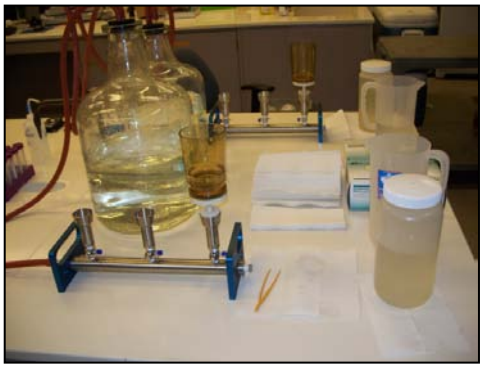
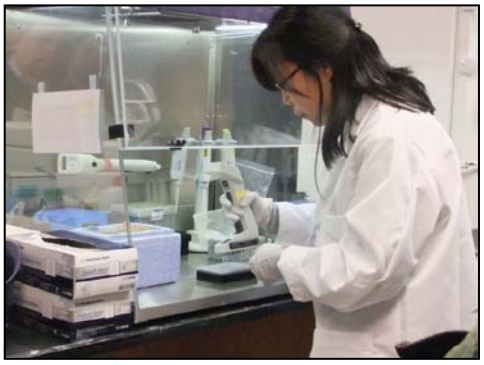
Environmental DNA Calibration Study

Interim Technical Review Report

March 2012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Preface

This report contains technical results through February 2012 from the Environmental DNA Calibration Study (ECALS). Due to the schedule of work for ECALS, there will be several sections in the document that will have no results to report until later in the study. The following have contributed to the ECALS project and the results in this report:

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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) and possibly Lake Michigan, an electrical barrier has been operating near Lockport to deter the advance of Asian carp. Although a few individuals have been detected in Lockport pool of the Illinois Waterway (IWW), the leading edge of the invasion is considered to be at RM 278 in Dresden Island Pool, 18 miles downstream from the barrier and 55 miles from Lake Michigan.

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 2011 Asian Carp Control Strategy Framework outlined major tasks to be completed for a better understanding of factors related to the advance of Asian carp populations towards the Great Lakes.

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

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

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

1.2 Project Goals, Objectives, and Products

Goal 1 is to determine the impacts of potential Asian carp vectors being a source of fish or eDNA movement in the CAWS (ACRCC Framework Item 2.5.3). The product of Goal 1 is a report and graphical representation of alternative avenues of eDNA transport with some broad conclusions on risks of positive eDNA hits from sources 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 alternate pathways
- 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.

Goal 2 is to develop high-fidelity, sensitive genetic markers for detecting the presence of Asian carp DNA in filtered water samples based on real time polymerase chain reaction (RT PCR) or quantitative PCR (qPCR) (ACRCC Framework 2.6.5). The product of Goal 2 is a report describing a set of highly polymorphic mitochondrial DNA (mtDNA) markers that provide some degree of inference as to minimum numbers of individual Asian carp within a pooled eDNA sample. Based on the ECALS WBS, the ECALS objectives under Goal 2 are:

- Objective 2.1: Sequence bighead and silver carp mitochondrial genomes

- Objective 2.2: Test new markers
- Objective 2.3: Detection of multiple alleles.

Goal 3 is to determine the relationship between the number and distribution of positive Asian carp eDNA detections with the density of Asian carp (ACRCC Framework 2.6.3). The products of Goal 3 include a robust protocol for rapid extraction and analysis of eDNA samples; detailed conversion of the current PCR band-based (i.e., presence-absence) assay to a TaqMan real-time qPCR assay; 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, zooplankton and microbial biomass, pH -- on eDNA degradation rates, with a focus on no flow water 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 (IACUC) for approval or modification); an updated/expanded hydrodynamic model of the CAWS for use as the basis to transport eDNA in the system, including influence of barges and the electrical barrier; and a model to estimate the probability that each of the potential sources of eDNA in a water body is, in fact, an actual source of eDNA in that water body, and derive the probability that an Asian carp population is present in that water body above the monitoring location. Based on the ECALS WBS, the ECALS objectives under Goal 3 are:

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

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

The purpose of this first 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.

