DEPARTMENT OF THE ARMY
U.S. ARMY CORPS OF ENGINEERS
441 G STREET, NW
WASHINGTON, DC 20314-1000

REPLY TO
ATTENTION OF

SEP 7 2011

CECW-LRD

MEMORANDUM FOR ASSISTANT SECRETARY OF THE ARMY (CIVIL WORKS)

SUBJECT: Environmental DNA (eDNA) Science and Methodology – Final USACE Response to Independent External Peer Review

1. References:

a. Memorandum, CELRD-RB, 6 July 2011, subject: eDNA IEPR Agency Response and the eDNA IEPR Revised Final Report.

b. Revised Final Independent External Peer Review Report Environmental DNA (eDNA) Science and Methodology, December 7, 2010.

2. An Independent External Peer Review (IEPR) was conducted for the subject science and methodology in accordance with Section 2034 of the Water Resources Development Act of 2007, EC 1165-2-209, and the Office of Management and Budget's Final Information Quality Bulletin for Peer Review (2004).

3. The IEPR was conducted by Batelle Memorial Institute. The IEPR panel consisted of four panel members with technical expertise in genetics and population ecology.

4. The final written responses to the IEPR are hereby approved. The enclosed document contains the final written responses of the Chief of Engineers to the issues raised and the recommendations contained in the IEPR Report. The IEPR Report and USACE responses have been coordinated with the vertical team and will be posted on the internet, as required in EC 1165-2-209.

5. If you have any questions on this matter, please contact me or have a member of your staff contact Mr. Jay Warren, Planner, Great Lakes and Ohio River Division Regional Integration Team at 202-761-4589.

FOR THE COMMANDER:

Encl

STEVEN L. STOCKTON, P.E.
Director of Civil Works

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Environmental DNA (eDNA) Science and Methodology
FINAL
USACE Response to Independent External Peer Review
September 2011

In accordance with Section 2034 of the Water Resources Development Act (WRDA) of 2007 and Engineer Circular 1165-2-209, the U.S. Army Corps of Engineers (USACE) conducted an Independent External Peer Review (IEPR) of the Environmental DNA (eDNA) scientific method developed and implemented by researchers at the University of Notre Dame (UND). USACE is employing the eDNA scientific method as a surveillance tool to indicate the genetic presence of invasive bighead carp (*Hypophthalmichthys nobilis*) and silver carp (*H. molitrix*), two species of Asian carp, as one component of an interagency monitoring plan implemented in the vicinity of the Aquatic Nuisance Species Barrier at Romeoville, Illinois. Currently, eDNA is used by the Asian Carp Regional Coordinating Committee (ACRCC) in the Monitoring and Rapid Response Plan (MRRP) developed by its interagency subgroup, the Monitoring and Rapid Response Work Group (MRRWG). The MRRP is an adaptive document and the sampling strategies are based on the data received and analyzed by the interagency team, of which USACE is a member.

USACE engaged Battelle Memorial Institute, a non-profit science and technology organization with experience in establishing and administering peer review panels, to coordinate the IEPR. The IEPR of the eDNA science and methodology focused on the scientific ideas, methods, and evaluations; it did not include an assessment of management decisions or actions.

USACE solicited review and comments from the IEPR Panel on specific sections of electronic versions of UND eDNA science and methodology documents. A teleconference and an in-person meeting between USACE, Battelle and the IEPR Panel were held to discuss the eDNA methodology. Overall, the IEPR Panel identified and documented 8 final comments in their final report. Of the 8 comments, two were identified as having high significance, and the remaining six were identified as having medium significance. Each review comment included an explanatory discussion and recommendation for resolution.

The eDNA method was developed and implemented by researchers at the University of Notre Dame (UND) as a monitoring tool for Asian carp in the Chicago Area Waterways System (CAWS) under a Cooperative Agreement with the U.S. Army Corps of Engineers (USACE), beginning in late summer 2009. Both USACE and UND contributed to the review of and response to comments provided by the IEPR Panel.

Comments from the IEPR Panel helped to shape the direction of the interagency eDNA Calibration study (ECALS) referenced in many of the responses to the comments. The ECALS, currently underway, aims to advance the usefulness of Asian carp eDNA forensics such that eDNA can be used to assess and manage uncertainty of the presence of Asian Carp in the CAWS. The goals of the ECALS are to evaluate alternative sources of eDNA, improve existing eDNA markers, reduce eDNA analytical turnover time, and calibrate the detection and fate of eDNA as an integral contribution to Asian carp management under the ACRCC agency task framework. UND has not been a participant in the development of ECALS, and statements in this IEPR document regarding the outcomes, significance, and deliverables of the ECALS are attributable solely to USACE.

