited, resulting in the same effect as described above. Because DNA extraction and purification protocols often have very low DNA recovery rates (e.g. 30% or less), the seemingly simple solution of undertaking additional purification efforts to more fully remove inhibitory cofactors could result in significant risks of reducing target eDNA to undetectable levels.

We surmised that if the apparent spikes in DNA abundance are the result of changing concentrations of PCR inhibitors then such changes would be due to some combination of microbially-mediated degradation and abiotic breakdown of inhibitory molecules. However, as microbial load had little apparent effect on the occurrence or magnitude of the spikes on Day 3 and Day 10 of our first microbial load trials (Figure 4.4.8), and that the microbially-depauperate 10X and 20X antibiotic treatment in second microbial load trial were the only samples to show the peak on Day 7, we now suspect that abiotic breakdown of inhibitors may be the primary factor driving this pattern. However, an initial assessment of the chemical characteristics of slurry at time points with and without peaks revealed no significant differences in chemistry.

Summary

We have completed trials of five different factors believed to possibly influence eDNA degradation, as well as a trial where eDNA-type material was exposed to a combination of some of these factors. We have, in each trial, observed an unexpected effect -- apparent DNA abundance spikes that are observed on Days 2 or 3, and in some cases Days 7 or 10 -- that we attribute to changing concentrations of qPCR inhibitors, as opposed to actual increases in DNA abundance. Identifying a mechanism to explain the DNA spike has been a challenge. Nonetheless, degradation rates and patterns observed in our studies are similar to those observed in the few eDNA degradation results published to date in that degradation is initially quite rapid, but that some DNA persists for longer periods. We have identified temperature, pH, and microbial load as factors that can affect degradation, particularly that higher temperature, higher pH, and higher microbial loads are associated with more rapid DNA degradation. In every case, DNA abundances capable of producing positive detections with qPCR or conventional PCR assays persisted beyond the length of trials (14, 15, 28 days, or 91 days).

4.5 Validation Trials

Artificial stream and outdoor mesocosm activities are scheduled to begin 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

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 4.6.1). 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 dur- ing 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.

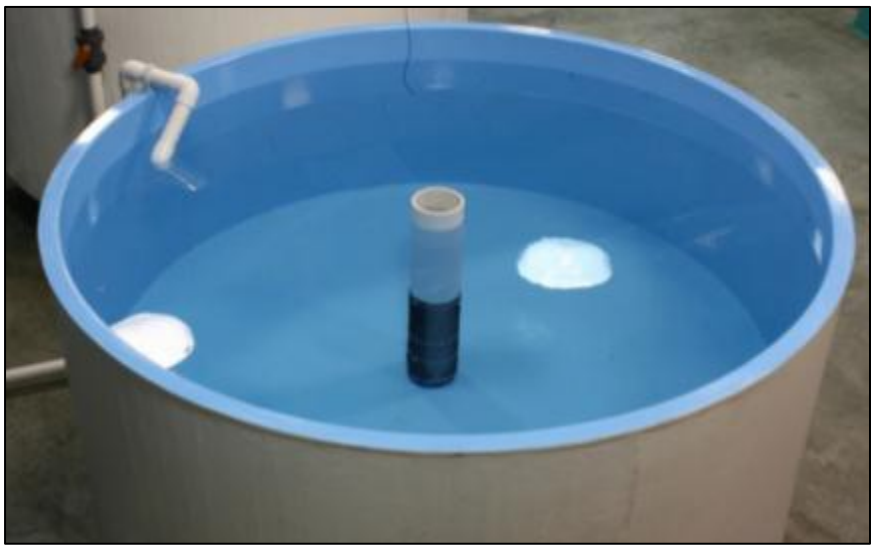
Holding Facilities – Fish are maintained in a secure laboratory facility (Figure 4.6.2) at ERDC with a closed-system of individual re-circulating tanks (Figure 4.6.3). The laboratory is approximately 400 m^2 in a building that is approximately 1672 m^2. A generator is automatically started during local electrical failures to ensure that 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 aq-

uaculture use; water leaving the building enters the municipal sewage system and receives tertiary treatment.

Figure 4.6.1. Transportation truck with aerated live wells.

Figure 4.6.2. Laboratory building where silver and bighead carp are housed.

Figure 4.6.3. 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 4.6.4). The inner 3" stand pipe is elevated 42" to protect from complete tank drainage. All tanks are re-circulating. There are two tanks per filtra- tion 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 4.6.5). 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 4.6.4. 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.

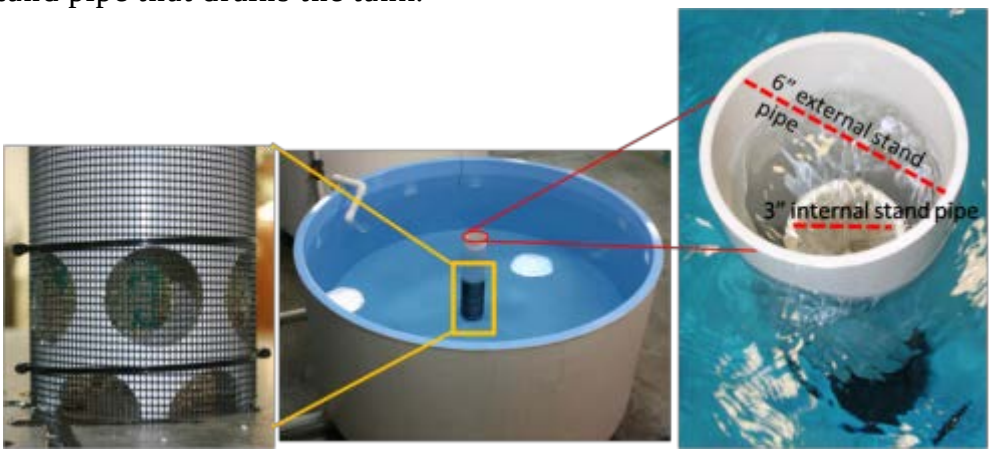
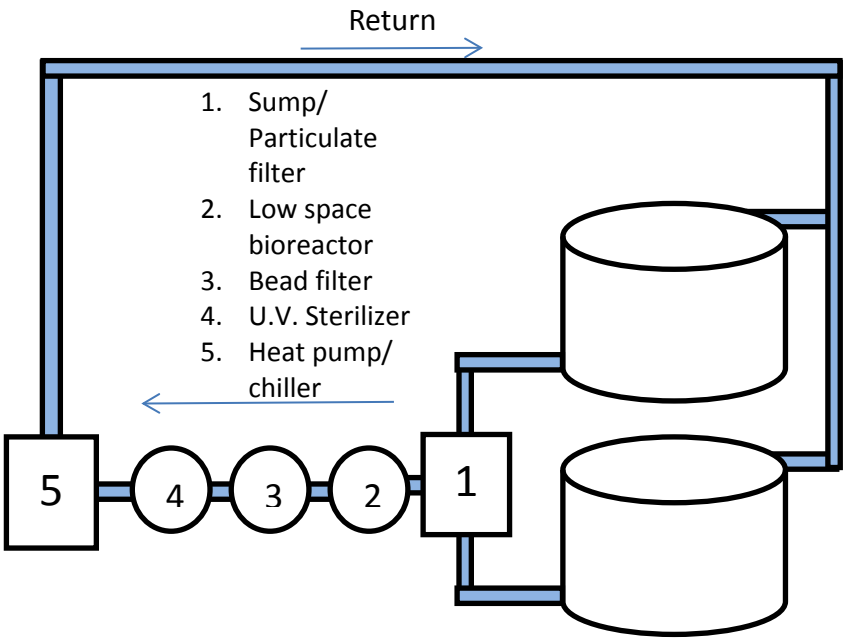


Figure 4.6.5. Recirculating aquaculture system flow drawing.



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 Probability Modeling

Activities are presently in progress and will be reported in December 2014 in separate report. Refer to section 2.1.2 for preliminary work.

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APPENDIX A

Table A.1: Description of variables in the conceptual model of eDNA occurrence and persistence. This information is preliminary and subject to change.

Variable	Description
ADS	Adsorption rate of eDNA marker to suspended sediment. This variable is defined as a discretized continuous random variable between 0 and 1. The information is being developed through ECALS studies on how eDNA interacts with sediment.
ASEG	Cross-sectional area of the reach (m^2). This is a discretized continuous random variable greater than or equal to 0. This information will be developed from information about stream geometry and hydrology as represented in ECALS CH3D model of the CAWS.
ASSIM	Assimilation rate of birds (kcal/day). This variable is defined as a discretized continuous random variable. This information will be developed by consulting peer-reviewed literature sources.
BBILGE	The number of eDNA markers imported in commercial boat and barge bilge water (copies/day). This variable is defined as a discretized continuous random variable greater than or equal to 0. This variable is calculated from BSAREA, BTRAF, UPFRACT, and CBILGE.
BDTY	Bird density in the reach (m^{-2}). This variable is defined as a discretized continuous random variable greater than or equal to 0. The Cornell Lab of Ornithology's eBIRD database (http://ebird.org/content/ebird/) contains voluntary reports of bird sightings and provides one potential source of information about the relative densities of birds along the CAWS.
BHULL	The number of eDNA markers attached per unit area of commercial boat and barge hulls (copies/m^2). This variable is defined as a discretized continuous random variable greater than or equal to 0. This node is parameterized using results of ECALS studies of eDNA on commercial boat and barge hulls.
BHULLS	The number of eDNA markers imported on commercial boat and barge hulls (copies/day). This variable is defined as a discretized continuous random variable greater than of equal to 0. The variable is calculated from BSAREA, BHULL, and BTRAF using information about the rate at which eDNA markers may be washed off hulls in transit. ECALS studies suggest that eDNA adhering to commercial boat and barge hulls is not easily washed off during transit.
BIL	Load of eDNA markers contributed to the reach by birds (copies/day). This variable is defined as a discretized continuous random variable greater than or equal to one. This variable is calculated from DROP and ROOK.
BIOM	Biomass of the supposed population of Asian carp in the reach (kg). This variable is defined as a discretized continuous random variable greater than 0. It is calculated from POP, FLEN, and a species-specific length-weight relationship.
BIRDS	Piscivorous bird species composition in the CAWS. This variable is defined as a discretized continuous random variable with at least four possible states (*Cormorants*, *Bald eagles*, *Pelicans*, *Other*). This information is based on reported bird sightings in the CAWS.
BP	Length of the genetic marker in nucleotide base pairs (bp). This variable is a constant given knowledge of which genetic marker for Asian carp is being tracked in the CAWS.
BSAREA	Surface area of commercial barges and boats (m^2/day). This is a discretized continuous random variable. The information is based on knowledge of commercial boat and barge characteristics in the waterway.
BTRAF	The number of commercial boats and barges transiting through a segment of the CAWS on any give day (boats and barges/day). This is a discretized continuous random variable. The information is based on records of commercial boat and barge movements.
CARC	Load of eDNA marker contributed from carcasses on barge decks (copies/day). This is a discretized continuous random variable greater than or equal to 0. The information is based on reports of carcasses on barge decks at USACE operated locks and dams on the waterway.
CATCH	The probability that a fish from the supposed population within the reach is caught. This is a discretized continuous random variable greater than or equal to 0. The variable is calculated from UEFFORT, POP, and Q.
CBILGE	Concentration of eDNA in bilge water of commercial boats and barges travelling upstream in the CAWS (copies/L). This variable is defined as a discretized continuous random variable greater than or equal to 0. ECALS presently has limited information on this variable.
CBL	Load of eDNA marker contributed from commercial boat hulls (copies/day). This variable is defined as a discretized continuous random variable. The variable is calculated from BHULLS, BBILGE, and CARC.
CHULL	Number of eDNA markers per unit area of commercial boat and barge hulls (copies/m^2). This variable is defined as a discretized continuous random variable. The information is obtained from ECALS studies on eDNA attached to commercial boat and barge hulls.
CNL	Load of eDNA originating from commercial fishing nets (copies/day). This variable is defined as a discretized continuous random variable. The variable is calculated from EFFORT and UNL.
COPKG	Copies of eDNA marker per kilogram of fish tissue (copies/kg). This variable is defined as a discretized continuous random variable with lower and upper bounds to be determined. The value is calculated based on information about the eDNA content of fish tissue.
CSEG	Concentration of an eDNA marker (copies/L) in a reach. CPTs can be calculated or derived from the outputs of ECALS eDNA fate and transport model in the CAWS.
CSOL	Load of eDNA markers contributed to the reach from combined sewer outfalls (copies/day). This variable is defined as a discretized continuous random variable greater than or equal to 0. This variable is calculated from CSOQ and FMKT.

