ECALS MILESTONE REPORT

ACRCC Framework Item: 2.6.3 eDNA Calibration and Increased Efficiency

ECALS Project Management Plan Tasks: 3.2.2.1, 3.2.2.3, 3.2.2.4, 3.2.2.5

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; Figs. 1, 2, and 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 address the following as factors influencing eDNA degradation: water temperature (3.2.2.1), light exposure (3.2.2.2), water pH (3.2.2.3), microbial loads (3.2.2.4), water energy (turbulence; 3.2.2.5), total organic content of water (3.2.2.6), and dissolved oxygen in the water (3.2.2.7).

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).

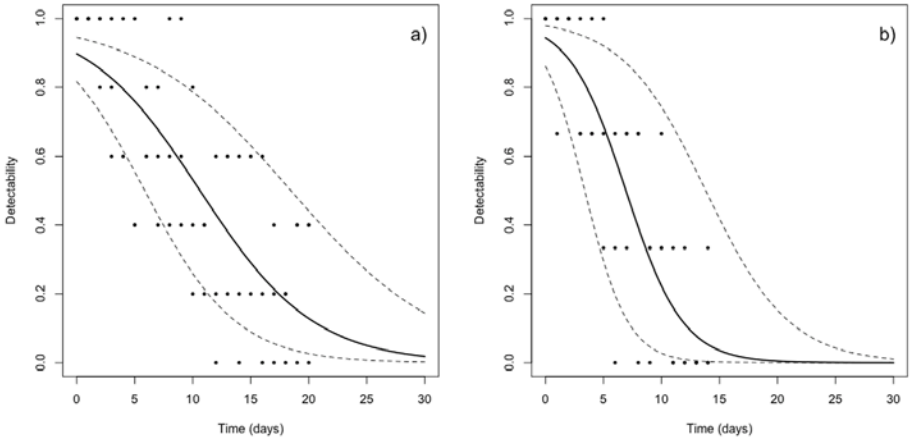


Fig. 1. From Dejean et al. 2011. DNA degradation rates following removal of a) frog tadpoles from beakers and b) sturgeon from naturalistic outdoor mesocosms. "Detectability" is the proportion of conventional PCRs with positive results (bands) on an agarose gel at each time point.

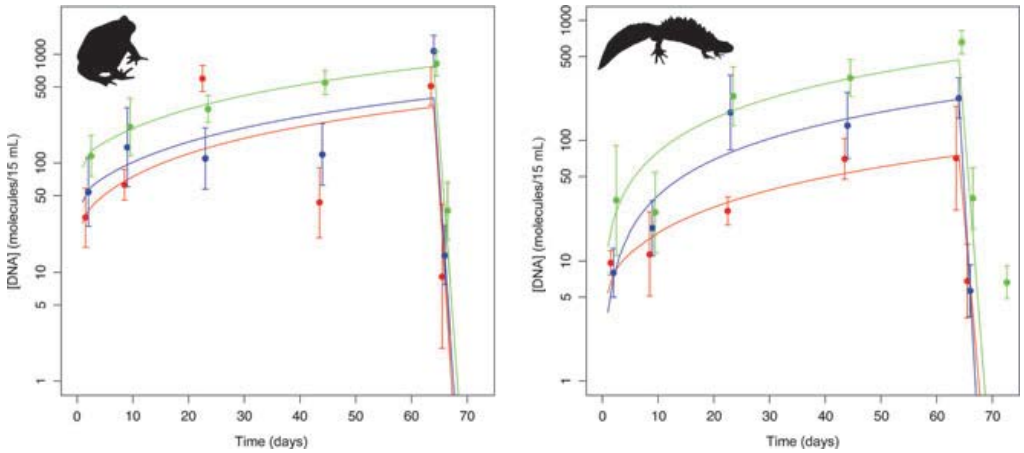


Fig. 2. From Thomsen et al. 2011. DNA degradation rates following removal (on Day 64) of larval toads and (left) and newts (right) from outdoor mesocosms.