2 Asian Carp eDNA Vectors

In addition to eDNA associated with living Asian carp, there are alternative Asian carp eDNA vectors that can transport eDNA into and within the CAWS. These alternative vectors will be the focus of this ECALS task. Initially, experts in various pertinent fields were tasked with identifying potential eDNA vectors. Field trials and experiments will be followed up on those vectors deemed most likely by experts. These tasks will determine, in some cases, whether detectable eDNA[1] can actually be transported by the proposed vector, and in other cases, the broad likelihood that eDNA would be moved upstream of the electrical barrier by the vector.

2.1 Conceptual Model

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 would not have such a need (supported by results from a USGS study on the issue). The only potential location that might be considered is a low railroad

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

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. Current US Coast Guard regulations prohibit ballast water from below the barrier to be released above 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).

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 eDNA 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 interfere with 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 eDNA survive the manufacturing process? Which companies in the region use Asian carp and how much? What is the likelihood that feed/meal-derived eDNA reaches the CAWS via livestock facilities or pet excrement? Would enough eDNA 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/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 genetic markers in the source population? Which storm sewers drain areas with fish markets? Is eDNA present within the sewers and

how long can it remain detectable? Are any eDNA hits in the CAWS upstream or downstream from the storm sewers draining the markets and was any water flowing in the sewer during/prior to the hit? Is the hit bighead or silver carp? Are relatively large fish parts being deposited into the sewer system?

- **Bait Trade**

Asian carp is popular for use as bait by trappers because it is inexpensive. A large amount of fish bait is needed for raccoons with lesser amounts for turtles. Anglers may also be using Asian carp for cut bait, with cleaning and disposing of cut bait directly into water bodies. Related pathways include bait shops, live wells, contaminated trailers, and disposal of angler-caught fish. Trapper/angler surveys in the CAWS might be useful, as well as checking with IL DNR 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 Recreational Boats**

Barge-associated activities may transport Asian carp eDNA 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 eDNA in live wells and/or bilge water.

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

- **Birds and Meso Predators**

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 it or eat it at the CAWS, or transport water contaminated with eDNA in their feathers. Research has shown that it is possible for eDNA to pass through a mammal's digestive system and preliminarily for birds (6 to 8 hour passage time). 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, and osprey. Rookery locations, home ranges, migration periods and routes, and distance from Asian carp spawning areas are important considerations of bird vectors.

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

- **Des Plaines River**

The Des Plaines River and its tributaries have isolated occurrences of Asian carp which may be a source of eDNA to the CAWS via flooding, pumping, or cracks in the karst. Flooding is a rare event, but transfer might take place during a combined sewer overflow (CSO) event when no eDNA sampling would be occurring. An ongoing USGS study is taking place in the area.

- **Bottom Sediments**

There is the possibility that a pre-existing reservoir of Asian carp tissue 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 eDNA for a while. Disturbance of the sediments would move sediment-associated eDNA into the water column. Suggested disturbances include barges stirring up the bottom, CSO events, and dredging. It was noted that minimal dredging occurs in the CAWS.

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

- **Fertilizer**

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

Additional Questions Posed: Are there fertilizer manufacturers using Asian carp in the region? Does eDNA 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 eDNA and brought into the CAWS where some eDNA could be sloughed off into the water. The extent to which these possible sources contribute 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 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 eDNA 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 very 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 eDNA 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, and waste operation areas of manufacturers who utilize Asian carp may be a source of eDNA to the CAWS.

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

- **Stock Ponds**

Ponds for recreational fishing have been stocked in the past with catfish, but may have unintentionally included bighead carp. These fish are now typically very large suggesting they've been there a long time. Records, if any, would likely be poor. Grass carp have been and continue to be stocked in golf courses; Asian carp may be unintentionally stocked here as well. Runoff from stock or golf course ponds may occur during flood conditions, transporting eDNA to the CAWS.

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

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

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

- **Environmental Degradation Factors**

Factors associated with eDNA degradation are quite numerous in the CAWS, but a number of major categories emerged from the workshop including temperature, UV 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, BOD), biological activity (eDNA bioavailability, microbial community), chloride and conductivity, pharmaceuticals, eDNA binding (e.g. sediments, eDNA-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 surface films where eDNA is known to concentrate) and water (flow rate, direction, turbulence due to flow rate and boats).

- **DNA Source Concentration**

The quantity of eDNA 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 eDNA are size-related, ranging from naked DNA to cells to larger chunks of tissue. An additional consideration is the release location of the eDNA 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 to the CAWS depends in part on factors that vary temporally. Examples include source input (e.g. barge traffic), collection ability, 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 eDNA in the sample might inhibit the extraction process (e.g. massive amounts of eDNA at a sewage plant). High concentrations of eDNA and/or extraneous material are also harder to detect with PCR (e.g. big tissue mass vs. filtered sample, interferences due to the presence of algae). 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.

