ECALS: Loading Studies Interim Report July 2013

ACRCC Framework Item: 2.6.3
ECALS Project Management Plan Tasks: 3.2.1, 3.2.3 and 3.2.4
Milestone: Part 1 of a report describing shedding/loading studies
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Objectives:

1. Describe how qPCR can quantify eDNA from Asian carp and describe assay/ primer sensitivity - minimum amount of DNA that this protocol can detect (LOD) and quantify (LOQ)
2. Understand how eDNA is shed into the environment and what factors can influence this shedding rate (loading studies)
    A. Fish density (number of fish/ liter of water)
    B. Environmental factors – temperature
    C. Diet effects
    D. Spawning - eDNA loading and degradation from sperm
3. Describe how long it takes to detect eDNA signal from a point source (pond studies)

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 environmental 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 (Quality Assurance Project Plan) for the Environmental DNA (eDNA) Monitoring of Invasive Asian carp in the CAWS. 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 information 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.

In this report, 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 experiment, 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 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 higher 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. All data collection and analyses should be completed by December.

1. **Objective 1: Description of qPCR protocol for measurement of eDNA shedding rates (protocol sensitivity and eDNA sample variability)**

   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 incorporates 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.

   A. Currently we have a set of primers and TaqMan® probes designed by 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.

B. 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.

2. **Objective 2: Loading Studies**

Background: In order to understand how eDNA behaves in the environment, we must understand how it enters the system. We addressed four hypotheses that could influence the shedding rate of eDNA by these fish.

A. 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).**

B. 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**.

C. 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 hypothesized 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.

D. 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:

1. **Objective 1: Description of qPCR protocol, protocol sensitivity, and eDNA sample variability**

A. 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. Appendices A – C list 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.

2. Objective 2: Loading Studies – quantification of eDNA shedding rates

A. Experimental set up
   a. 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.
   b. 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.
   c. 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.
   d. For the diet study, a total of four treatments: no food, low feeding rate of algae (soft food), high feeding rate algae (soft food), and low feeding rate of brine shrimp (rough crustacean food). Each treatment had three replicates. Three fish were placed in each tank.
   e. 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 and sperm counts were calculated. 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”) before running sperm counts. After the sperm counts, 500 ul 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. Below surface samples (50 ml) were collected using a clean serological pipette.

B. qPCR

a. Sample processing:

i. 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.

ii. 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 ul of the extraction TDS0 buffer (AutoGen Inc., Holliston, MA). Samples were then frozen until extracted.

iii. Samples were digested using Proteinase K (AutoGen Inc. Holliston, MA) and left overnight in 55°C water bath.

iv. Samples were extracted with an AutoGen (AutoGen Inc. Holliston, MA) automated robot, using a phenol chloroform extraction method.

b. 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).

C. 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:

1. **Objective 1: Description of qPCR protocol for measurement of eDNA shedding rates (protocol sensitivity and eDNA sample variability)**

    A. We found the UMESC primers to be more sensitive than the Jerede et al. (2011) markers. As shown in Table 1, the UMESC primers amplified genomic DNA extracts more efficiently. 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 ul reaction), thus we can quantify ~40 copies/ul. Our limit of detection (LOD) for both Silver Carp and Bighead assays was our 10^1 standard (~20 copies/ 5 ul reaction). Therefore, we can detect around 4 copies of DNA/ul. These numbers vary slightly depending on the concentration of the plasmid stock. For further analyses, any samples with detectable eDNA at concentration below the LOQ were assigned a quantity of half the LOQ.

    B. For the preliminary study, a scatter plot (Figure 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 2).

2. **Objective 2: Loading Studies – quantification of eDNA shedding rates**

    A. Fish Density

    All four density experiments showed a significant correlation between eDNA shedding rate and density (Figure 2a) (Table 3). Running a one-way ANOVA using fish density as a factor, all analyses showed significant or near significant treatment differences. Post-hoc pairwise comparisons using the Bonferroni correction showed that 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 3). We also looked at eDNA shedding rates and total length or total weight of all fish in each tank (Figures 2b and 2c). 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 asses this.

B. Temperature

Shedding rate did not correlate with temperature for either carp species (Figure 3), 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 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 treatment.

C. Diet

These studies are currently being analyzed. During preliminary analyses, we discovered that the polymerase chain reaction was inhibited in samples from the tanks with the algae-fed 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 fish fed brine shrimp were not inhibited. Currently we are diluting the inhibited samples which can remove the inhibitory effect. This will allow us to quantify these samples; however, quantification sensitivity is reduced. Results from these studies will be reported in fall 2013.