The following discussions present the USACE Final Response to the 8 IEPR comments. Further details on the IEPR process, Review Panel selection and charge questions, as well as basis for each of the 8 comments, determination of significance and recommendations for resolution can be found in the *Revised Final Independent External Peer Review Report Environmental DNA (eDNA) Science and Methodology*. The following discussions take into account the exchange of comments from the IEPR Panel, USACE, and UND generated as part of the IEPR process.

1. IEPR Comment - High Significance: The current mitochondrial DNA (mtDNA) methodology cannot distinguish pure silver or bighead carp from hybrids of the two species.

USACE Response: Not Adopted.

USACE concurs that the method, as evaluated by the Review Team, cannot distinguish pure silver carp or pure bighead carp from hybrids of these two species.

The recommendations for resolution called for developing a protocol for nuclear markers that would differentiate hybrid individuals from pure-species individuals; efforts to use nuclear markers in positive water samples to differentiate pure silver and bighead carp from hybrids of the two species; and an examination of downstream populations for the amount and pattern of hybridization.

USACE and UND did not concur with the Review Panel that this is a high significance level comment because the purpose of the eDNA methodology in the application of monitoring in the Chicago Area Waterways System is to assist federal agencies in evaluating the leading edge of Asian carp presence. In this case, data gathered indicating the presence of eDNA from bighead, silver, or a hybrid meets this objective. Therefore, USACE has not adopted the recommendations for resolution in this context.

2. IEPR Comment - High Significance: The eDNA methodology does not unequivocally indicate the physical presence of live bighead or silver carp.

USACE Response: Adopted.

Both USACE and UND agree that:

- Alternative pathways could transport DNA.
- These alternative pathways may serve as less likely sources of DNA.
- No studies on alternative pathways have yet been conducted.

UND also concludes that:

- Published studies have documented a correlation between the proportion of positive eDNA detections and the presence of live target organisms (Ficetola et al. 2008, Jerde et al. 2011).

Action to be Taken: Both USACE and UND agree with the recommendation that alternative sources of DNA be more directly tested to provide more definitive conclusions. The eDNA calibration study will directly test the viability of eDNA through other vectors (e.g. piscivorous birds, sewers, and dead fish).

3. IEPR Comment - Medium Significance: The assumption that the eDNA methodology is of limited use in the winter months should be evaluated.

USACE Response: Adopted.

Although UND originally suggested that winter sampling might have limited utility, other factors that encourage limited winter sampling were safety and access. Having crews leaning over the sides of boats in freezing cold

water is a safety hazard (especially since the sampling requires contact with the water). Although the safety risk posed by sampling in the winter can be minimized by safety gear, it cannot be completely eliminated. Therefore, sampling in the winter should only be conducted once studies are complete to verify the viability of eDNA in winter conditions. Additionally, many of the backwaters or side eddies and tributary waters that are targeted for sampling are commonly frozen over the winter, inhibiting both aquatic species use of these waters as well as limiting access for sampling.

The recommendations for resolution called for a methodology to include:

1. A determination as to whether temperature affects eDNA detection and, if so, at what temperature is the technique ineffective.
2. A continuation of sampling protocols for eDNA during winter and early spring months.
3. Use of the documented natural behavior of the fish (i.e., tributary movement for bighead and pool aggregation for silver carp) to establish an appropriate sampling strategy.

Action to be Taken: USACE will forward this recommendation to the Monitoring and Rapid Response Work Group (MRRWG) for consideration in the eDNA sampling strategy. Specifically, the eDNA calibration study (ECALS) is an interagency-led study (USGS, USACE, and USFWS) that will, as one objective, investigate the viability of eDNA in colder water temperatures. The ECALS is investigating the viability of eDNA under different environmental variables (temperature, light, dissolved oxygen, flow, and sampling at different water depths). If eDNA is found to be viable in colder water temperatures, USACE will submit this result to the MRRWG with an endorsement to include winter sampling as part of the set sampling strategy. Priority will be given to areas that are known not to freeze completely, and that crews can have safe access to during winter conditions. All results from ECALS that will enhance or improve current eDNA sampling will be evaluated for implementation by USACE. All results from ECALS that will better inform sampling strategy (to include winter season sampling) will be sent to the MRRWG with a USACE endorsement to implement the recommendation.