Initial size of DNA in the sample. For example feces, urine, blood, slime, sperm, or larger tissue sizes.

Presence/absence of various inhibitors. Examples of inhibitors include lignins, tannins, humic acids, sewage, gut and fecal materials, algae, 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 you would seek to influence your belief that any one potential source of eDNA is the actual source of eDNA?

High Importance

- Ability of eDNA to exist in a potential vector source
- Persistence of eDNA in vector
- Quantity of eDNA present within vector
- Documented observation of AC specimens from reliable sources
- Capture of AC specimens
- Genotype assay for source identification.

Moderate Importance

- Distance of source from point of detection

- Frequency of potential release of eDNA
- Environmental conditions (e.g. water chemistry, hydrology and hydraulics, UV 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 and there is a probability for the presence of Asian carp in sites normally inaccessible to the Asian carp.
- Might want to consider more sampling, even in these areas which might not have had carp presence in the past.
- May be most logical to assess the top contributing vectors and based on their presence 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.

Using the information discussed at the November 2011 Vectors workshop, a graphical conceptual eDNA model has been drafted by the PDT (Figure 1) and represents a work in progress. Once eDNA is released from its point of origin, it is transported to another location (receiving point) by a vector. From the receiving point, a release mode for the eDNA might include a live fish, fish carcasses, or sediment, to name a few. From the release point the eDNA is subject to several environmental degradation factors through time and space. Examples of such factors include water temperature, organic matter, pH, dissolved oxygen, UV light, microbial activity, and turbulence. Finally the eDNA reaches a detection point where a monitoring sample is taken for analysis at a laboratory.

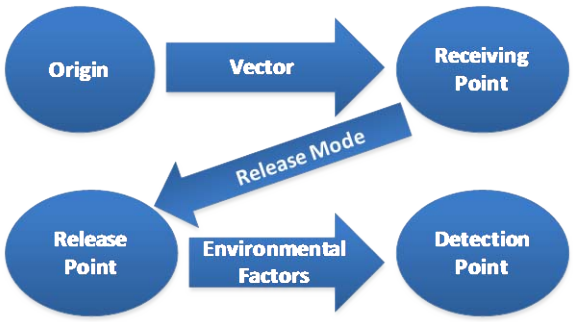

Figure 1. Asian carp eDNA draft conceptual model. The model represents the most likely possible avenues for Asian carp eDNA to be deposited upstream of electrical barriers (aside from actual fish passage of the barriers).

An example of this chain of events might be a live Asian carp at the Dresden Lock (origin) being transported to Lake Calumet (receiving point) by a gull scavenger (vector). At the receiving point the eDNA enters the environment via bird scat (i.e. a release mode). The scat eDNA's release point could be the southwest shore of Lake Calumet. The eDNA might be subject to environmental stresses including cold water temperatures and no flow conditions until a sample is taken for analysis.

The conceptual model will be refined in the future as studies addressing components of the model are studied as part of the ECALS scope of work.

2.2 Alternative Pathways

Task 1.2 activities focused on two alternative pathways for eDNA, storm sewers and fertilizers. These two pathways were chosen in part as a result of the November 2011 Vectors workshop (see section 2.1 above).

2.2.1 Storm Sewers

In October and November 2011 we executed a trial 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. 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. In the trial, 10 ice chests (volume) were half-filled with ice from ice machines at ERDC. One

Asian carp head (either silver or bighead carp) was placed in each chest. Chests were set outside and moderately shaken once every hour until roughly half the ice appeared to be melted. One liter of the “fishy ice” water was collected, and 500ml were filtered through a 934-AH glass microfiber filters following protocols described in the Asian carp monitoring Quality Assurance Project Plan (QAPP). All cooler samples were tested for the presence of Asian carp eDNA (following QAPP protocols). All 10 samples exhibited very strong eDNA bands and were confirmed via DNA sequencing. All control samples were negative for Asian carp DNA.