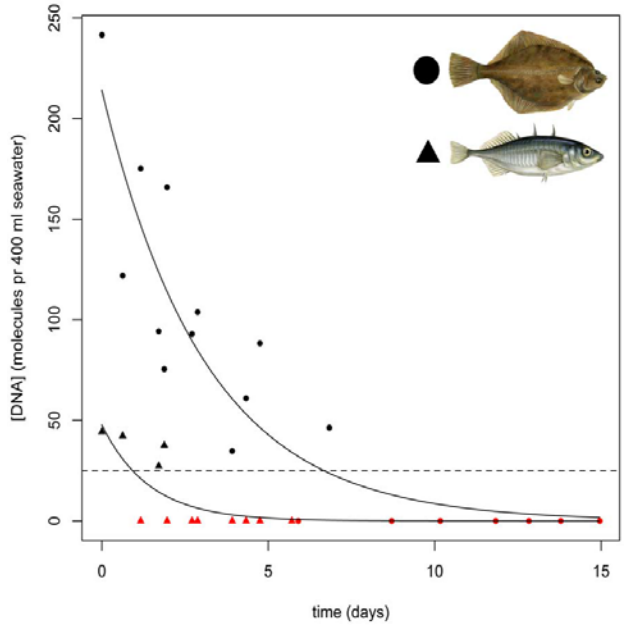


Fig. 3. From Thomsen et al. 2012. Temporal change in estimated eDNA abundance in large water sample. Aliquots from larger sample removed and assayed with quantitative PCR (qPCR) for two species of marine fish (*Platichthys flesus* and *Gasterosteus aculeatus*) at several intervals over 15 days. Red symbols 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.

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. Also, DNA and eDNA are used somewhat interchangeably in this report

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". 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 the 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 negative controls, was then subjected to 3 replicate qPCRs using primers and a probe developed by the eCALS team for bighead carp (Table 1), a commercial qPCR kit reagent mix (Table 2), and a standard qPCR thermal-cycle program (Fig. 4).

Table 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 2. Taqman qPCR Master Mix and Cycling Conditions:

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

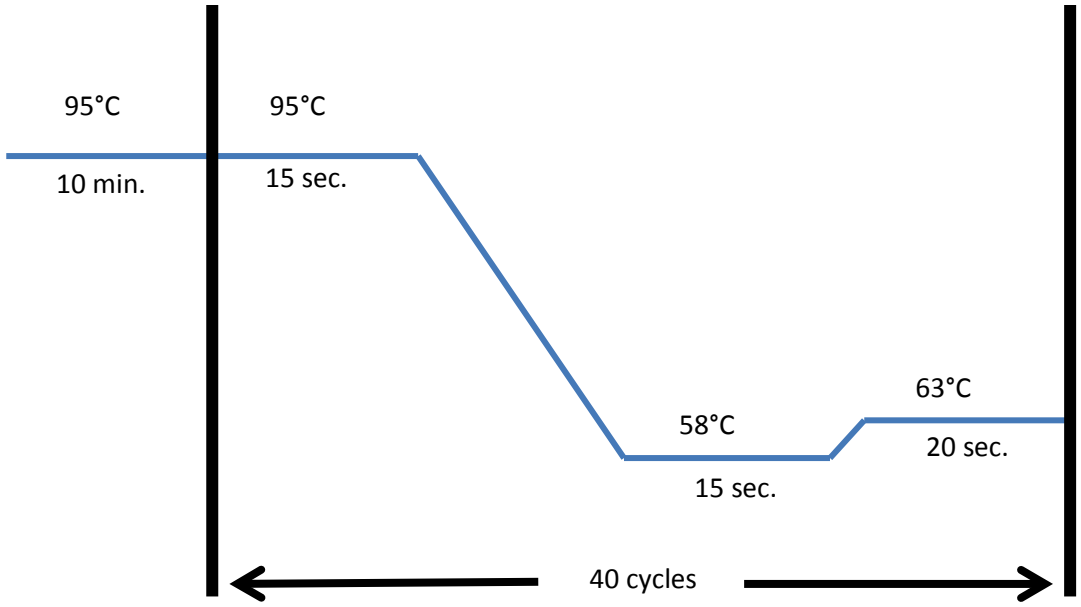


Fig. 4. Thermal-cycle program utilized for qPCR assays of target DNA abundance in degradation trials.