CSOQ	Reach inflows from CSO discharges (m^3/day). This variable is defined as a discretized continuous random variable greater than or equal to 0. The variable is estimated from PRECIP and DAREA using relationships developed by MWRD and incorporated into ECALS CH3D model of the CAWS.
DAREA	Drainage area to the reach (m^2). This variable is defined as a discretized continuous random variable greater than or equal to 0. The information is based on data provided by MWRD and used in developing ECALS CH3D model of the CAWS.
DEP	Deposition rate of sediment from the water column to the sediment layer (day^{-1}). This variable is defined as a discretized continuous random variable greater than or equal to 0. The value is calculated from GSIZE.
DROP	Quantity of eDNA marker excreted per day (copies/day). This variable is defined as a discretized continuous random variable greater than or equal to 0. The value is calculated from INGEST, ASSIM, RESP, SASEG, and BDTY.
DSEG	Depth of the reach (m). This is a discretized random variable greater than or equal to 0. This information will be developed from information about stream geometry and hydrology as represented in ECALS CH3D model of the CAWS.
EFFORT	Level of fishing effort by gear type. This variable is a discretized continuous random variable greater than or equal to 0. This information is based on records of fishing effort for conventional surveillance in the CAWS.
FLEN	Size distribution (mm) of the supposed population in the reach. This variable is a discretized continuous random variable greater than or equal to 0.
FMKT	Load of eDNA markers contributed by CSOs by fish markets (copies/day). This variable is a discretized continuous random variable greater than or equal to 0. The value is calculated from COPKG, SALES, and WMKT.
GEARTYPE	Gear type used during conventional surveillance (e.g. Fixed and random site monitoring, Planned intensive surveillance, and Rapid response actions). A discrete random variable representing types of fishing gear used in the course of conventional surveillance (*Electrofishing*, *Gill nets*, *Fyke nets*, *Trawl nets*, etc.).
GSIZE	Sediment grain size distribution (mm). This variable is a discretized continuous random variable representing sediment grain sizes in the CAWS. This probability table is developed from available data on sediment size in the CAWS.
INGEST	Quantity of food ingested by birds per day (kcal/day). This is a discretized continuous random variable greater than or equal to 0. This information will be developed by consulting peer-reviewed literature sources.
K	Degradation rate of eDNA marker (day^{-1}). This is a discretized continuous random variable between 0 and 1. The CPT for this variable is developed based on ECALS degradation studies.
LIGHT	The amount of light, UV radiation, microbial activity, or other factors influencing degradation rates in the CAWS. Estimated from external data sources. This is a discretized continuous random variable greater than or equal to 0. The probability table for this variable is developed based on results of ECALS degradation studies.
LOAD1	Load of an eDNA marker originating from a live fish with the reach (copies/day). This variable is a discretized continuous random variable greater than or equal to 0. This value is calculated from SHED and BIOM.
LOAD2	Load of an eDNA marker originating from secondary sources within the reach (copies/day). This variable is a discretized continuous random variable greater than or equal to 0. This value is calculated from CNL, CBL, RBL, UPL, CSOL, and BIL.
LSEG	Length of the reach (m). The information is based on data provided by MWRD and used in developing ECALS CH3D model of the CAWS.
MANHRS	Man hours of conventional surveillance (electro-fishing and netting)(hours). This variable is a discretized continuous random variable greater than or equal to 0. The information is based on records of fishing effort for conventional surveillance in the CAWS.
MPCAL	eDNA marker per calorie of bird tissue by Asian carp species (copies/kcal). This variable is a discretized continuous random variable greater than or equal to 0. The CPT for this variable is developed from external sources in the peer-reviewed literature and CAWS databases.
NETDEP	Net deposition rate of eDNA to the sediment layer (day^{-1}). This variable is a discretized continuous random variable greater than or equal to 0. The value is calculated from DEP and RESUSP.
POP	Number of individuals in the supposed bighead or silver carp population in the reach. This variable is a discretized continuous random variable greater than or equal to 0.
PRECIP	Precipitation (mm/day). This variable is a discretized continuous random variable greater than or equal to 0. The probability table is developed from National Weather Service records from the Chicago area during the period coincident with the hydrologic modeling period.
PUSE	The extent to which fishing gear has been used previously in waters where bighead and silver carp are present.
Q	Probability that a single fish of a given species and length is caught using a specific gear type. This variable is a discretized continuous random variable between 0 and 1. Catchability is a difficult quantity to measure, but can be related to fishing mortality and fishing effort.
QROF	Surface runoff to the reach (m^3/day). This variable is a discretized continuous random variable greater than or equal to 0. The CPT is estimated from CH3D outputs.
QSEG	Flow to the stream segment (m^3/day). This variable is a discretized continuous random variable greater than or equal to 0. The CPT is estimated from CH3D outputs.
QUPSEG	Inflows to the reach from all upstream reaches (m^3/day). This variable is a discretized continuous random variable greater than or equal to 0. The value is calculated from the QSEG node for upstream reaches.
QWRP	Inflows to the reach from water reclamation plants (WRP)(m^3/day). This variable is a discretized continuous random variable greater than or equal to 0. The CPT is estimated from CH3D outputs.
RAREA	Recreational boat hull area (m^2). This variable is a discretized continuous random variable greater than or equal to 0. The node is parameterized using external data sources documenting fleet characteristics in the CAWS.
RBILGE	The number of eDNA markers imported in recreational bilge water (copies/day). This variable is a discretized continuous random variable greater than or equal to 0. This value is calculated from RTRAF and RVOL.

RBL	Load of eDNA marker imported from recreational fishing vessels (copies/day). This variable is a discretized continuous random variable greater than or equal to 0. This value is calculated from RHULLS and RBILGE.
RES	Residence time of water in the reach (day). This variable is a discretized continuous random variable greater than or equal to 0. This node is parameterized using the outputs of ECALS CH3D model for the CAWS.
RESP	Respiration rate of birds (kcal/day). This variable is a discretized continuous random variable greater than or equal to 0. This node is parameterized by using external peer-reviewed literature sources.
RESUSP	Resuspension rate of sediment from the sediment layer to the water column (day^{-1}). This variable is a discretized continuous random variable greater than or equal to 0. The node is calculated from BTRAF, GSIZE, and USEG.
RHULL	The number of eDNA markers attached per unit area of recreational boat hulls (copies/m^2). This variable is a discretized continuous random variable greater than or equal to 0. The value is calculated from RHULL, RTRAF, and RAREA.
RHULLS	The number of eDNA markers imported on recreational boat hulls (copies/day). This variable is a discretized continuous random variable greater than or equal to 0. The value is calculated from RHULL, RTRAF, and RAREA.
ROOK	The number of eDNA markers contributed by bird feces runoff from known bird rookeries. This variable is a discretized continuous random variable greater than or equal to 0. The value is calculated from DROP and RSIZE.
RSIZE	Population of birds roosting at the rookery. This variable is a discretized continuous random variable greater than or equal to 0. Estimated from external data sources.
RTRAF	Recreational boat traffic (boats/day). This variable is a discretized continuous random variable greater than or equal to 0. Estimated from external data sources.
RVOL	Recreational boat bilge or ballast tank water (m^3). This variable is a discretized continuous random variable greater than or equal to 0. The probability table will be estimated from data on recreational boat characteristics.
SALES	Wet weight of silver and bighead carp sold by fish markets (kg). This variable is a discretized continuous random variable greater than or equal to 0. External data sources.
SASEG	Surface area of the reach (m^2). This variable is a discretized continuous random variable greater than or equal to 0. This value is calculated from WSEG and LSEG as reported in CH3D stream geometry.
SEASON	Season of the year (*Winter, Spring, Summer, Fall*).
SED	The number of eDNA markers stored in the sediment layer (copies/day). This variable is a discretized continuous random variable greater than or equal to 0. This value is calculated from NETDEP and SUSP.
SHED	The shedding rate from live fish in the reach (copies/kg/day). This variable is a discretized continuous random variable greater than or equal to 0. This value is estimated from ECALS studies of eDNA shedding rates.
SOPP	The opportunity available for casual sighting of Asian carp in the reach (man hours/day). This variable is a discretized continuous random variable greater than or equal to 0. This information is developed based on information about the types and levels of activity in each reach.
SPECIES	Target Asian carp species (*Bighead carp, Silver carp*).
SUSP	The number of eDNA markers adsorbed to suspended sediment (copies/mg TSS). This variable is a discretized continuous random variable greater than or equal to 0. This value is calculated from LOAD1, LOAD2, ADS, TSS, and QSEG.
TSS	Total suspended sediment concentration (mg/L). This variable is a discretized continuous random variable greater than or equal to 0. This value is estimated from external databases.
UEFFORT	Unit effort calculated for gear type and reported effort. This variable is a discretized continuous random variable greater than or equal to 0. This value is based on records of conventional surveillance in CAWS reaches.
UNL	The number of eDNA markers per unit length of fishing gear (commercial nets) by gear type (copies/m^2). This information is developed from ECALS studies on the level of gear contamination.
UPFRACT	Fraction of commercial barge and boat traffic headed upstream. External data sources.
UPL	The number of eDNA marker contributed by flow from upstream reaches (copies/day). This variable is a discretized continuous random variable greater than or equal to 0. This value can be derived from CE-QUAL-ICM outputs or calculated.
USEG	Velocity of water in the reach (m/s). This variable is a discretized continuous random variable greater than or equal to 0. The value is estimated using CH3D outputs.
VCRED	Credibility given to the reported sighting of an Asian carp. This variable is a discretized continuous random variable between 0 and 1. This value reflects the degree of belief in the truth of a report.
VIS	A discrete variable indicating that an Asian carp has been sighted in the reach (*True, False*).
VRPT	A discrete variable indicating that a report stating that an Asian carp has been sighted in the reach has been received (*True, False*).
WMKT	Fraction of fish market sales discarded to the sewer system. This variable is a discretized continuous random variable between 0 and 1. This information is based on external data sources.
WSEG	Width of the reach (m). This variable is a discretized continuous random variable greater than or equal to 0. Estimated from CH3D outputs.
WTEMP	Water temperature (deg. C) in the reach. This variable is a discretized continuous random variable greater than or equal to 0. Estimated from external data sources.
XBILG	The exchange of commercial boat and barge bilge water during transit or while tied up at dock (L/barge/day). ECALS presently has little information about where and how commercial boats and barges exchange bilge water. This information will be developed by consulting industry experts.