In a second trial, ice (approximate volume) from a chest (volume) that had held Asian carp carcasses was dumped into a storm sewer in Chicago and the sewer was flushed with water from a fire hydrant. Ten water samples were then taken from the CAWS at the point where the sewer water entered the system a few minutes later, as well as 10 samples from 1 meter distance from that point, and ten samples from the entry point 20 minutes after the end of the flush. Ten water samples had been taken from the CAWS entry prior to the experiment and after the sewer was initially flushed prior to the “fishy ice” dump. In all, 50 samples were taken.

Interestingly, the initial water samples taken prior to the fishy ice dump showed positive hits for both species, and an increased number of hits after the initial flush. This would indicate that eDNA was already present in the system, and that the amount of eDNA sampled increased after flushing the sewer (but before adding fishy ice). We suspect that either the sewer already contained eDNA, which was detected in the post-flush samples, or that the action of the water pouring out of the sewer stirred up eDNA in the sediment on the bottom of the water column. The sewer used in this effort does pass through an area with Asian restaurants that likely serve Asian carp.

The samples from the entry point after the flush, from the 1-m distance from the entry point (post-ice dump), and from the 20-minute post ice dump all showed a significant increase in eDNA signal relative to the samples taken before the ice dump. However, the extraction control and field blank sample associated the point of entry and 1-m distance samples showed evidence of contamination. Not all samples showed positive results, so it is evident that not all samples were contaminated, and the contamination would not appear to account for the increase in DNA band intensity for these samples.

In Spring 2012 we plan to expand on these efforts by conducting a new set of trials. These trials will largely follow the format of the 2011 effort, but will include direct sampling of the water flushing out of the storm sewer prior to dumping fishy ice, sampling at greater distances from the sewer outlet, and sampling at a series of time-points from 1 day to 3 weeks after the fishy ice dump and flush.

2.2.2 Fertilizers

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

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

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

We were able to filter and test volumes of both fertilizers ranging from 4.2 – 7.5 ml. Protocols for assaying the fertilizer for eDNA followed the QAPP. We did not detect eDNA. However, the volume of fertilizer we tested was very small – for example, we tested the same volume of the soil booster that would be applied to only 39 ft^2 of lawn. Significantly larger volumes could not be filtered within reasonable time frames (8 hours required to filter 7.5 ml of soil booster) and we are currently unaware of any protocols or kits that allow for efficient DNA extraction from very large volumes of viscous liquid.

It is apparent that neither of these two fertilizers contains high concentrations of detectable eDNA. However, tests of larger volumes of fertilizer may provide stronger evidence for or against such fertilizers being vectors for eDNA. Currently, we are searching for additional brands of fertilizer that may contain Asian carp. We are also seeking means by which larger volumes of liquid fertilizer can be assayed. We are unsure if we will be able to proceed beyond the trials that have already been completed.

2.3 Bird Transport and Deposition

Task 2.1 activities are scheduled to begin in April 2012.

Recently, a small number of obscure or new scientific papers demonstrating that eDNA can be detected in the excrement of birds were identified by the ECALS team. Examples of these papers included:

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

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

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

These papers will not significantly alter future subtasks addressing piscivorous birds as vectors of eDNA. 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 samples, its degradation properties, and piscivorous bird feeding and movement patterns in the Chicago region.

2.4 Asian Carp Carcasses on Barges

Task 2.1 activities are scheduled to begin in June 2012. US EPA is currently developing a Best Management Practice (BMP) for vessels that cross the barrier 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 BMP will outline the protocol for documenting these occurrences, verifying the species, and ensuring removal before the vessel crosses. This type of practice will also inform the ECALS team on the frequency of these events.

3 Asian Carp eDNA Genetic Marker Development

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

3.1 Sequencing the Asian Carp Genome

Task 2.1 activities are scheduled to begin in early 2012.

In order to develop new markers for Asian carp we need to identify the range of genetic variants that might occur in the CAWS and associated waters. We plan to assay Asian carp of both species from 9 North American populations each and one Chinese population (50 fish from each species from each population). To date we have obtained sample sets for both Asian carp species from China and partial sample sets for silver carp from the LaGrange Reach of the Illinois River and Alton Reach of the Mississippi River, and a partial sample set for bighead carp from the Alton Reach. We are currently identifying target populations, as well as other researchers that may already have samples that we can use to help meet our goals.