D. 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). However, even up to the 21st day, some eDNA was detectable.

Discussion:

1. **Objective 1: Description of qPCR protocol, protocol sensitivity, and eDNA sample variability**

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 conditions. 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 peak in eDNA shedding rates observed during the first 1.5 weeks in the preliminary study (Figure 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.

Objective 2: Loading Studies – quantification of eDNA shedding rates

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.

Although the diet studies are still underway, preliminary analyses found that PCR inhibition can be a major contributor to false negatives. Interpretation of current field sampling efforts may be hindered by false negatives. Research in water and food quality use positive internal controls to assess PCR inhibition in samples, as the consequences of false negatives could lead to human health hazards. We view the incorporation of a positive internal control as a vital component to eDNA monitoring efforts, as false negatives are detrimental to detection efforts. Inhibition controls must be used to determine which samples are inhibited and should not be used for inference of carp presence. Dilutions and further purification of samples may allow for recovery of the polymerase chain reaction, but both of these options also lead to loss of sensitivity. Field monitoring programs should address the identification of inhibited samples and how to deal with these samples.

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. 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.

Summary:

1. 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).

2. The eDNA signal can be highly variable, likely reflecting clumped eDNA distribution.

3. We found no correlation between water temperature and eDNA shedding rates.

4. We observed a correlation between eDNA loading and fish density. We expect to see a stronger correlation when larger treatment differences are used in upcoming pond studies.

5. 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.

Acknowledgments:

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Literature Cited

Dejean, T., Valentini, A., Miquel, C., Taberlet, T., Bellemain, E., and Miaud, C. 2012. Improved detection of an alien species through environmental DNA barcoding: the example of the American Bullfrog Lithobates catsebeianus. Journal of Applied Ecology. 49(4): 953-959.

Ficetola, G. F., Miaud, C., Pompanon, F., and Taberlet, P. 2008. Species detection using environmental DNA from water samples. Biol. Lett. 4(4): 423-425.

Foote, A.D., Thomsen, P. F., Sveegaard, S., Wahlberg, M., Kielgast, J., Kyhn, L. A., Salling, A. B., Galatius, A., Orlando, L., and Gilbert, M. T. 2012. Investigating the potential use of environmental DNA (eDNA) for genetic monitoring of marine mammals. PLOS one. 7(8).

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

Takahara, T., Minamoto, T., Yamanaka, H., and Kawabata, Z. 2012. Estimation of fish biomass using environmental DNA. PLOS One. 7(4).

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

Genomic DNA

		Medium SC	Low SC	Medium BH	Low BH
	Concentrations	1.1 ng/ reaction	0.11 ng/ reaction	1.2 ng/ reaction	0.12 ng/ reaction
Primers					
Jerde SC		26.36 cycles at 53.9°C	30.32 cycles at 53.9°C	36.38 at 53.9°C	N/A
Jerde BH		> 40 cycles at 50.0°C	N/A	33.05 cycles at 50.0°C	>40 cycles at 50.0°C
UMESC SC		23.75 cycles at 58.0°C	27.27 cycles at 58.0°C	25.29 cycles at 58.0°C	N/A
UMESC BH		28.03 cycles at 58.0°C	N/A	22.29 cycles at 58.0°C	27.17 cycles at 58.0°C

Table 2. Amounts of eDNA (copies of eDNA/ L) and shedding rates (copies of eDNA/Hr) for fish kept at three different flow through rates.

Flow Rate	1L/Hr	2 L/Hr	3 L/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

Table 3. Regression and ANOVA statistics for all temperature and density loading studies. Significant post-hoc pairwise comparisons are shown with high, medium and low representative of the temperature or density treatment.* statistically significant ($p < 0.05$)

		Temperature Silver Carp Sub-Adult	Temperature Bighead Carp Sub-Adult	Density Silver Carp Juvenile	Density Silver Carp Sub-Adult	DensityBighead Carp Juvenile	DensityBighead Carp 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	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 comparisons		Medium v. Low		High v. Low	High v. Low	High v. Low	High v. Low
	p	0.05		0.03*	0.04*	0.06	<0.01*
							Medium v. Low
							<0.01*