APPENDIX A

Table A.2: Description of variables in the conceptual model of eDNA detectability.

Variable	Description
ACTCT	The CT-value at which fluorescence exceeds critical fluorescence during the qPCR assay. This variable is a discretized continuous random variable greater than or equal to 0. The CPT for this variable is based on the results of the cPCR assay with three replicates.
CALIQ	Concentration of the cPCR marker in the aliquot withdrawn for a PCR assay (copies/µl). This variable is a discretized continuous random variable greater than or equal to 0. This variable is calculated as a function of CELUTC.
CELUTC	Concentration of the cPCR marker in the elutriate (copies/µl). This variable is a discretized continuous random variable greater than or equal to 0. This variable is calculated from EEXT, SVOL, LRATIO, and CSAMP.
CELUTQ	Concentration of the qPCR marker in the elutriate (copies/µl). This variable is a discretized continuous random variable greater than or equal to 0. This variable is calculated from EEXT, SVOL, LRATIO, and CSAMP.
CEPCR	Efficiency of the cPCR reaction, which is the ratio of the number of copies detected in an aliquot and the number of copies present in the aliquot. This variable is a discretized continuous random variable between 0 and 1. The CPT is constructed by running the assay on a bank of standards with known concentrations.
CINHIB	The fraction of cPCR markers that cannot be detected because of the presence of one or more inhibitors. This variable is a discretized continuous random variable between 0 and 1. The CPT is constructed from standards run to characterize inhibition in water samples taken from the CAWS.
CPCR	A binary variable describing the outcome of the cPCR assay (*True, False*). This variable is the detection sensitivity (probability of detection given the concentration) and is adjusted for beliefs about the specificity of the marker (CSPEC) and the efficiency of the PCR reaction (CEPCR).
CPRIMER	The ease with which a cPCR primer binds to the target marker.
CRITCT	The CT-value at which fluorescence should exceed critical fluorescence during the qPCR assay given prior knowledge of the concentration. This node is parameterized by running a bank of standards with known concentrations.
CSAMP	Concentration of the target eDNA marker in the monitoring sample taken from the CAWS (copies/L). This variable is a discretized continuous random variable greater than or equal to 0. This value is calculated from CSEG, SAFF, and SITE.
CSEG	Concentration of the target eDNA marker in the reach (copies/L). This variable is a discretized continuous random variable greater than or equal to 0. This value is based on outputs of the CE-QUAL-ICM model.
CSPEC	Specificity of the cPCR marker. This variable is a discretized continuous random variable between 0 and 1. The variable represents the degree of belief in the uniqueness of the target marker to the target species.
CURR	A variable characterizing flow conditions at the site where the monitoring sample was collected (*Backwater, Bank, Mid-Channel*).
EEXT	Efficiency with which eDNA markers are extracted from the sample to the elutriate. This variable is a discretized continuous random variable between 0 and 1. The node is parameterized by running a set of experiments to assess the extraction efficiency in the laboratory doing the assay.
HABITAT	A variable characterizing habitat at the location where the monitoring sample was collected.
LRATIO	Ratio of the length of the cPCR marker to the length of the qPCR marker. This is a discretized variable between 0 and 1. The value is calculated from the lengths of the cPCR and qPCR markers.
QALIQ	Concentration of the qPCR marker in the aliquot withdrawn for a qPCR assay (copies/µl). This variable is a discretized continuous random variable greater than or equal to 0. This variable is calculated as a function of CELUTQ.
QEPCR	Efficiency of the qPCR reaction, which is the ratio of the number of copies detected in an aliquot and the number of copies present in the aliquot. This variable is a discretized continuous random variable between 0 and 1. The CPT is constructed from standards run to characterize the efficiency of the assay.
QINHIB	The fraction of qPCR markers that cannot be detected because of the presence of one or more inhibitors. This variable is a discretized continuous random variable between 0 and 1. The CPT is constructed from standards run to characterize inhibition in water samples taken from the CAWS.
QPCR	The concentration of the qPCR marker in the aliquot withdrawn for the assay (copies/µl). This variable is defined as a discretized continuous random variable greater than or equal to 0. This variable is calculated from CRITCT, ACTCT, and QSPEC.
QSPEC	Specificity of the qPCR marker. This is a discretized continuous random variable between 0 and 1. The variable represents the degree of belief in the uniqueness of the target marker to the target species.
QPRIMER	The ease with which a qPCR primer binds to the target marker.
SAFF	A variable describing the surface affinity of eDNA. The fraction of eDNA in a water body that is located at the surface. This variable captures the belief that there is a tendency for eDNA to be located on the surface rather than mid-water. This is a continuous random variable between 0 and 1.
SITE	A variable that describes how much more likely it is that eDNA would be encountered at the site within the reach where the monitoring sample was collected than elsewhere within the reach.
SVOL	The volume of the monitoring sample (L).
WIND	Wind conditions at the time the sample was collected. If eDNA has a high surface affinity, eDNA may be blown across the surface and have a tendency to collect in backwaters and along banks.

APPENDIX B

SEDIMENT SAMPLE PREPARATION

DRYING PROCEDURES

Estimating required sample mass

Estimate the total dry sample mass required for the testing, accounting for sample mass required for individual samples, number of replicates, and testing intervals. Prepare approximately twice this amount of dry sediment, to permit for tests to be re-run as necessary; calculate the corresponding wet sediment mass based on a representative water content (in this case, assume in-situ water content is approximately 150%).

Water content is defined as:

$$w = \frac{W_W}{W_S} \qquad \text{(A1)}$$

Where:

w = water content

W_W = weight water (g)

W_S = weight solids (g)

Calculate a value for W_w by substituting assumed water content (w) and dry sediment mass required (Ws) into equation X1. Total wet sediment mass required is then:

$$W_T = W_W + W_S \qquad \text{(A2)}$$

Water content determination

Sorbed eDNA concentrations must be expressed as mass DNA per unit (dry) weight of sediment. Because the mass of sediment utilized in the batch testing is very small; all measurements must be very precise to minimize potential error in the measured water content. A sample size of 0.2g dry sediment has been specified for all batch tests; the entire sediment sample will be extracted along with a residual volume of DNA solution.

Sediment intended for use in the sorption testing also must be dried at temperatures low enough to minimize destruction of organic materials in the samples. The drying procedure described below will also be used to prepare the sediment for sorption testing.

Equipment needed:

- Weighing tins for drying and for weighing out dry sediment
- Scoops or spoons for taking homogenized sediment from buckets
- Small spatula for manipulating and mixing dry samples
- Mortar and pestle for grinding dry samples
- Plastic sample bottles
- Ball point pen for marking weighing tins (marking should be done prior to taring)

1. Prepare a list of samples to be dried. Include date, site name, sample identification (e.g. test, sampling time/interval, and replicate) and approximate wet weight required.

2. Set oven temperature at 60 degrees C and verify with internal thermometer.

3. Wash all utensils with liquinox and rinse with RO water. Decontaminate with a 10% bleach solution, as described in Appendix B. Air or oven dry, as appropriate.

4. Mark the underside of an aluminum weighing tin with date, site name, sample ID and PI.

5. Weigh the tin and record the weight. Tare the tin.

6. Weigh out the required amount of wet sediment into the tared tin.

7. Verify oven temperature. Place weighed samples in the oven for 24 hours.

8. Remove samples from oven and cool in desiccator. Weigh and record.

9. Place samples back in oven for another 1 - 2 hours, remove, cool and weigh.

10. Repeat until samples are sufficiently dry that the weight does not change by more than 0.005g between weighing.

11. Once samples are determined to have been dried to a consistent weight, grind to a uniform consistency in a clean mortar and pestle (cleaned and decontaminated using the previously described procedure). Mix well to ensure homogeneity, then place in a clean, decontaminated, plastic sample bottle and label with project name, site name, date, and PI. This sediment is ready to be used for sorption testing.

12. Record all tares and weights in record book for water content determination. Calculate water content (w), using Equation A1, substituting the weight of the sample after drying (W_S) and difference in wet and dry weights (W_W). Record.

13. To calculate the dry solids of the extracted sample:

Solve Equation A1 for W_W, which gives:

$$W_W = w * W_S \qquad \text{(A3)}$$

Substitute the expression for W_W from Equation A3, into Equation A2, and rearrange to solve explicitly for W_S. Enter the water content (w) obtained from the corresponding sample aliquot, and the wet weight of the extracted sample (W_T) into Equation A2:

$$W_T = W_W + W_S \qquad \text{(A2)}$$

Solve for the value of W_S. This is the dry weight of the extracted sample.

Sample Homogenization

Prior to taking subsamples from the dried, homogenized sediment for the sorption testing, the sediment must be re-homogenized to ensure that subsamples contain a representative distribution of all particle sizes. A small square of plastic should be decontaminated using the 10% Clorox solution and allowed to air dry. When dry, the plastic should be laid out in the clean area and the previously dried, homogenized sediment poured out onto the center of the plastic. Form the sediment into a cone by picking up all four corners of the plastic. Then flatten the sediment cone with a spatula. Remove two opposing quarters and repeat the process with the remaining quarters. Repeat the process until approximately the required amount of sediment for 5 replicates remains; measure out 0.2g subsamples for the sorption testing from this remaining volume. (It would be acceptable to separate the dry sediment mass by quartering into seven approximately 2g subsamples and store in small plastic sample bottles which can then be quickly re-homogenized prior to obtaining the 0.2g subsamples for testing.)

Sorption Sample Preparation and Labeling

For the sorption studies, sterile or decontaminated centrifuge tubes will be labeled, then placed in a small, decontaminated glass beaker and tared, prior to weighing the dry sediment mass specified by the test matrix into the tubes. Sediment weight will be recorded to three decimal places. All replicates will be weighed out prior to addition of DNA solution, in order to minimize differences in contact time between samples.