4. IEPR Comment - Medium Significance: The sampling design used is not statistically based.

USACE Response: Adopted.

USACE and UND do not concur with the assessment made by the Review Panel; the approach was intentional and statistically sound given the goals of using eDNA data to help define the leading edge of the Asian carp invasion front. USACE concurs with the panel that future efforts, guided by subsequent goals and data from the eDNA Calibration study, may need a different statistical design.

The recommendations for resolution called for a methodology to include:

1. A clarification of the goals of the sampling program to set the proper context for evaluating the sampling design.
2. A clearer description of the sampling program, with an emphasis on the overall design, sample sizes by time and location, the method used to statistically handle replicate samples, and the method used to estimate measures of variability (e.g., standard error). The rationale for the sampling design should also be included, and if a non-statistical sampling design is used, this choice should be justified.
3. A report of measures of variability where appropriate (e.g., proportion of samples testing positive).

Locating the leading edge of an incipient invasion is problematic (Pielaat et al. 2006). The appropriate sampling regime, which is the one employed by USACE and UND, incorporates effort (number of samples collected) that is inversely related to presumed abundance of the target species (Asian carp). That is, more samples were taken from reaches of the Chicago Area Waterways System where Asian carp were expected to be at low abundance (or presumably absent); within those reaches, samples were taken from specific locations (e.g., eddies, backwaters) that have the highest probability of detection (Thompson 2004). The Panel advocates sampling designs that are used in monitoring population abundances of fisheries or wildlife, which are not the same as those used for helping in uncovering the leading edge of range expansions and spread (see, for example, Pielaat et al. 2006). Simple random sampling, for example, would not be optimal for delimitation for rare and/or difficult to detect species. In fact random sampling would have increased the occurrence of false negatives (i.e., concluding Asian carp are absent when in fact they are present) (Gu and Swihart 2004).

USACE also concurs with the panel recommendation in so far that different sampling designs may be more appropriate if the objectives of the sampling change. For example, a different sampling design would be appropriate to monitor changes in abundance and distribution of positive eDNA detections or correlating detections with known occurrence of fish in experimental calibration studies.

Action to be Taken: Moving forward with the eDNA sampling approach: The current sampling design is set by the Monitoring and Rapid Response Work Group (MRRWG). USACE will coordinate with the workgroup to develop a plan consistent with the methodology laid out in the recommendations. The current strategy would be classified as a systematic sampling design – where 5 locations are targeted on a weekly rotation at 120 samples per location (so 120 samples per week are processed). The MRRWG should specify clear goals and objectives for this design, justify the collection of 120 samples and justify the location selection. However, the MRRWG will ultimately determine what sampling plan and design best meets the relevant goals.

5. IEPR Comment - Medium Significance: The eDNA methodology should be used to screen ichthyoplankton and egg samples to provide a means to identify sites with successful reproduction.

USACE Response: Adopted.

Ichthyoplankton and egg screening using the eDNA method could provide incredibly useful information for early detection monitoring and enhance the monitoring plan. The inclusion of this sampling would fill a void in the current monitoring regime that does not directly sample for these life stages, and could provide evidence of natural reproduction in the pools that may be missed using other techniques.

The recommendations for resolution called for a methodology to include:

1. An ichthyoplankton sampling protocol during the spring of the year when bighead and silver carp are known to spawn.
2. A provision for the preservation of collections in a fixative that will not compromise the DNA or ability to qualify and quantify egg/larval development from a sample, if warranted.
3. The incorporation of nuclear DNA (nucDNA) markers to estimate numbers of spawners contributing to collected eggs/larvae.

Action Taken: This recommendation has been forwarded by USACE to the Monitoring and Rapid Response Work Group (MRRWG) for consideration and inclusion in the monitoring plan. Recommendation #1 was included in the

latest version of monitoring plan, scheduled for 2011 sampling. The other two recommendations are under consideration by the MRRWG, but would need to be funded to explore this methodology.

6. IEPR Comment - Medium Significance: The current eDNA methodology should be modified to estimate the number of individual fish contributing eDNA to a positive sample.

USACE Response: Adopted.

USACE concurs with all the recommendations of the panel.