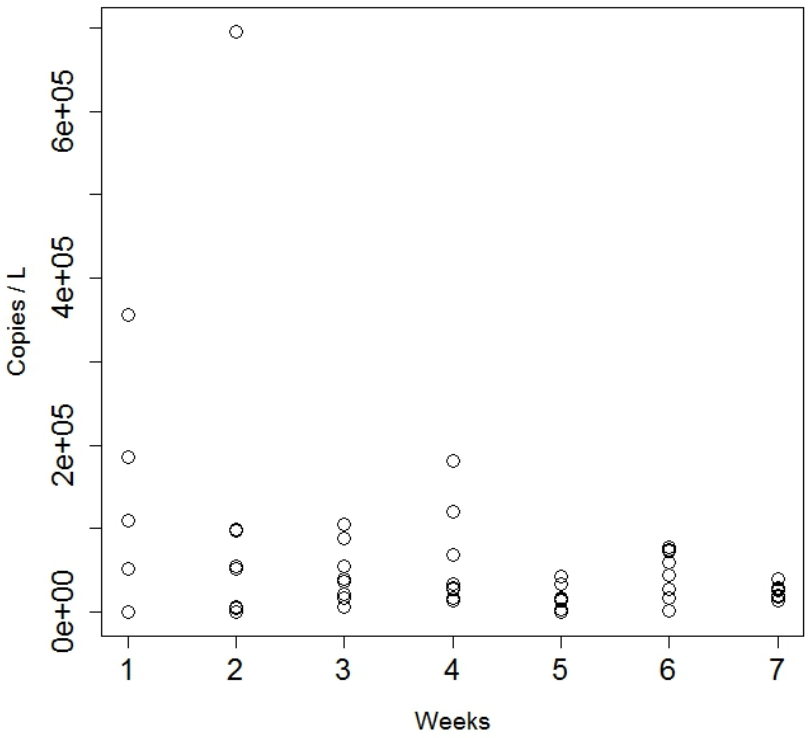

Figure 1. Scatter plot from preliminary study showing variation of eDNA sample quantification (copies of eDNA / L versus sampling week). Fish were added to the tank at the start of week 1.

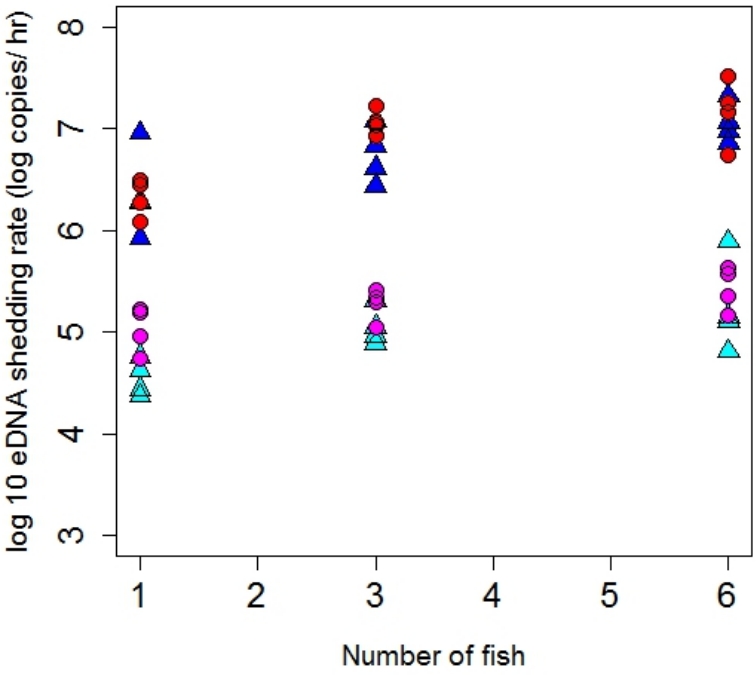

Figure 2a. Scatterplot of fish density (number of fish per tank) and eDNA shedding rates for Silver Carp and Bighead Carp. Dark Blue Triangles – Silver Carp sub-adults; Light Blue Triangles – Silver Carp juveniles; Red Circles- Bighead Carp sub-adults; Pink circles – Bighead Carp juveniles