DNA solution (0.5 ml, or as otherwise specified for the testing matrix) will be added to the sediment in the centrifuge tubes using a digital pipette and decontaminated or sterile pipette tips, to ensure precise volume delivery; solution will be added to all replicates at once to ensure consistent contact times. Samples will be capped and placed immediately on a sample shaker (this is particularly critical for the shortest contact times), and start time noted. Samples will be removed promptly at the end of the specified contact time, and centrifuged at 3000 RPM[1] for 12

[1] Determine maximum safe operating speed for the equipment being used considering any necessary de-rating factors.

minutes to achieve clarification of the supernatant. For most of the sorption testing, exactly 0.4 ml of supernatant will be pipetted off of each sample and placed in a pre-labeled, sterile centrifuge tube; this is the “supernatant” sample. The remaining supernatant and sediment will be recapped and submitted as the “sediment” sample. The wet sediment sample will be remixed by the analytical lab and extracted as a whole; DNA concentration on the solids will be corrected for the contribution associated with the water, based on the concentration of DNA measured in the clear supernatant sample.

A sufficient amount of sediment will be dried to support an entire testing matrix (e.g. equilibrium, partitioning, or release testing matrices); sediment will be dried in accordance with the procedure previously outlined. Dried sediment will be ground in a decontaminated mortar and pestle just enough to break up agglomerated particles and permit thorough homogenization. Dried sediment will be homogenized by quartering on a decontaminated plastic sheet, as previously described, and stored in a decontaminated plastic jar.

The outside of all containers must also be decontaminated by wiping down with 10% Clorox solution and clean KimWipes. Each batch of samples will be bagged in decontaminated zip lock bags, with decontaminated gel packs to keep them cold, and immediately transported to the DNA analytical lab for freezing.

Centrifuge tubes will be pre-labeled to minimize delays in sample preparation and changes in weight due to the addition of labeling. Sample preparation should be done in batches, by time interval, starting with the longest time interval. In this manner it may be possible to initiate multiple tests on the same day without exceeding the specified contact time for the samples with shorter contact times; only as many samples as can be processed without delay will be prepared on one day, however. It is imperative to conform to the specified contact times as closely as possible for all samples.

The time at which the test was initiated (sediment and solution placed in containers), and the time at which water and sediment were separated, will be noted in the lab book. These times will determine the actual contact time.

Additional samples will be made up and processed as for the other samples; final pH and salinity of the supernatant will be measured using the designated probes and the values recorded. These samples can then be discarded.

APPENDIX B

EQUIPMENT DECONTAMINATION/LABORATORY PROCEDURES

Decontamination

These decontamination procedures have three objectives:

- Prevent the introduction of foreign substances into the test samples from contaminated equipment
- Prevent the introduction of bacteria into the samples, which would result in degradation of the DNA in the samples
- Prevent contamination of the clean rooms where samples will extracted and analyzed

Prior to testing, all non-sterilized equipment must be washed with a solution of liquinox, rinsed with DI water, soaked in a 10% solution of Clorox for 10 minutes, rinsed again with DI water, and allowed to air dry on a clean, drying rack which has been decontaminated using the same procedure. All washing should be done in a plastic tub dedicated to the purpose, and cleaned and decontaminated prior to use. Dry equipment will be packaged in clean, decontaminated zip lock bags or glass sample jars to preserve for future use. Be vigilant about cross contamination; do not touch potentially contaminated surfaces with clean gloves and then touch the decontaminated equipment.

It will be necessary to create a "clean-zone" in which to conduct the testing; contact with any equipment or surfaces outside of the clean-zone will necessitate a change of gloves before further work in the clean-zone. The countertop will be washed with liquinox, then wiped down with Clorox solution, and allowed to air dry, then covered with clean bench paper before testing begins daily. The boundaries of the clean zone will be clearly marked with tape. Any equipment to be used during each day's testing will be first decontaminated and then placed in the clean zone. This includes all surfaces (including the feet) of balances, the exterior of pipetters, beakers, etc. A clean drawer or cabinet will be established for storage of clean equipment, preferably immediately above or below the clean zone. This area will be decontaminated and marked off in the same manner as the clean work area; clean equipment will be covered with a clean paper towel or stored in decontaminated zip lock bags to prevent airborne recontamination.

A new box of Kim Wipes, clean paper towels and sterile gloves will be placed in the clean work or storage area for ready access (a separate box of gloves should be kept outside of the clean zone for use in equipment decontamination). Hands will be washed, dried on clean paper towels, and sterile gloves donned before touching anything in the clean zone/storage area. Equipment washing and decontamination will be done in a cleaned and decontaminated plastic tub. The tub will be stored outside of the clean zone but decontaminated prior to each use. Liquinox and

Clorox will be stored outside the clean zone near the sink to be used for decontamination; the necessary volume of Clorox solution will be made up daily and excess disposed of at the end of the day. The decontaminated drying rack will be placed within the clean zone for drying decontaminated equipment. The exterior of all sample bottles must also be decontaminated prior to bagging in decontaminated zip lock bags for freezing immediately after testing is completed; sample bottles will be wiped down with Clorox solution prior to bagging and kept cool with decontaminated frozen gel packs. Samples will be transported immediately to the DNA analytical lab for freezing and subsequent analysis.

Clorox Solution

Make up a 10% solution of Clorox by measuring 900 ml of DI water into a clean 1L glass bottle, using a clean graduated cylinder. Measure out 100 ml of Clorox and add to the DI water. Place a clean (Liquinox followed by DI) stir bar in the bottle and stir the solution for 10 minutes. Pour a portion of the Clorox solution into a clean squeeze bottle for use in equipment decontamination. Label both containers; store the Clorox bottle outside of the clean zone and the Clorox squeeze bottle near the sink (also outside of the clean zone).

APPENDIX B

MASS BALANCE CALCULATIONS

DNA concentration is measured in a subsample of liquid extraction media; mass balance calculations are required to extrapolate DNA concentrations in the supernatant and sorbed to the sediment. A cursory understanding of the major steps in the CTAB extraction is necessary in order to construct a mass balance. As described in the Methods and Materials section, sediment samples of approximately 0.2g dry weight were combined with 500 ul of DNA marker solution for the sorption testing. Following the specified contact time, the samples were separated into a "sediment" sample and a "supernatant" sample, consisting of (with a some exceptions as noted in the report):

- Sediment, plus 100 ul of residual DNA marker solution
- Supernatant, 400 ul pipetted off of the sediment after centrifuging

Both the sediment and the supernatant samples were extracted in their entirety, in accordance with the CTAB extraction procedure. The following describes the major steps and reagent additions:

1. Add 500µl of CTAB buffer to the sample.

2. Incubate samples at 55°C for 1hr to overnight.

3. Add 500µl of 24:1 Chloroform :Iso Amyl Alcohol and mix well by shaking tubes.

4. Centrifuge for 5-10 minutes at maximum speed.

5. Pipette off the 400 ul of aqueous phase

6. Add 32ul of cold 7.5 M ammonium acetate-see attached table.

7. Add 233ul of cold isopropanol.

8. Mix well.

9. Let sit in freezer for 15 min to overnight.

10. Centrifuge for 3 min at maximum speed.

11. Pour or pipette off the liquid, being careful not to lose the pellet with your DNA.

12. Add 500µl of cold 70% Ethanol and mix

13. Centrifuge for 1 min at maximum speed.

14. Pour or pipette off the liquid, being careful not to lose the pellet with your DNA.

15. Add 500µl of cold 95% Ethanol and mix

16. Centrifuge for 1 min at maximum speed.

17. Pour or pipette off the liquid, being careful not to lose the pellet with your DNA.

18. Dry the pellet

19. Add 100ul of ddH2O.

The following figure (B1) illustrates graphically the movement of the DNA through the extraction phases.

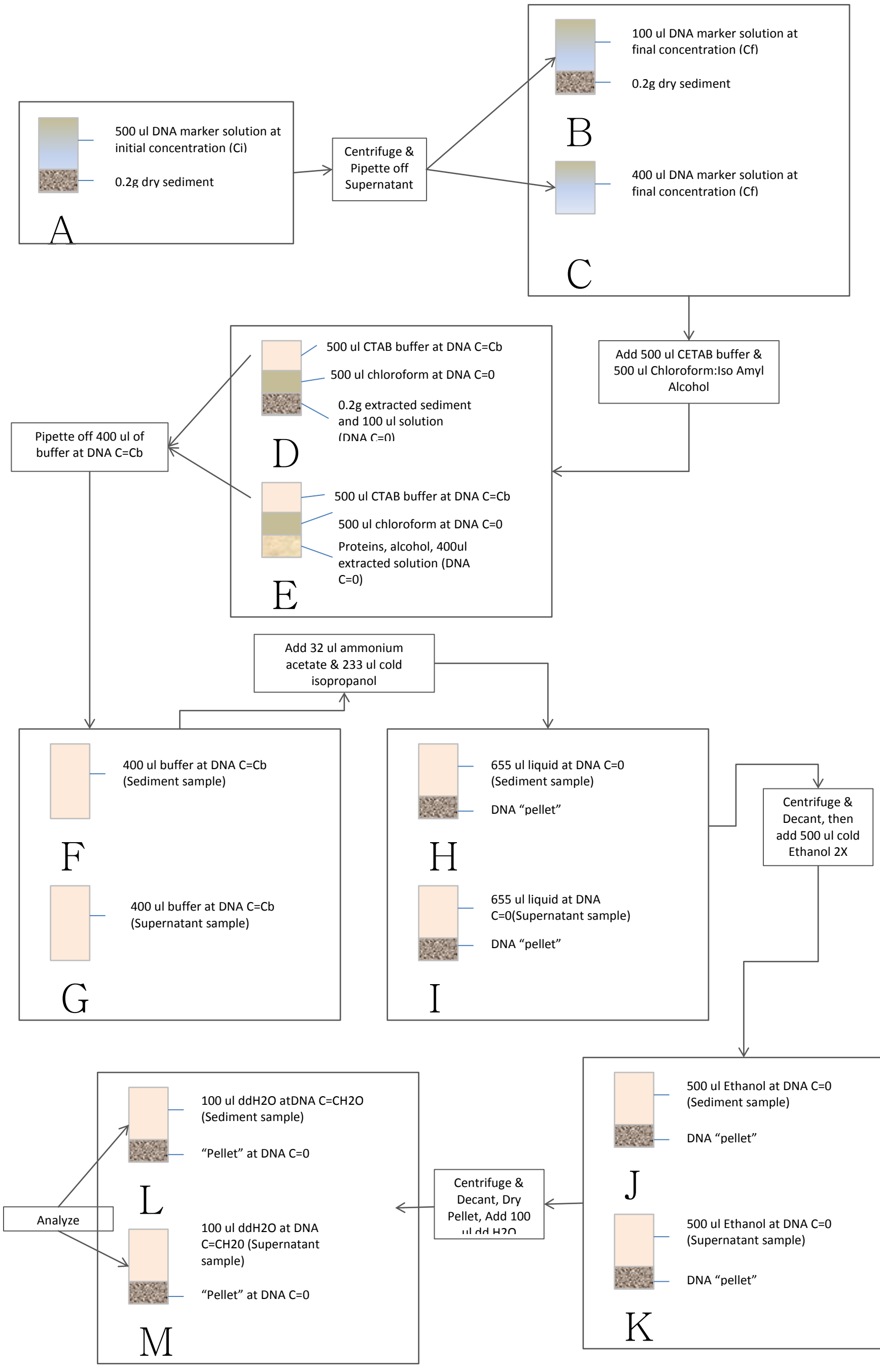

Figure B1. Mass balance

APPENDIX B

AMPLICON PREPARATION PROCEDURE

In order to measure DNA sorption accurately in the sediment, PCR amplicon was used in the equilibrium study instead of genomic DNA (gDNA). PCR product used for the sediment equilibrium study was amplified with the USGS silver carp primer. About 1ng/ul of SC gDNA was used as a template to amplify the PCR product.