3.2.2.1. 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° 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, paired t-tests with treatment, day, and treatment x day interactions as factors, and with a pairwise Tukey Honest Significant Difference calculations using the

stats package of R (version 2.15.0; R Development Core Team 2012). This same basic analytical approach was applied in all trials.

3.2.2.3. 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.

3.2.2.4. Characterize Influence of Microbial Loads on eDNA Degradation

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 non-eutrophic 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.

3.2.2.5. 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 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^{o} C until DNA extraction. Samples were extracted using the CTAB protocol and then assayed with qPCR as described above.

RESULTS

Baseline eDNA Degradation

DNA degraded quickly under baseline conditions (Fig. 5), 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.

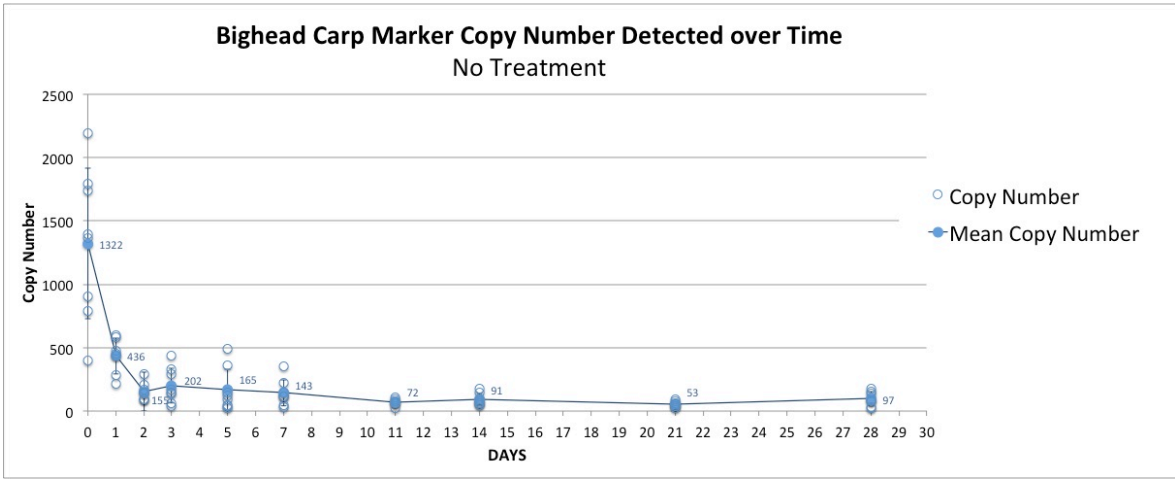


Fig. 5. Degradation of DNA from Asian carp slurry over 28 day span. The slurry was comprised of mixed filtrate (slime, feces, food, etc.) from tanks holding bighead carp.

3.2.2.1. Characterize Influence of Temperature on eDNA Degradation

DNA degraded quickly under all four temperature treatments (Fig. 6), 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 3), with a strong trend for slower degradation (more DNA across time) in the 4^{o} C class and more rapid degradation in the 30^{o} C class.