We have also begun to test the use of commercial kits for isolation of mitochondria from fish tissues. While not a necessary step, isolation of the mitochondria would enhance our ability to identify new regions of DNA (or "loci") for marker development. One of the challenges of working with mitochondrial DNA (mtDNA) is that there are often "junk DNA" copies of mtDNA loci located within the nuclear chromosomes (the vast bulk of each species DNA); these are sometimes known as pseudogenes or numts (*from* nuclear mitochondrial DNA). Isolation of mitochondrial DNA from the nuclear DNA (nDNA) reduces the chance that any new markers might actually be based on numt loci, not the more

desirable[1] mitochondrial loci. So far, commercial kits have not proven to be completely effective in completely isolating the mtDNA from the nDNA. We will continue to work to develop an approach for isolating mtDNA. Also note that not all new markers will be targeted for mtDNA, but will also include some nDNA loci, which will, under a limited scenario, allow us to identify hybrid fish.

Once we have the required Asian carp population samples, we will sequence the entire mitochondrial genomes and select nDNA loci of several individuals of both species. This DNA sequence data will provide the material for new marker development, and each new marker will be tested on the acquired samples.

We have also acquired multiple samples from a large number of non-target fish species (Table 1). These non-target fish will allow us to directly test new markers for specificity in detecting Asian carp, and will compliment database searches for non-target species with problematic DNA sequences[2]. We are currently seeking additional fish, both in already existing collections (e.g. university research collections) and via new collection efforts.

3.2 Testing the Asian Carp Genome

Task 2.2 activities are scheduled to begin in early 2012.

3.3 Detection of Multiple Alleles

Task 2.3 activities are scheduled to begin in July 2012.

[1] Mitochondrial DNA loci are excellent targets for eDNA markers because this kind of DNA is much more abundant in each cell than nuclear DNA and, hence, more likely to be detected.

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

Table 1. Non-target fish collections. Most were obtained during recent ACRCC Asian carp Rapid Response on Lake Calumet, IL. The ECALS goal for non-target fish is 10 individuals from each of 30 species. Currently, 29 species are represented in our collection.

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

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, which is inadequate for rapid response action in the CAWS. Fieldwork is very intensive, followed by laborious sample filtering that can take several hours. Laboratory extraction and analysis is also presently a long, intensive process. ECALS is evaluating ways to reduce time and effort for this process.

ECALS calibration will consist of sensitivity studies to determine the relationship between Asian carp size, number, and behavior on eDNA quantity and PCR detection and dispersion of eDNA in non-flowing or sluggish water. Degradation studies will evaluate the effect of environmental factors on eDNA, with validation trials taking place in artificial streams and outdoor mesocosms. Study results will be incorporated into a hydrodynamic model of the CAWS.

4.1 Increasing Efficiency and Throughput of eDNA Processing

As part of ECALS, we are seeking to identify more efficient means for obtaining eDNA isolates from water samples. In particular, there may be ways to increase the throughput of DNA extraction. Currently, DNA extraction from 24 samples requires 4 hours. The ECALS team is currently testing the use of a mini bead-beater instrument that reduces the time required for the DNA extraction lysis step by approximately 66% and provides a more pure supernatant for extraction. Additional tests of potentially more efficient extraction matrices will follow, as will independent tests of alternative protocols by the USGS Upper Mississippi Environmental Sciences Center (UMESC) and the USFWS Northeast Fishery Center (NEFC).

4.2 Optimizing Field Sampling Methods

In November 2011 the ECALS team visited the Brandon Pool of the Illinois River for two days to 1) test the relative efficacy of two sampling alternatives (QAPP protocol and fine-mesh sieving) and 2) test the relative efficacy of sampling from the top, middle, and bottom of the water column. A total of 15 sample sites were

visited. At each site we took a 2-L grab sample (QAPP protocol), a 10-L sample of water from the top of the water column pumped or poured into a PVC pipe with a fine mesh sieve cloth attached to the bottom (sieve sample), a 6-L sieve sample from the top, a 2-L sieve sample from the top, and either a 10-L sieve sample taken from the mid-column (N = 7) or bottom of the water column (N = 8). Each of these 75 samples and a few negative control samples (e.g. water blanks) were processed according to QAPP protocols. The results (Figure 2) indicated that the sieve samples worked as well or better than the grab sample, and that increasing volumes of water sampled increases the probability of detecting eDNA. The results also indicated that sampling from the top of the water column was as effective as sampling from mid-column or from the bottom of the water column. Sampling from the bottom of the water column, or along the river bottom, was problematic in that large amounts of sediment, which rapidly clogged the sieve cloth, were occasionally encountered.