Silver carp (108 bp)	**Sequence (5'-3')**	**Tm**
Forward	GGTGGCGCAGAATGAACTA	59.81
Reverse	TCACATCATTTAACCAGATGCC	59.83

Each PCR 1X reaction (25ul) should contain:

- 2.5 µL 10X PCR buffer
- 0.5 µL dNTP (= 10 mM mixed dNTP)
- 0.75 µL Mg2+ solution (25mM)
- 0.5 µL forward primer (= 10mM working dilution)
- 0.5 µL reverse primer (= 10mM working dilution)
- 0.25 µL Platinum® *Taq* polymerase (= 1.25 U)
- 19.0 µL sterile water

Cycling Conditions:

95^{o}C – 10min

95^{o}C -15S

50^{o}C – 30S 45 cycles

72^{o}C – 30S

72^{o}C- 7 min

PCR products were then purified with EtBr stained 2% size select gel and measured with Qubit. As EtBr was co-purified with PCR products which prevented accurate measurement of the DNA concentration, there were some difficulties initially in determining how best to quantify the DNA concentration. Following some preliminary testing, Qubit was selected to measure the DNA concentration for future testing.

APPENDIX B

SEDIMENT PARTICLE SIZE DISTRIBUTION

Lake Calumet

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LS Particle Size Analyzer

22 Nov 2013 9:01

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File name:	C:\Users\U4EERRW9\Documents\A-PROJECT\eDNA\Coulter-LakeCalumetComp_01_01.$ls Coulter-LakeCalumetComp_01_01.$ls		
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Run number:	1		
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Residual:	1.51%		
LS 100Q	Fluid Module		
Start time:	14:32 9 May 2013	Run length:	60 seconds
Pump speed:	45		
Obscuration:	10%		
Fluid:	,0		
Software:	3.29	Firmware:	2.02

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Sample ID:	Sediment		
Operator:	R. Hudson		
Run number:	2		
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Residual:	1.56%		
LS 100Q	Fluid Module		
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Pump speed:	45		
Obscuration:	10%		
Fluid:	,0		
Software:	3.29	Firmware:	2.02

LS Particle Size Analyzer

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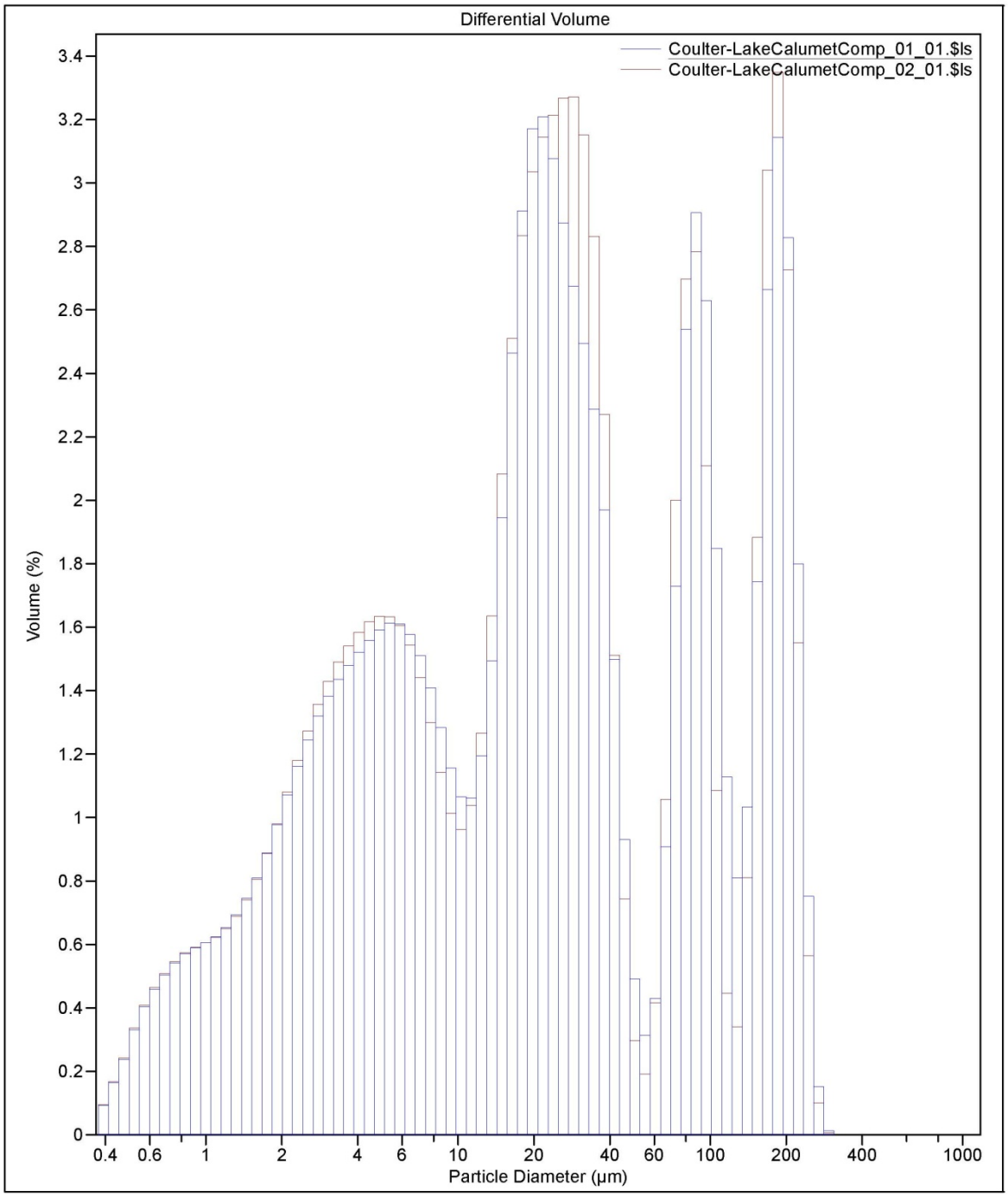

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Volume %	Coulter -LakeCalumet Comp_01_01.$ls Particle Diameter	Coulter -LakeCalumet Comp_02_01.$ls Particle Diameter
10	2.031	2.027
25	5.526	5.388
50	21.26	21.26
75	79.34	71.30
90	169.2	168.1

Volume Statistics (Arithmetic) Coulter-LakeCalumetComp_01_01.$ls

Calculations from 0.375 µm to 948.3 µm

Volume:	100%		
Mean:	50.55 µm	S.D.:	63.91 µm
Median:	21.26 µm	Variance:	4084 µm^2
Mean/Median ratio:	2.378	C.V.:	126%
Mode:	21.70 µm	Skewness:	1.481 Right skewed
		Kurtosis:	1.036 Leptokurtic

<10%	<25%	<50%	<75%	<90%
2.031 µm	5.526 µm	21.26 µm	79.34 µm	169.2 µm

Volume Statistics (Arithmetic) Coulter-LakeCalumetComp_02_01.$ls

Calculations from 0.375 µm to 948.3 µm

Volume:	100%		
Mean:	48.23 µm	S.D.:	62.38 µm
Median:	21.26 µm	Variance:	3891 µm^2
Mean/Median ratio:	2.268	C.V.:	129%
Mode:	185.4 µm	Skewness:	1.575 Right skewed
		Kurtosis:	1.291 Leptokurtic

<10%	<25%	<50%	<75%	<90%
2.027 µm	5.388 µm	21.26 µm	71.30 µm	168.1 µm

Volume Statistics (Arithmetic) Calculations from 0.375 µm to 948.3 µm

Nominal	Lower Limit	Upper Limit	Coulter-LakeCalumetComp_01_01.$ls

Selected sample statistics not present

Volume Statistics (Arithmetic) Calculations from 0.375 µm to 948.3 µm

Nominal	Lower Limit	Upper Limit	Coulter-LakeCalumetComp_02_01.$ls

Selected sample statistics not present

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Volume Statistics (Arithmetic)
Calculations from 0.375 µm to 948 µm

	Amount %	Mean µm	S.D. µm
Coulter-LakeCalumetComp_01_01.$ls	100	50.5	63.9
Coulter-LakeCalumetComp_02_01.$ls	100	48.2	62.4
(Average)	100	49.4	63.1
(C.V.)	0.0%	3.3%	1.7%
(Maximum)	100	50.5	63.9
(Minimum)	100	48.2	62.4

Channel Diameter (Lower) µm	Coulter -LakeCalumetComp_01_01.$ls Diff. Volume	Coulter -LakeCalumetComp_02_01.$ls Diff. Volume	Channel Diameter (Lower) µm	Coulter -LakeCalumetComp_01_01.$ls Diff. Volume	Coulter -LakeCalumetComp_02_01.$ls Diff. Volume
0.375	0.093	0.095	36.24	1.97	2.27
0.412	0.16	0.17	39.78	1.50	1.51
0.452	0.24	0.24	43.67	0.93	0.74
0.496	0.33	0.34	47.94	0.49	0.30
0.545	0.40	0.41	52.63	0.31	0.19
0.598	0.46	0.46	57.77	0.43	0.42
0.657	0.50	0.51	63.42	0.91	1.06
0.721	0.54	0.55	69.62	1.73	2.00
0.791	0.57	0.57	76.43	2.54	2.70
0.869	0.59	0.59	83.90	2.91	2.78
0.954	0.61	0.61	92.10	2.63	2.11
1.047	0.62	0.62	101.1	1.85	1.09
1.149	0.65	0.65	111.0	1.13	0.45
1.261	0.69	0.69	121.8	0.81	0.34
1.385	0.74	0.74	133.8	1.03	0.81
1.520	0.81	0.81	146.8	1.74	1.88
1.669	0.89	0.89	161.2	2.66	3.04
1.832	0.98	0.98	176.9	3.14	3.35
2.011	1.07	1.08	194.2	2.83	2.73
2.208	1.16	1.18	213.2	1.80	1.55
2.423	1.25	1.27	234.1	0.75	0.56
2.660	1.32	1.36	256.9	0.15	0.10
2.920	1.38	1.43	282.1	0.013	0.0067
3.206	1.44	1.49	309.6	0	0
3.519	1.48	1.54	339.9	0	0
3.863	1.52	1.58	373.1	0	0
4.241	1.56	1.62	409.6	0	0
4.656	1.59	1.63	449.7	0	0
5.111	1.61	1.63	493.6	0	0
5.610	1.61	1.61	541.9	0	0
6.159	1.58	1.54	594.9	0	0
6.761	1.51	1.44	653.0	0	0
7.422	1.41	1.30	716.9	0	0
8.148	1.28	1.14	787.0	0	0
8.944	1.16	1.01	863.9	0	0
9.819	1.06	0.96	948.3		
10.78	1.06	1.04			
11.83	1.19	1.27			
12.99	1.49	1.63			
14.26	1.95	2.08			
15.65	2.46	2.51			
17.18	2.91	2.83			
18.86	3.17	3.03			
20.71	3.21	3.14			
22.73	3.08	3.21			
24.95	2.87	3.27			
27.39	2.67	3.27			
30.07	2.49	3.15			
33.01	2.29	2.83			