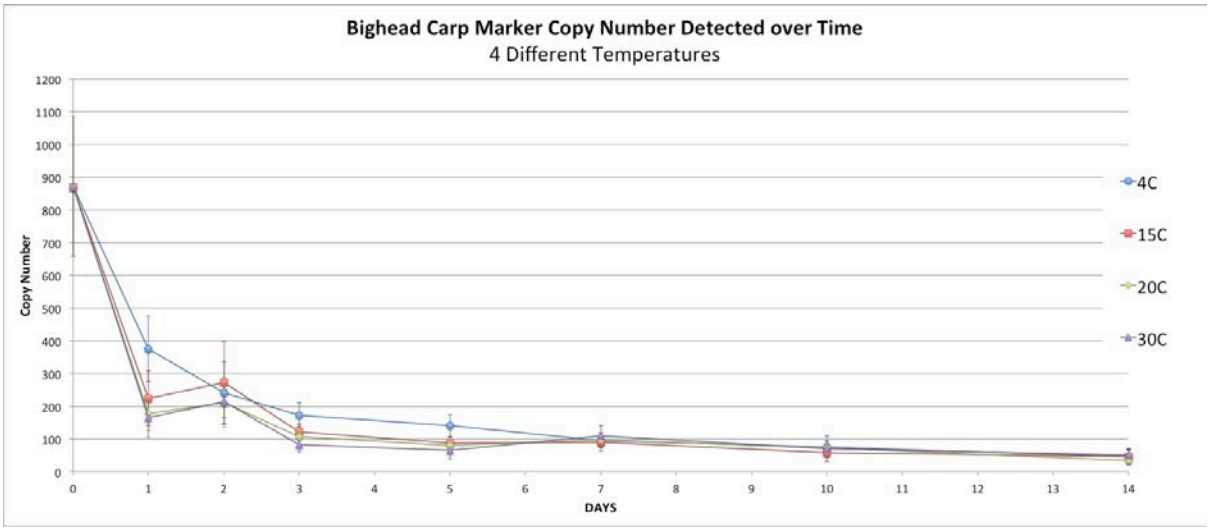


Fig. 6. Degradation of DNA from Asian carp slurry over 14 day span at 4 different water temperatures. DNA abundance for the 4° C (4C) treatment class at Day 1 was significantly greater than any other temperature class, while DNA abundances for the 4° C treatment class were significantly greater than DNA in the 30° C (30C) treatment class on Day 3 and Day 5.

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

Table 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.

An apparent increase in DNA abundance on Day 2, likely due to changing concentrations 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 degrading 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.

3.2.2.3. Characterize Influence of pH on eDNA Degradation

DNA degraded less rapidly in these trials (Fig. 7) 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, likely due, again, to changing influence of PCR inhibitors. Overall, pH significantly influenced degradation ($p = 0.004$), and in particular, the pH class 6.5 did exhibit significantly slower degradation than the pH classes 7.5 ($p = 0.024$) and 8.0 ($p = 0.003$).

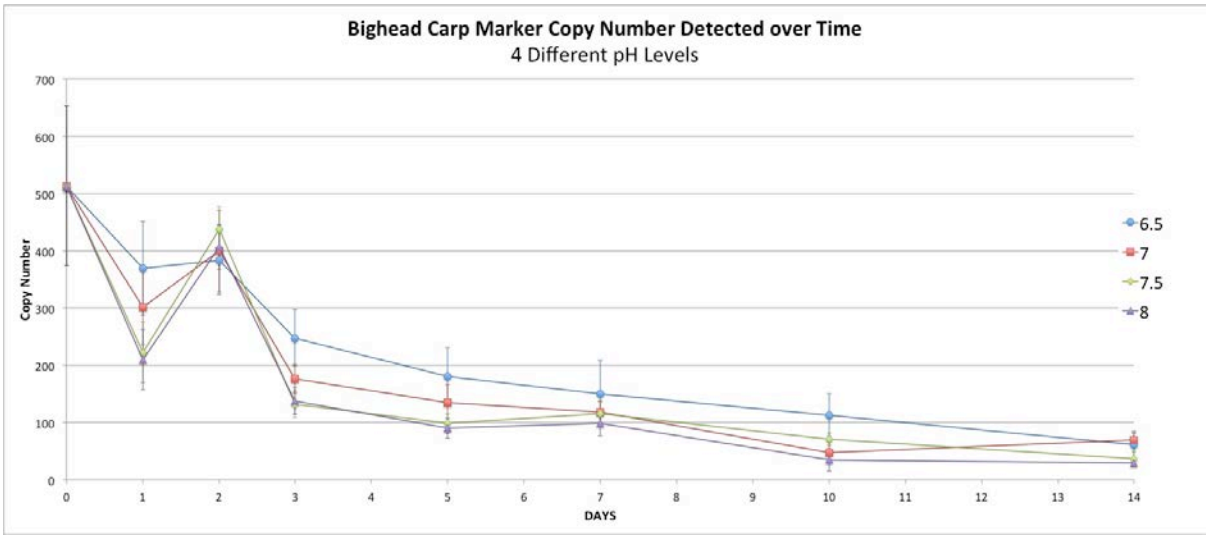


Fig. 7. Degradation of DNA from Asian carp slurry over 14 day span at 4 different pH levels. DNA abundance for the pH = 6.5 treatment tended to degrade at a slower rate than the other treatment classes.

3.2.2.4. Characterize Influence of Microbial Loads on eDNA Degradation

DNA degraded very rapidly in these trials (Fig. 8) 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 ($p = 0.655$).