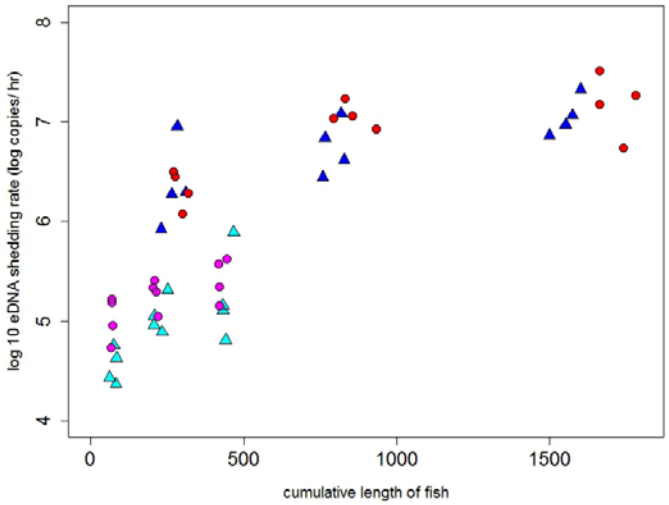

Figure 2b.

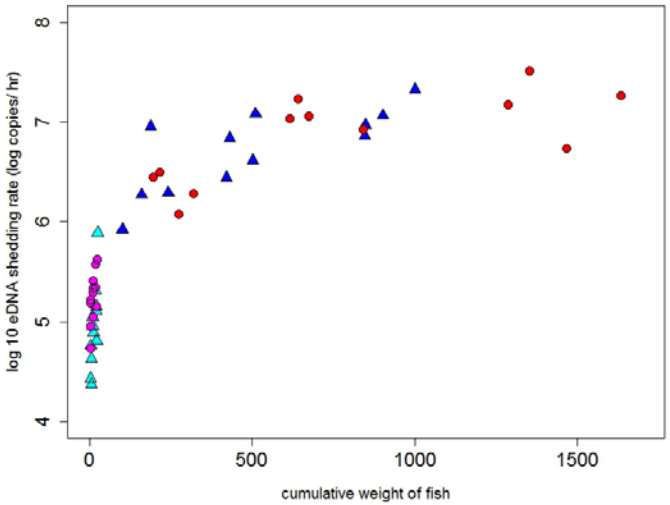

Figure 2 c.

Figure 2 b and c. Shedding rates of eDNA for two age classes of two species. Figure 2 b shows mean eDNA shedding rates versus total length of fish per tank. Figure 2 c shows mean eDNA shedding rate versus total weight of fish per tank. Dark blue triangles – Silver Carp sub-adults; Light blue triangles – Silver Carp juveniles; Red circles- Bighead Carp sub-adults; Pink circles – Bighead Carp juveniles

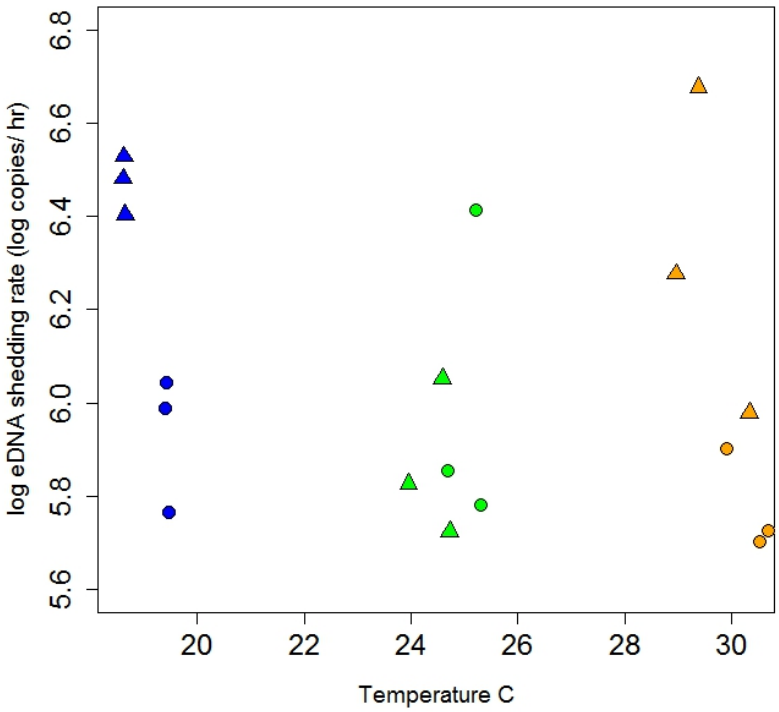

Figure 3. Scatterplot for the regression of eDNA shedding rate and temperature for both Silver Carp (colored triangles) and Bighead Carp (colored circles). Color is indicative of the treatment type (low , medium, high temperature), but the regression was run using average tank temperature as a continuous variable.