The alternative sampling study provided some strong indications about the different strategies employed, but is limited by small sample size (N = 15). Additional sampling events are planned for Spring 2012. These future sampling events will provide an opportunity to optimize sieve sampling to reduce the time required to take each sample and to employ revised protocols to minimize chances for sample contamination.

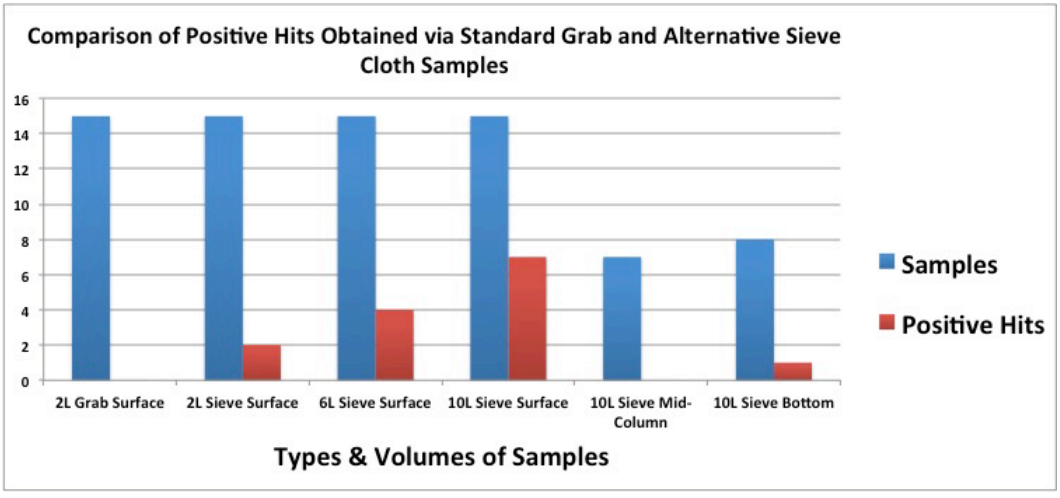

Figure 2. Results of alternative sampling trials on Brandon Pool, IL. 2-L grab samples correspond to current standard sample types (QAPP), while sieve samples employ a 40 micron mesh cloth to immediately filter water samples as they are taken.

4.3 Sensitivity Studies

Task 3.2.1 activities are scheduled to begin in early 2012.

4.4 Degradation Studies

Task 3.2.2 activities are scheduled to begin in July 2012.

4.5 Validation Trials

Task 3.2.3 (artificial streams) and Task 3.2.4 (outdoor mesocosms) activities are scheduled to begin in March 2013.

4.6 Fish Supply

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

Transportation Containers – Fish are transported from point the hatchery to ERDC in commercially manufactured "live haul" tanks carried in the bed of a ¾ ton pickup truck (Figure 3). 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 (DO), water temperature, and condition of fish are checked at 2-hour intervals. Low DO or elevated temperatures will be mitigated with compressed oxygen and ice. Any fish that die in-route are removed from the tank, placed in ice chests, and brought to ERDC for documentation. No fish is discarded during the trip. Voucher specimens of dead fish are preserved in formalin and deposited in a museum collection (e.g., Mississippi Museum of Natural Science, University of Louisiana Museum of Natural History). The remainder are desiccated and buried on-site at ERDC.

Holding Facilities – Fish are maintained in a secure laboratory facility (Figure 4) at ERDC with a closed-system of individual re-circulating tanks (Figure 5). The laboratory is approximately 400 m^2 in a building that is approximately 1672 m^2. A generator is automatically started during local electrical failures to ensure that

Figure 3. Transportation truck with aerated live wells.

Figure 4. Laboratory building where silver and bighead carp will be housed.

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

Figure 5. 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 6). The inner 3" stand pipe is elevated 42" to protect from complete tank drainage. All tanks are re-circulating. There are two tanks per filtration system with a 2.86 times per hour turnover rate. Six 800 µm bag filters act as the primary mechanical filter, 4.4 ft^3 bead filter acts as the fine particulate mechanical filter, a low space bioreactor acts as the biological filtration, and two UV sterilizers outputting 42,667 $\mu Ws/cm^2$ dosage are capable of killing microorganisms such as bacteria, viruses, molds, and algae (Figure 7). 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.