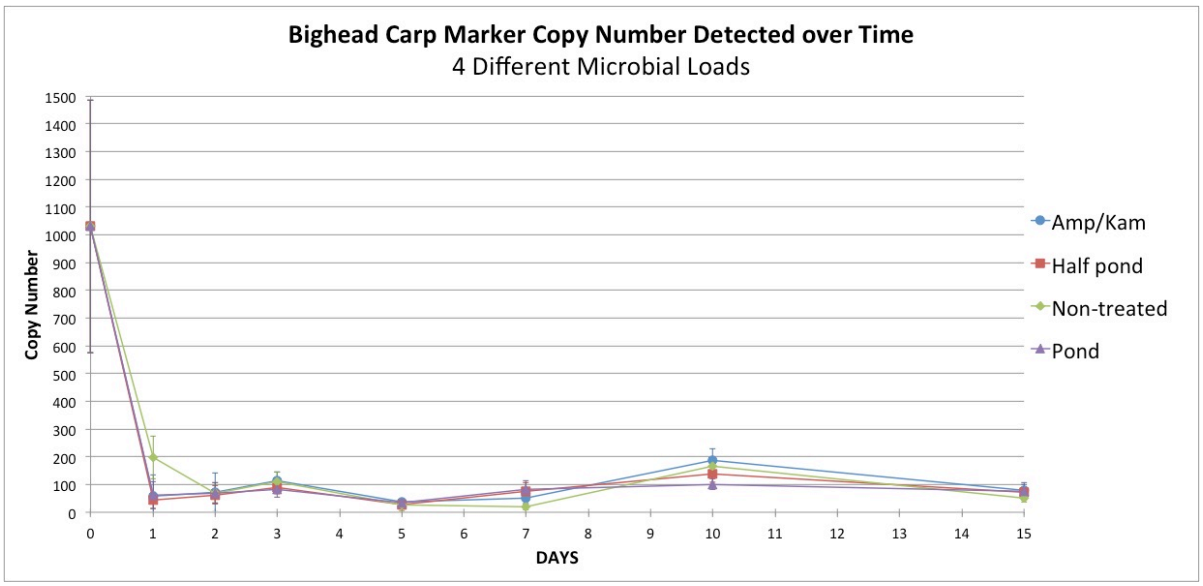
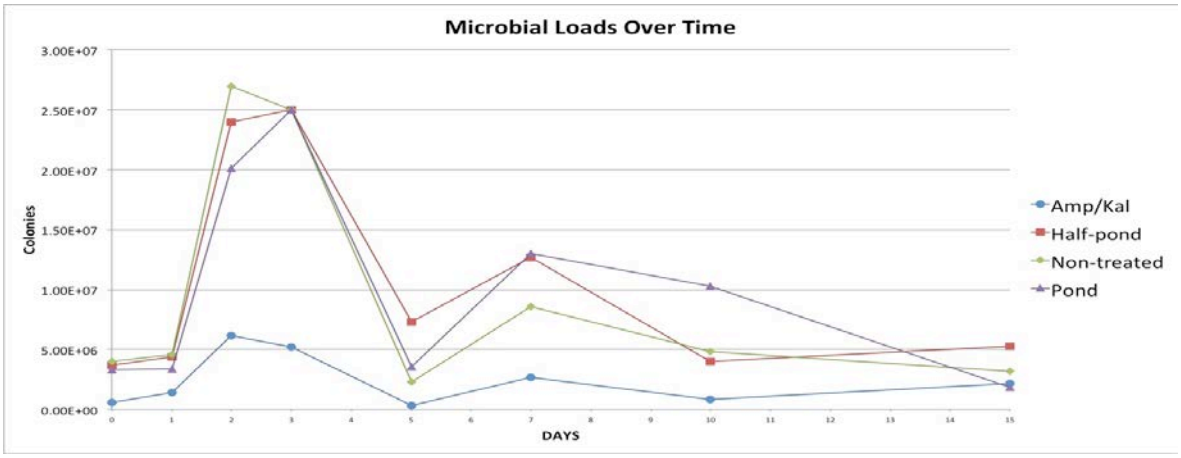


Fig. 8. Degradation of DNA from Asian carp slurry over 14 day span at 4 different microbial load levels.