LS Particle Size Analyzer

22 Nov 2013 9:54

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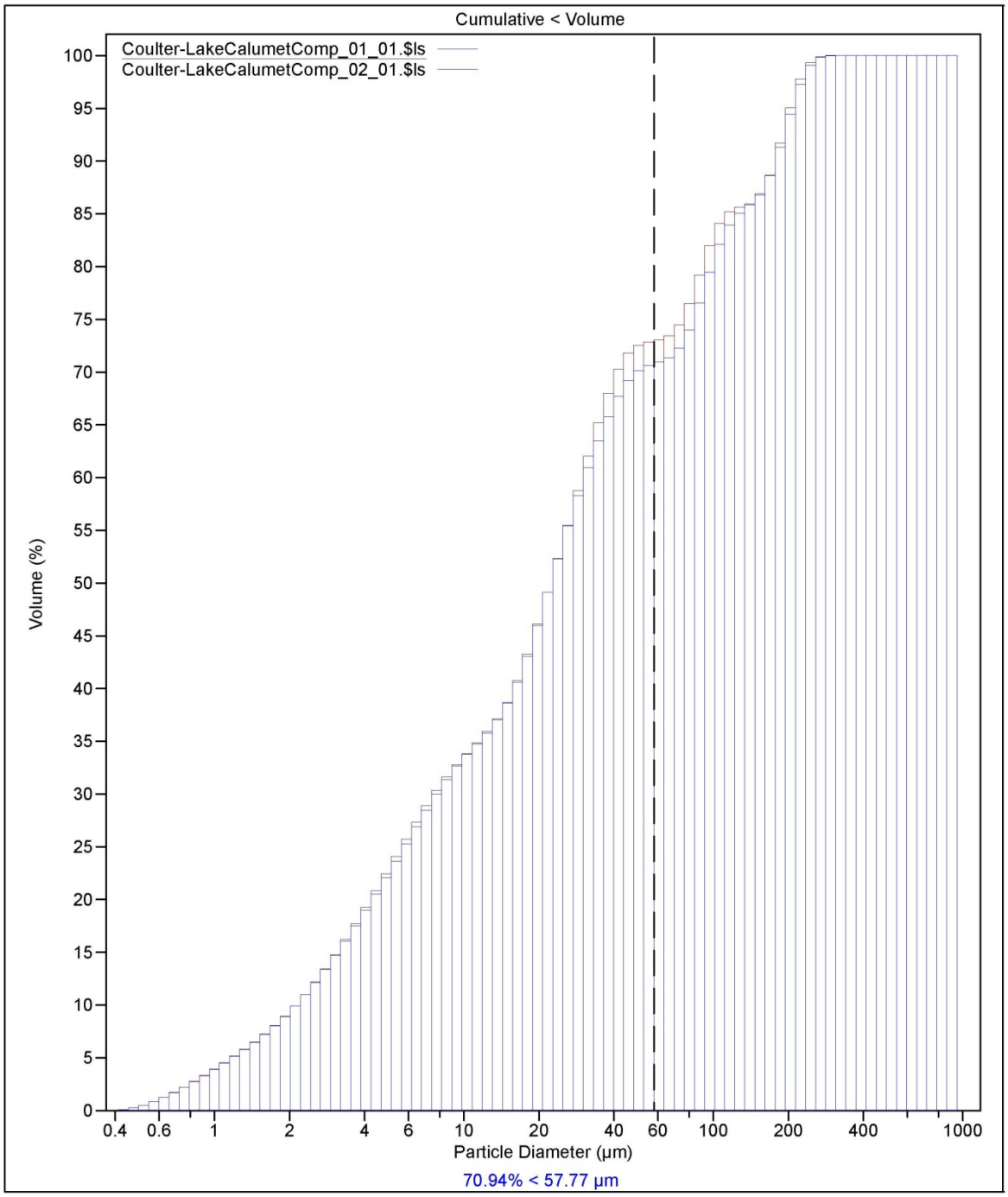
Sample ID:	Sediment		
Operator:	R. Hudson		
Run number:	1		
Optical model:	Fraunhofer.rf780z		
Residual:	1.51%		
LS 100Q	Fluid Module		
Start time:	14:32 9 May 2013	Run length:	60 seconds
Pump speed:	45		
Obscuration:	10%		
Fluid:	,0		
Software:	3.29	Firmware:	2.02

File name:	C:\Users\U4EERRW9\Documents\A-PROJECT\eDNA\Coulter-LakeCalumetComp_02_01.$ls Coulter-LakeCalumetComp_02_01.$ls		
File ID:	Lake Calumet Comp		
Sample ID:	Sediment		
Operator:	R. Hudson		
Run number:	2		
Optical model:	Fraunhofer.rf780z		
Residual:	1.56%		
LS 100Q	Fluid Module		
Start time:	14:33 9 May 2013	Run length:	60 seconds
Pump speed:	45		
Obscuration:	10%		
Fluid:	,0		
Software:	3.29	Firmware:	2.02

LS Particle Size Analyzer

22 Nov 2013 9:54

Beckman Coulter LS 13 320



BECKMAN COULTER™

LS Particle Size Analyzer

22 Nov 2013 9:54

Beckman Coulter LS 13 320

Volume Statistics (Arithmetic)			Coulter-LakeCalumetComp_01_01.$ls	
Calculations from 0.375 µm to 948.3 µm				
Volume:	100%			
Mean:	50.55 µm		S.D.:	63.91 µm
Median:	21.26 µm		Variance:	4084 µm^2
Mean/Median ratio:	2.378		C.V.:	126%
Mode:	21.70 µm		Skewness:	1.481 Right skewed
			Kurtosis:	1.036 Leptokurtic
<10%	<25%	<50%	<75%	<90%
2.031 µm	5.526 µm	21.26 µm	79.34 µm	169.2 µm

Volume Statistics (Arithmetic)			Coulter-LakeCalumetComp_02_01.$ls	
Calculations from 0.375 µm to 948.3 µm				
Volume:	100%			
Mean:	48.23 µm		S.D.:	62.38 µm
Median:	21.26 µm		Variance:	3891 µm^2
Mean/Median ratio:	2.268		C.V.:	129%
Mode:	185.4 µm		Skewness:	1.575 Right skewed
			Kurtosis:	1.291 Leptokurtic
<10%	<25%	<50%	<75%	<90%
2.027 µm	5.388 µm	21.26 µm	71.30 µm	168.1 µm

Volume Statistics (Arithmetic)
Calculations from 0.375 µm to 948 µm

	Amount %	Mean µm	S.D. µm
Coulter-LakeCalumetComp_01_01.$ls	100	50.5	63.9
Coulter-LakeCalumetComp_02_01.$ls	100	48.2	62.4
(Average)	100	49.4	63.1
(C.V.)	0.0%	3.3%	1.7%
(Maximum)	100	50.5	63.9
(Minimum)	100	48.2	62.4

Channel Diameter (Lower) µm	Coulter -LakeCalumetComp_01_01.$ls Diff. Volume	Coulter -LakeCalumetComp_02_01.$ls Diff. Volume	Channel Diameter (Lower) µm	Coulter -LakeCalumetComp_01_01.$ls Diff. Volume	Coulter -LakeCalumetComp_02_01.$ls Diff. Volume
0.375	0.093	0.095	1.261	0.69	0.69
0.412	0.16	0.17	1.385	0.74	0.74
0.452	0.24	0.24	1.520	0.81	0.81
0.496	0.33	0.34	1.669	0.89	0.89
0.545	0.40	0.41	1.832	0.98	0.98
0.598	0.46	0.46	2.011	1.07	1.08
0.657	0.50	0.51	2.208	1.16	1.18
0.721	0.54	0.55	2.423	1.25	1.27
0.791	0.57	0.57	2.660	1.32	1.36
0.869	0.59	0.59	2.920	1.38	1.43
0.954	0.61	0.61	3.206	1.44	1.49
1.047	0.62	0.62	3.519	1.48	1.54
1.149	0.65	0.65	3.863	1.52	1.58

Channel Diameter (Lower) µm	Coulter -LakeCalume tComp_01 _01.$ls _Diff. Volume	Coulter -LakeCalume tComp_02 _01.$ls _Diff. Volume
4.241	1.56	1.62
4.656	1.59	1.63
5.111	1.61	1.63
5.610	1.61	1.61
6.159	1.58	1.54
6.761	1.51	1.44
7.422	1.41	1.30
8.148	1.28	1.14
8.944	1.16	1.01
9.819	1.06	0.96
10.78	1.06	1.04
11.83	1.19	1.27
12.99	1.49	1.63
14.26	1.95	2.08
15.65	2.46	2.51
17.18	2.91	2.83
18.86	3.17	3.03
20.71	3.21	3.14
22.73	3.08	3.21
24.95	2.87	3.27
27.39	2.67	3.27
30.07	2.49	3.15
33.01	2.29	2.83
36.24	1.97	2.27
39.78	1.50	1.51
43.67	0.93	0.74
47.94	0.49	0.30
52.63	0.31	0.19
57.77	0.43	0.42
63.42	0.91	1.06
69.62	1.73	2.00
76.43	2.54	2.70
83.90	2.91	2.78
92.10	2.63	2.11
101.1	1.85	1.09
111.0	1.13	0.45
121.8	0.81	0.34
133.8	1.03	0.81
146.8	1.74	1.88
161.2	2.66	3.04
176.9	3.14	3.35
194.2	2.83	2.73
213.2	1.80	1.55
234.1	0.75	0.56
256.9	0.15	0.10
282.1	0.013	0.0067
309.6	0	0
339.9	0	0
373.1	0	0
409.6	0	0
449.7	0	0
493.6	0	0
541.9	0	0
594.9	0	0
653.0	0	0
716.9	0	0
787.0	0	0
863.9	0	0
948.3		