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, DO, and pH are measured using a Hydrolab multi-parameter water quality probe dedicated to laboratory use (i.e., not used in

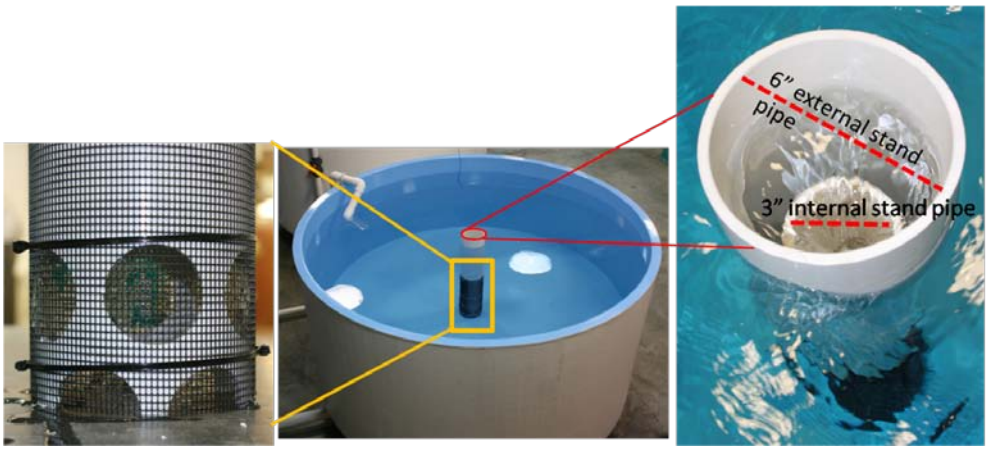

Figure 6. Recirculating tank details. From left to right: A close up image of the 1/16" mesh that covers the external stand pipe. The middle photo is an overview photo of the tank. The right image shows the 6" external stand pipe covering the 3" internal stand pipe that drains the tank.

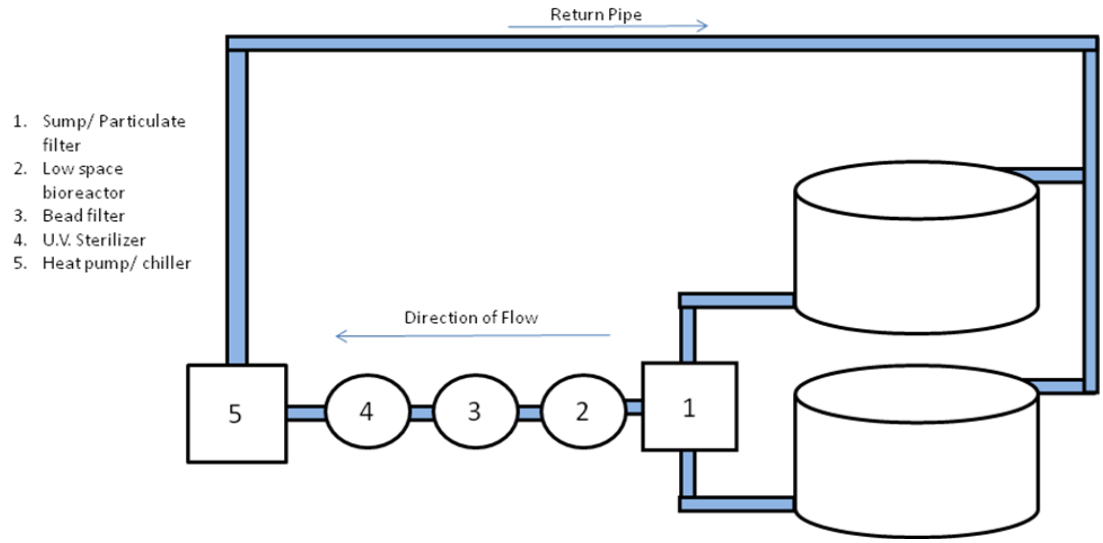

Figure 7. Recirculating aquaculture system flow drawing.

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 is food is removed after that. Fish feeding on dry foods (flakes, pellets) are fed smaller quantities 2-4 additional times during the day by mechanical battery-powered feeders (Eheim Model 3582000). Carps are fed flakes and pellets. Time of feeding is recorded and entered on experiment data sheets. Frozen and live foods and kept in a laboratory micro-fridge dedicated to that purpose.

4.7 Hydrodynamic Modeling

Task 3.4 activities are scheduled to begin in July 2012.

4.8 Probability Modeling

Task 3.5 activities are presently not funded.