The ampicillin-kanamycin treatment class had a much smaller estimated microbial load relative to the other treatment classes (Fig. 9). 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 biases 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 assume inter-treatment biases to be minimal.

Fig. 9. Differences in microbial loads (colony forming units) across treatments and days.



3.2.2.5. Characterize Influence of Water Energy (Turbulence) on eDNA Degradation

DNA degraded less rapidly in this trial (Fig. 10) 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, 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).

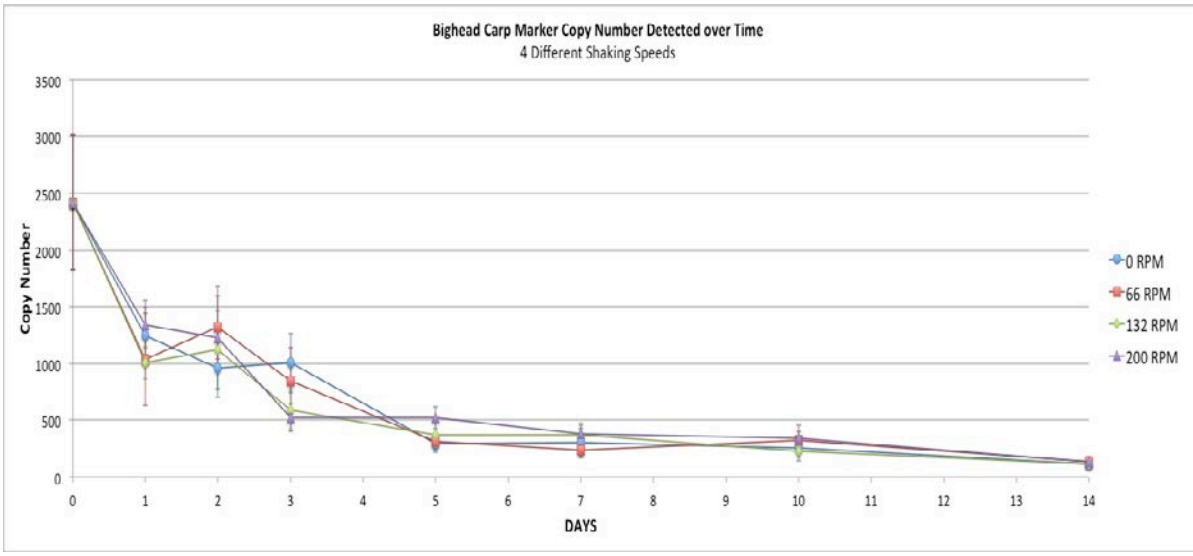


Fig. 10. Degradation of DNA from Asian carp slurry over 14 day span at 4 shaking speeds on orbital shakers.

DISCUSSION

Degradation rates in our trials exhibited similar patterns to those observed in Thomsen et al. (2011) where eDNA abundance declined rapidly over the course of a 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[1] for successful detection (Jerde et al. 2012), we would have observed a gradual decrees in detections over the course of a number of days, as seen in Dejean et al. 2011. There were two interesting features of the combined trials that have been completed – 1) the persistence of a small percentage of DNA (~ 10%) for longer than two to four weeks and 2) a frequently observed "spike" in DNA abundance within the first 2-3 days of each trial, as well as, in fewer cases, a spike around Day 7 or Day 10.

The persistence of a small percentage of the eDNA over longer time spans in our trials reflects the observed detection of eDNA over the course of 2-3 weeks in other trials (Dejean et al. 2011). In our case, we did not observe a complete eradication or degradation of eDNA to undetectable levels, even after 2-4 weeks, depending on the trial length. The persistence of eDNA when multiple environmental factors are acting in concert could be much shorter than we observed, though Dejean et al. 2011 might indicate otherwise. This question will be in part answered by upcoming Task 3.2.2.8, in which slurry will be exposed to what would likely be the "most degradative" conditions – combined high temperature, high pH, etc. The capacity of a slowly degrading fraction of eDNA to accumulate over time or to be protected from degradation through binding with other substrates will be of particular importance to future applications of this tool. The potential for eDNA to become sequestered and less bioavailable in sediment is the focus of another ongoing eCALS task.