Lockport Pool

LS Particle Size Analyzer

22 Nov 2013 8:59

Beckman Coulter LS 13 320

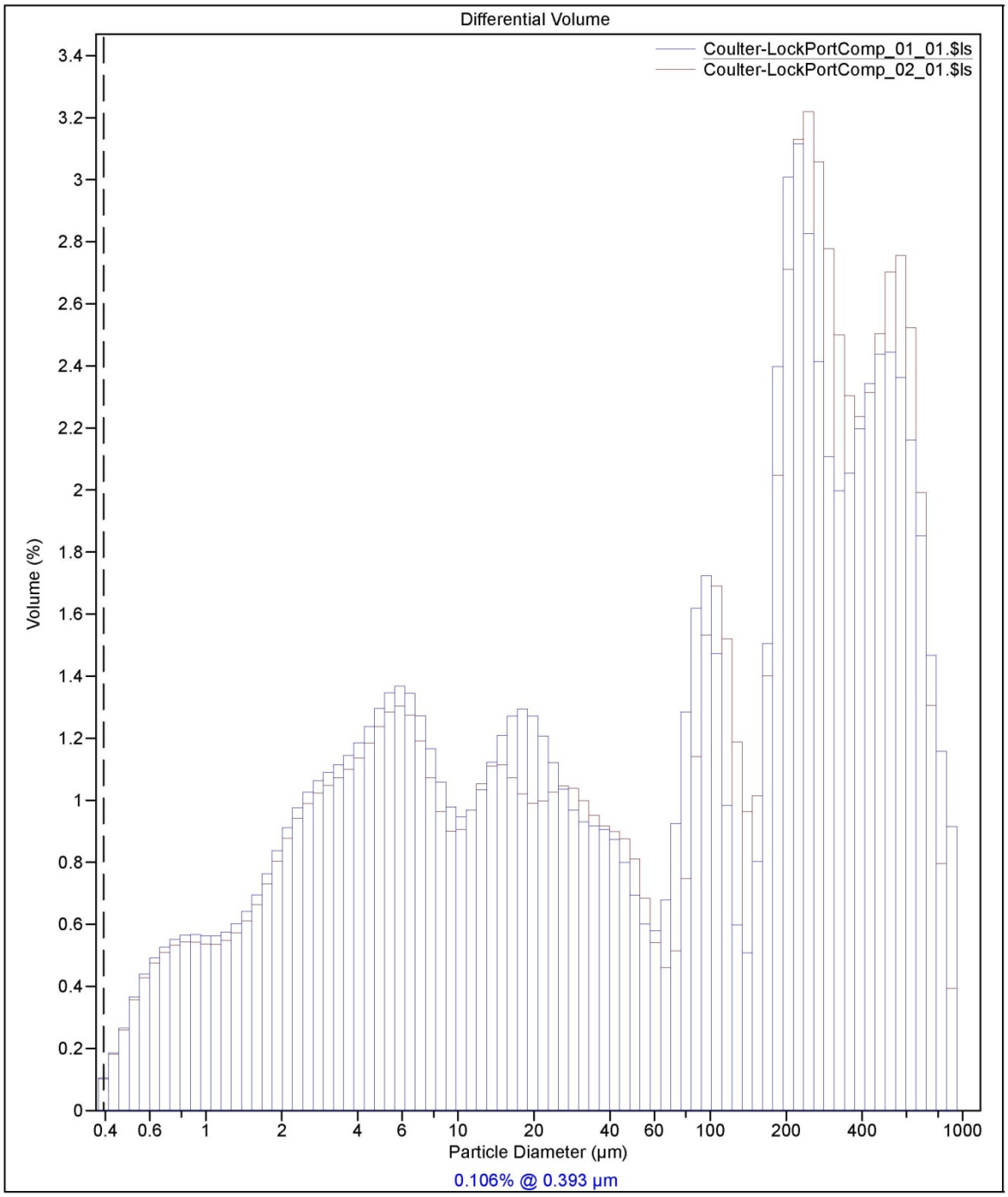
File name:	C:\Users\U4EERRW9\Documents\A-PROJECT\eDNA\Coulter-LockPortComp_01_01.$ls Coulter-LockPortComp_01_01.$ls		
File ID:	Lockport Comp		
Sample ID:	Sediment B		
Operator:	R. Hudson		
Run number:	1		
Optical model:	Fraunhofer.rf780z		
Residual:	1.91%		
LS 100Q	Fluid Module		
Start time:	15:04 9 May 2013	Run length:	60 seconds
Pump speed:	45		
Obscuration:	11%		
Fluid:	,0		
Software:	3.29	Firmware:	2.02

File name:	C:\Users\U4EERRW9\Documents\A-PROJECT\eDNA\Coulter-LockPortComp_02_01.$ls Coulter-LockPortComp_02_01.$ls		
File ID:	Lockport Comp		
Sample ID:	Sediment B		
Operator:	R. Hudson		
Run number:	2		
Optical model:	Fraunhofer.rf780z		
Residual:	2.20%		
LS 100Q	Fluid Module		
Start time:	15:05 9 May 2013	Run length:	60 seconds
Pump speed:	45		
Obscuration:	11%		
Fluid:	,0		
Software:	3.29	Firmware:	2.02

LS Particle Size Analyzer

22 Nov 2013 8:59

Beckman Coulter LS 13 320



Beckman Coulter LS 13 320

Volume %	Coulter -LockPortCo mp_01_01 .$ls Particle Diameter	Coulter -LockPortCo mp_02_01 .$ls Particle Diameter
10	2.161	2.251
25	7.068	7.696
50	74.64	95.52
75	288.6	303.0
90	540.2	537.7

Volume Statistics (Arithmetic) Coulter-LockPortComp_01_01.$ls

Calculations from 0.375 µm to 948.3 µm

Volume:	100%		
Mean:	181.6 µm	S.D.:	224.4 µm
Median:	74.64 µm	Variance:	50335 µm^2
Mean/Median ratio:	2.433	C.V.:	124%
Mode:	223.4 µm	Skewness:	1.299 Right skewed
		Kurtosis:	0.797 Leptokurtic

<10%	<25%	<50%	<75%	<90%
2.161 µm	7.068 µm	74.64 µm	288.6 µm	540.2 µm

Volume Statistics (Arithmetic) Coulter-LockPortComp_02_01.$ls

Calculations from 0.375 µm to 948.3 µm

Volume:	100%		
Mean:	186.6 µm	S.D.:	217.0 µm
Median:	95.52 µm	Variance:	47108 µm^2
Mean/Median ratio:	1.954	C.V.:	116%
Mode:	245.2 µm	Skewness:	1.147 Right skewed
		Kurtosis:	0.379 Leptokurtic

<10%	<25%	<50%	<75%	<90%
2.251 µm	7.696 µm	95.52 µm	303.0 µm	537.7 µm

Volume Statistics (Arithmetic) Calculations from 0.375 µm to 948.3 µm

Nominal	Lower Limit	Upper Limit	Coulter-LockPortComp_01_01.$ls

Selected sample statistics not present

Volume Statistics (Arithmetic) Calculations from 0.375 µm to 948.3 µm

Nominal	Lower Limit	Upper Limit	Coulter-LockPortComp_02_01.$ls

Selected sample statistics not present

LS Particle Size Analyzer

22 Nov 2013 8:59

Beckman Coulter LS 13 320

Volume Statistics (Arithmetic)
Calculations from 0.375 µm to 948 µm

	Amount %	Mean µm	S.D. µm
Coulter-LockPortComp_01_01.$ls	100	182	224
Coulter-LockPortComp_02_01.$ls	100	187	217
(Average)	100	184	221
(C.V.)	0.0%	1.9%	2.3%
(Maximum)	100	187	224
(Minimum)	100	182	217

Channel Diameter (Lower) µm	Coulter -LockPortComp_01_01 .$ls Diff. Volume	Coulter -LockPortComp_02_01 .$ls Diff. Volume
0.375	0.11	0.10
0.412	0.19	0.18
0.452	0.27	0.26
0.496	0.37	0.36
0.545	0.44	0.43
0.598	0.49	0.48
0.657	0.53	0.51
0.721	0.55	0.53
0.791	0.57	0.54
0.869	0.57	0.54
0.954	0.56	0.54
1.047	0.56	0.54
1.149	0.58	0.55
1.261	0.60	0.57
1.385	0.64	0.61
1.520	0.70	0.66
1.669	0.76	0.73
1.832	0.84	0.80
2.011	0.91	0.88
2.208	0.98	0.94
2.423	1.03	0.99
2.660	1.06	1.02
2.920	1.09	1.05
3.206	1.11	1.07
3.519	1.14	1.10
3.863	1.18	1.14
4.241	1.24	1.18
4.656	1.30	1.24
5.111	1.35	1.28
5.610	1.37	1.30
6.159	1.34	1.27
6.761	1.27	1.19
7.422	1.17	1.07
8.148	1.06	0.96
8.944	0.98	0.90
9.819	0.95	0.91
10.78	0.97	0.97
11.83	1.03	1.05
12.99	1.12	1.11
14.26	1.21	1.11
15.65	1.27	1.07
17.18	1.29	1.02
18.86	1.27	0.99
20.71	1.21	1.00
22.73	1.12	1.03
24.95	1.04	1.05
27.39	0.97	1.04
30.07	0.93	1.00
33.01	0.92	0.95
36.24	0.91	0.92
39.78	0.87	0.90
43.67	0.80	0.88
47.94	0.69	0.81
52.63	0.60	0.68
57.77	0.58	0.54
63.42	0.68	0.46
69.62	0.93	0.51
76.43	1.28	0.75
83.90	1.62	1.14
92.10	1.72	1.53
101.1	1.47	1.69
111.0	0.98	1.52
121.8	0.60	1.19
133.8	0.51	0.96
146.8	0.80	1.01
161.2	1.51	1.40
176.9	2.40	2.05
194.2	3.01	2.71
213.2	3.12	3.13
234.1	2.83	3.22
256.9	2.41	3.06
282.1	2.11	2.78
309.6	2.00	2.50
339.9	2.05	2.30
373.1	2.20	2.24
409.6	2.34	2.31
449.7	2.44	2.50
493.6	2.44	2.70
541.9	2.36	2.76
594.9	2.16	2.52
653.0	1.85	1.99
716.9	1.47	1.31
787.0	1.16	0.80
863.9	0.92	0.39
948.3		