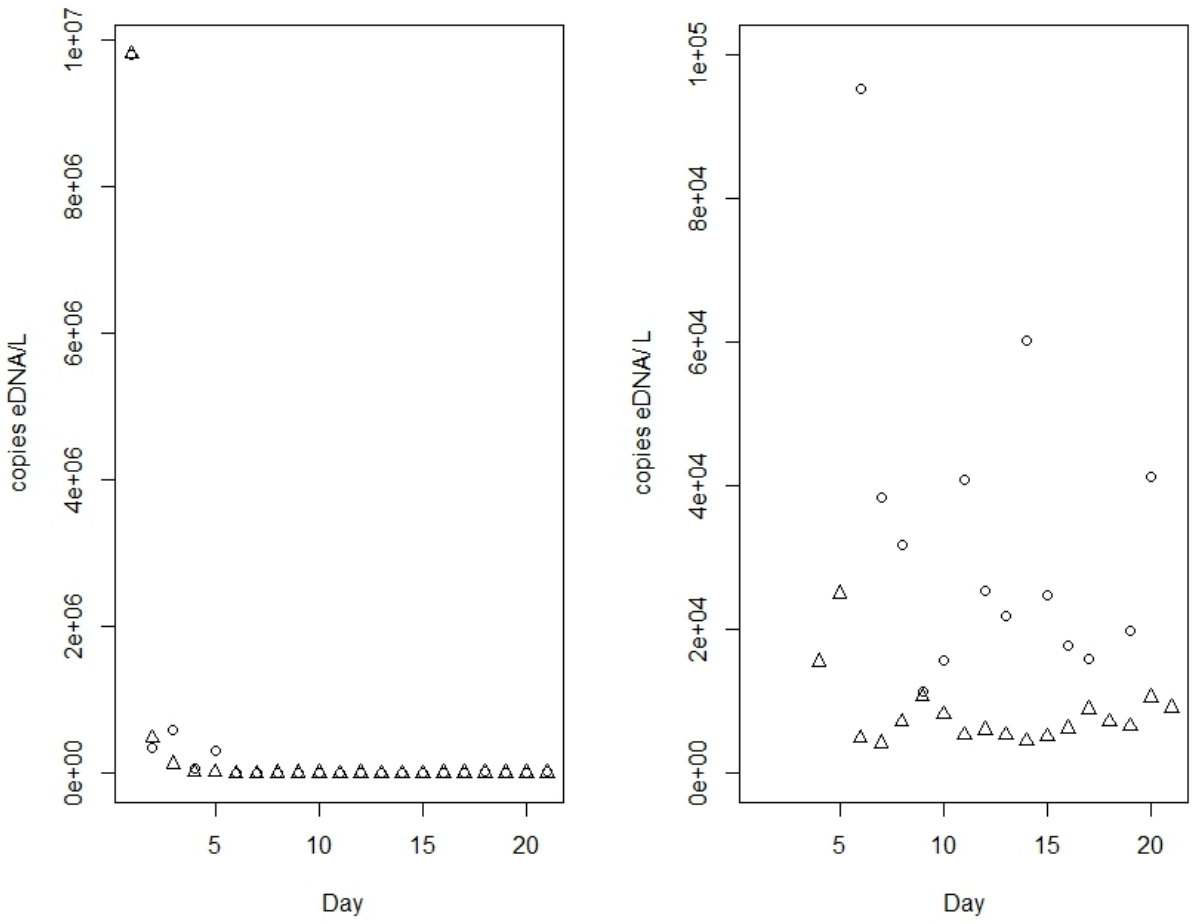

Figure 4.

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

Appendix A: 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

Appendix B: Thermocycler Protocols

SYBR Green

Step	Temperature	Time
Initial Denature	95°C	10 min
Denature	95°C	15 sec

TaqMan

Step	Temperature	Time
Initial Denature	95°C	2 min
Denature	95°C	5 sec

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

Appendix C: Reagent Mixes

SYBR Green

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

TaqMan

10.0 ul	Sso Fast Supermix (BioRad, 172-5231)
2.0 ul	Primer Mix (Forward and Reverse, 7.5 uM each)
2.0 ul	RNase-free dH20

1.0 ul	Probe (2.5 uM)
5.0 ul	Sample
20.0 ul	Total Volume