Of all the factors tested to date, -- water temperature, pH, microbial loads, and turbulence, only water temperature and pH appear to have significantly influenced on degradation rates. Warmer water and

[1] Caution must be taken in extrapolating these results to the amount of target eDNA in a sample that would be required for detection. DNA extraction and purification typically recover much less than 100% of target DNA in a sample (e.g. Mumy and Findlay 2004) and environmental samples may have particularly low yields. In a scenario with 30% target DNA recovery after extraction and purification, which is common, a solution of 200 copies per microliter (µl) in a standard 100 µl solution of purified DNA resulting from extraction would require over 66,000 copies of target DNA be present in a sample. In a standard 2 liter eDNA sample, this number corresponds to 33 copies/ml.

higher pH (within the range of 6.5 to 8.0) were associated with higher degradation rates. On a basic level, colder temperatures reduce the rates of both molecular interactions and biological activity – reducing the rates of both abiotic and biotic factors in degradation. DNA is generally stable at moderate pH, though in our case we appear to see an increase in degradation at higher pH. One factor may be the stabilization of the DNA molecule or it's protection from microbial nucleases following binding to other environmental molecules or particles, such clays, humic acids, or fulvic acids (*reviewed in* Levy-Booth et al. 2007). The binding affinity of DNA to such particles is influenced by pH, with, in many cases, greater binding affinities at lower pH (Levy-Booth et al. 2007).

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 sampling 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 affect on the occurrence or magnitude of the spikes on Day 3 and Day 10 of our microbial load trials (Fig. 7), we now suspect that abiotic breakdown of inhibitors may be the primary factor driving this pattern. To evaluate this hypothesis we are conducting baseline assessments of the changing chemistries of slurry and extract samples from different time points.

Understanding the degree to which our degradation results to date have been influenced by PCR inhibitors and the nature of those inhibitors (if present) will be important components of our remaining efforts.

Remaining Efforts

There are still four planned tasks that have yet to be executed:

- Characterize Influence of Light on eDNA Degradation (3.2.2.2)
- Characterize Influence of Water Organic Matter on eDNA Degradation (3.2.2.6)
- Characterize Influence of Dissolved Oxygen on eDNA Degradation (3.2.2.7)
- eDNA Degradation Under Extreme Degradative Conditions (3.2.2.8)

Additionally, we have identified two tasks that we believe are salient to the work done to date and the interpretation of results.

- Determination of the degree to which the results of degradation trials (baseline degradation, 3.2.2.1-7) may have been influenced by PCR/qPCR inhibitors and adjust DNA abundance estimates.
- A chemical analysis of degradation trial-type samples in an attempt to discover any cofactors that may be responsible for qPCR inhibition.

Study designs for 3.2.2.2 and 3.2.2.7 are in the planning stage. Neither trial will be able to utilize the design (numerous individual 14-ml samples randomly selected at different sampling points) used for the preceding trials, because of the need for equivalent exposure of all samples to limited light sources, the potential for tube walls to block some wavelengths of light, and the need to regulate dissolved oxygen (DO) in each sample. Currently we are experimenting with means for achieving DO levels within of the range recorded for the CAWS in 2011, some of which are low enough (average minimum ≈ 3.3 mg/L, average maximum ≈ 10.8 mg/L) that simple regulation of oxygen input may be inadequate. Task 3.2.2.8 will be the final trial.

We suggest that effort that might be spent in pursuing 3.2.2.6 (water organic matter) might be better employed in examining and accounting for the influence of inhibitors on our results. Water organic matter would largely influence degradation indirectly though its influence on microbial populations, which have been shown to have little effect in our trials. Understanding inhibitors, however, will improve the accuracy and interpretation of the results of our degradation studies, as well as provide salient information for future eDNA studies and monitoring efforts.

Summary

We have completed five of nine tasks initially planned for degradation studies. Degradation rates and patterns observed in our studies are similar to those observed in the few eDNA degradation results published to date. We have identified temperature and pH as factors that can affect degradation, particularly that low temperature and pH are associated with slower 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 or 28 days). We have also, 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. We have identified 2 additional tasks focused on qPCR inhibitors that should enhance study results and recommend that these take the place of degradation trials focused on water organic content (which would likely provide redundant information).

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