LS Particle Size Analyzer

22 Nov 2013 9:51

Beckman Coulter LS 13 320

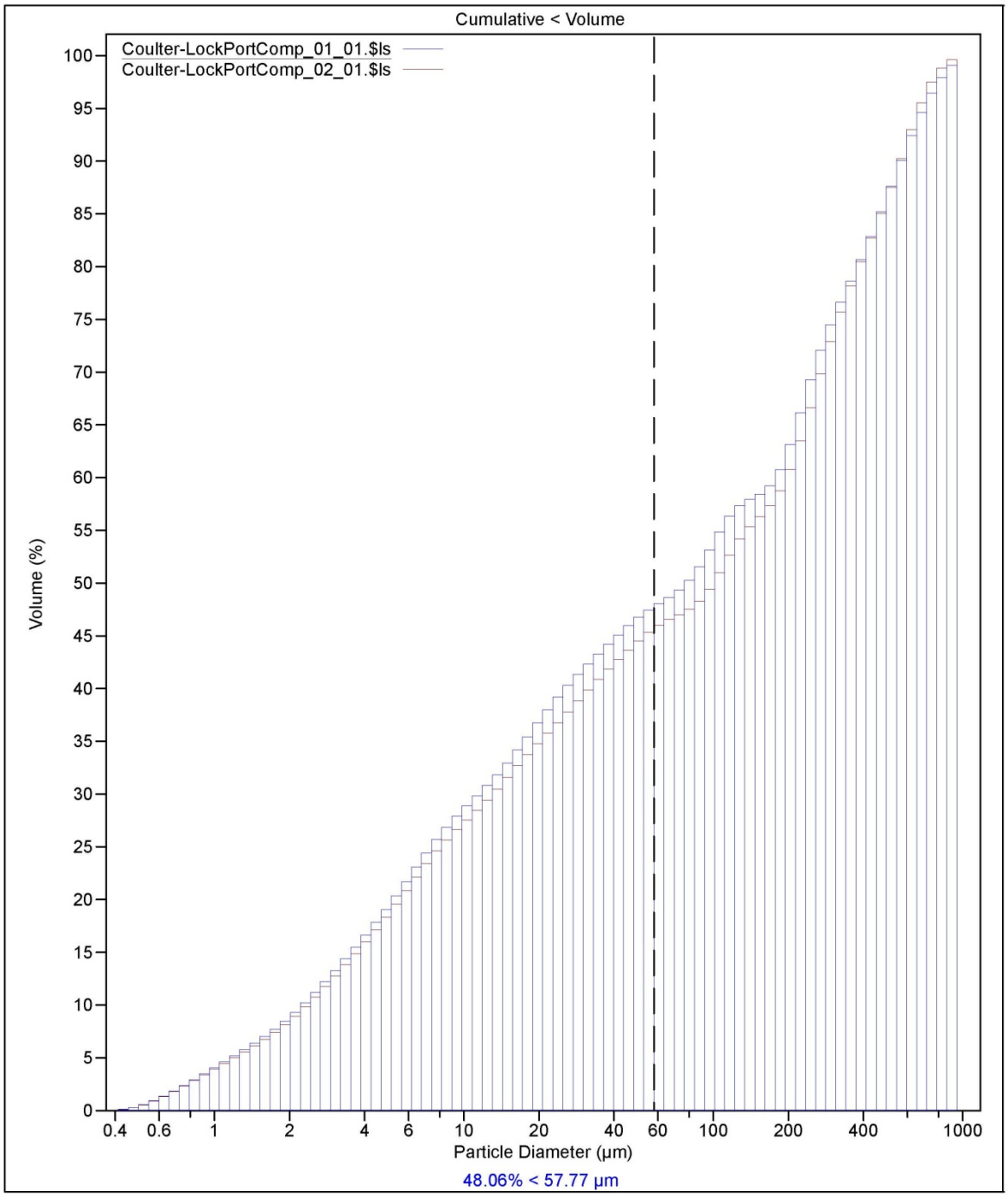
File name:	C:\Users\U4EERRW9\Documents\A-PROJECT\eDNA\Coulter-LockPortComp_01_01.$ls Coulter-LockPortComp_01_01.$ls		
File ID:	Lockport Comp		
Sample ID:	Sediment B		
Operator:	R. Hudson		
Run number:	1		
Optical model:	Fraunhofer.rf780z		
Residual:	1.91%		
LS 100Q	Fluid Module		
Start time:	15:04 9 May 2013	Run length:	60 seconds
Pump speed:	45		
Obscuration:	11%		
Fluid:	,0		
Software:	3.29	Firmware:	2.02

File name:	C:\Users\U4EERRW9\Documents\A-PROJECT\eDNA\Coulter-LockPortComp_02_01.$ls Coulter-LockPortComp_02_01.$ls		
File ID:	Lockport Comp		
Sample ID:	Sediment B		
Operator:	R. Hudson		
Run number:	2		
Optical model:	Fraunhofer.rf780z		
Residual:	2.20%		
LS 100Q	Fluid Module		
Start time:	15:05 9 May 2013	Run length:	60 seconds
Pump speed:	45		
Obscuration:	11%		
Fluid:	,0		
Software:	3.29	Firmware:	2.02

LS Particle Size Analyzer

22 Nov 2013 9:51

Beckman Coulter LS 13 320



BECKMAN COULTER™

LS Particle Size Analyzer

22 Nov 2013 9:51

Beckman Coulter LS 13 320

Volume Statistics (Arithmetic)			Coulter-LockPortComp_01_01.$ls	
Calculations from 0.375 µm to 948.3 µm				
Volume:	100%			
Mean:	181.6 µm		S.D.:	224.4 µm
Median:	74.64 µm		Variance:	50335 µm^2
Mean/Median ratio:	2.433		C.V.:	124%
Mode:	223.4 µm		Skewness:	1.299 Right skewed
			Kurtosis:	0.797 Leptokurtic
<10%	<25%	<50%	<75%	<90%
2.161 µm	7.068 µm	74.64 µm	288.6 µm	540.2 µm

Volume Statistics (Arithmetic)			Coulter-LockPortComp_02_01.$ls	
Calculations from 0.375 µm to 948.3 µm				
Volume:	100%			
Mean:	186.6 µm		S.D.:	217.0 µm
Median:	95.52 µm		Variance:	47108 µm^2
Mean/Median ratio:	1.954		C.V.:	116%
Mode:	245.2 µm		Skewness:	1.147 Right skewed
			Kurtosis:	0.379 Leptokurtic
<10%	<25%	<50%	<75%	<90%
2.251 µm	7.696 µm	95.52 µm	303.0 µm	537.7 µm

Volume Statistics (Arithmetic)
Calculations from 0.375 µm to 948 µm

	Amount %	Mean µm	S.D. µm
Coulter-LockPortComp_01_01.$ls	100	182	224
Coulter-LockPortComp_02_01.$ls	100	187	217
(Average)	100	184	221
(C.V.)	0.0%	1.9%	2.3%
(Maximum)	100	187	224
(Minimum)	100	182	217

Channel Diameter (Lower) µm	Coulter -LockPortComp_01_01.$ls Diff. Volume	Coulter -LockPortComp_02_01.$ls Diff. Volume	Channel Diameter (Lower) µm	Coulter -LockPortComp_01_01.$ls Diff. Volume	Coulter -LockPortComp_02_01.$ls Diff. Volume
0.375	0.11	0.10	1.261	0.60	0.57
0.412	0.19	0.18	1.385	0.64	0.61
0.452	0.27	0.26	1.520	0.70	0.66
0.496	0.37	0.36	1.669	0.76	0.73
0.545	0.44	0.43	1.832	0.84	0.80
0.598	0.49	0.48	2.011	0.91	0.88
0.657	0.53	0.51	2.208	0.98	0.94
0.721	0.55	0.53	2.423	1.03	0.99
0.791	0.57	0.54	2.660	1.06	1.02
0.869	0.57	0.54	2.920	1.09	1.05
0.954	0.56	0.54	3.206	1.11	1.07
1.047	0.56	0.54	3.519	1.14	1.10
1.149	0.58	0.55	3.863	1.18	1.14

Channel Diameter (Lower) µm	Coulter -LockPortCo mp_01_01 .$ls Diff. Volume	Coulter -LockPortCo mp_02_01 .$ls Diff. Volume
4.241	1.24	1.18
4.656	1.30	1.24
5.111	1.35	1.28
5.610	1.37	1.30
6.159	1.34	1.27
6.761	1.27	1.19
7.422	1.17	1.07
8.148	1.06	0.96
8.944	0.98	0.90
9.819	0.95	0.91
10.78	0.97	0.97
11.83	1.03	1.05
12.99	1.12	1.11
14.26	1.21	1.11
15.65	1.27	1.07
17.18	1.29	1.02
18.86	1.27	0.99
20.71	1.21	1.00
22.73	1.12	1.03
24.95	1.04	1.05
27.39	0.97	1.04
30.07	0.93	1.00
33.01	0.92	0.95
36.24	0.91	0.92
39.78	0.87	0.90
43.67	0.80	0.88
47.94	0.69	0.81
52.63	0.60	0.68
57.77	0.58	0.54
63.42	0.68	0.46
69.62	0.93	0.51
76.43	1.28	0.75
83.90	1.62	1.14
92.10	1.72	1.53
101.1	1.47	1.69
111.0	0.98	1.52
121.8	0.60	1.19
133.8	0.51	0.96
146.8	0.80	1.01
161.2	1.51	1.40
176.9	2.40	2.05
194.2	3.01	2.71
213.2	3.12	3.13
234.1	2.83	3.22
256.9	2.41	3.06
282.1	2.11	2.78
309.6	2.00	2.50
339.9	2.05	2.30
373.1	2.20	2.24
409.6	2.34	2.31
449.7	2.44	2.50
493.6	2.44	2.70
541.9	2.36	2.76
594.9	2.16	2.52
653.0	1.85	1.99
716.9	1.47	1.31
787.0	1.16	0.80
863.9	0.92	0.39
948.3		

Appendix C

Primer/Probe Sequences, qPCR Protocols and Reagents Used in Loading Studies

Primer/ Probe Sequences

Primers	Species	Region	Forward	Reverse	Probe	*Annealing Temperature (°C)	Amplicon Length (bp)
Jerde et al. (2011)	SC	D-loop	CCTGARAAAAGARKTRTTCCACTATAA	GCCAAATGCAAGTAATAGTTCATTC		50.0	191
	BH	D-loop	TAACTTAAATAAACAGATTA	TAAAAGAATGCTCGGCATGT		53.9	312
UMESC Amberg	SC	D-loop	GGTGGCGCAGAATGAACTA	TCACATCATTTAACCAGATGCC	CCATGTCCGTGAGATTCCAAGCC	58.0	108
	BH	D-loop	GGTGGCGCAAAATGAACTAT	GCAAGGTGAAAGGAAACCAA	CCCCACATGCCGAGCATTCT	58.0	190

SC-Silver Carp
BH-Bighead Carp
*Annealing Temperature according to Thermal Gradient qPCR

Thermocycler Protocols

SYBR Green

Step	Temperature	Time
Initial Denature	95°C	10 min
Denature	95°C	15 sec
Anneal	See primer table	30 sec
Extension	70°C	1 min 30 sec
Repeat from Denature through Extension a total of 40 cycles		
Final Extension	70°C	5 sec
	95°C	5 sec

TaqMan

Step	Temperature	Time
Initial Denature	95°C	2 min
Denature	95°C	5 sec
Anneal/ Extension	See primer table	10 sec
Repeat from Denature through Extension a total of 40 cycles		

Reagent Mixes

SYBR Green

12.5 µl	SYBR GN Master Mix (ABI, # 4309155)
2.0 µl	Primer Mix (Forward and Reverse, 7.5 µM each)
5.5 µl	RNase-free dH20
5.0 µl	Sample
25.0 µl	Total Volume

TaqMan

10.0 µl	Sso Fast Supermix (BioRad, 172-5231)
2.0 µl	Primer Mix (Forward and Reverse, 7.5 µM each)
2.0 µl	RNase-free dH20
1.0 µl	Probe (2.5 µM)
5.0 µl	Sample
20.0 µl	Total Volume