The recommendations for resolution called for a methodology to include:

1. An estimate of the number of fish present. The easiest way to do this is to remove the diagnostic PCR fragment bands of mtDNA from the gel and sequence them. They then could be aligned and examined for intraspecific variation.
2. The use of another region (or regions) of mtDNA other than that used for identifying species. This could be determined either by a literature search and/or by sequencing the entire mtDNA control region, which tends to be highly polymorphic in many species. The literature search should include the published literature, GenBank, and contacting labs known to be working on one or both of these species. Other regions of mtDNA could also be sequenced for intraspecific variation.
3. The collection of baseline data from both species. Many individual samples (perhaps 50) should be taken from the populations of each species below the barrier. These should then be screened for intraspecific genetic variation at mtDNA. Sequencing all positive eDNA samples and comparing them to this baseline information would make it possible to estimate the minimum number of individuals present in the eDNA samples.
4. The development of a suite of nucDNA markers to estimate the number of individuals present in the eDNA samples. mtDNA provides very limited information because it is a maternally inherited haploid marker. Ideally, a set of microsatellites would be developed to do this analysis because the genotype of each individual is potentially unique. There are some technical issues to overcome to make the nucDNA approach possible. First, there is roughly 100 to 1,000 times more mtDNA in a sample than nucDNA. Therefore, there might not be enough nucDNA present for this approach. A pre-amplification process prior to PCR might make it possible to PCR nuclear markers from eDNA (Piggott et al., 2004; Hedmark and Ellegren, 2006). Second, a positive eDNA sample could contain DNA from more than one individual. This could make it difficult to identify the genotypes of separate individuals, though still permitting the recognition of samples with two to five individuals present. This approach will require the use of acrylamide gels in either slab or capillary formats.
5. The development of a new set of microsatellites especially designed for the low amounts of DNA present in eDNA, if the available microsatellite primer sequences do not work. Such special microsatellites would have small fragment size and the primer sequences could be especially designed to be sensitive with small amounts of DNA. As with using mtDNA to estimate the number of individuals from eDNA, data from populations of both species below the barriers would be needed as baseline information.

Action Taken: USACE has developed an interagency eDNA calibration study (ECALS) that is underway to address these issues.

The approaches described by the IEPR (Recommendations 1 and 2), which would be based on minimum possible number of individual genotypes, is an estimate limited by the numbers of marker variants (=alleles) and markers (=loci) within a sample. This would be an improvement over current practice (no estimate of carp numbers).

However, it should be noted there is not a strict equivalency between numbers of genotypes and numbers of carp. For example, if available markers have a maximum of 16 possible genotypes (combinations of loci and alleles at each loci) and eDNA data consistent with at least 8 genotypes are found, there could actually be 8 fish, or there might be 800 or some other number. Many fish will share genotypes and so, again, only a minimum possible number can be determined, not an actual number of carp. Appropriate caveats on data interpretation would be required whenever releasing data on fish population size estimates.

As described by the IEPR, development of additional, relatively short mitochondrial and nuclear markers, essentially increasing the number of possible genotypes, would increase the power of eDNA surveys for estimating minimum numbers of individuals.

A potential difficulty with this approach would be the possibility for cross-amplification of markers among carp species. Although bighead and silver carp do hybridize and it may be more challenging to find species-distinct markers, the utility of identifying even a hybrid of the two species is still useful for identifying the invasion front in the Chicago Area Waterways System (as indicated by Comment 1). As noted in Recommendation 3, considerable sequence data from many carp below the barrier will be needed to optimize marker development.

7. IEPR Comment - Medium Significance: The current PCR methodology should be changed to a quantitative PCR (qPCR) approach to estimate the quantity of silver and bighead DNA in a sample and to speed detection of eDNA in water samples.

USACE Response: Adopted.

Action Taken: The interagency eDNA Calibration Study (ECALS) has been scoped to address this issue.

The recommendations for resolution called for developing a protocol for qPCR (qPCR, Ayra et al., 2005) in the SOPs substituted for the current PCR protocol. This protocol would include TaqMan assays specific for silver and for bighead carp mtDNA. qPCR permits the quantification of DNA in the sample because it monitors the amplification each cycle (real-time amplification), not just at the end of PCR as currently practiced using agarose gels. The positives could then be repeated 16 times and sent directly for sequencing. This alternate process removes the use of agarose gels to detect the presence of silver or bighead carp mtDNA and the current approach of excising the DNA band from the gel for sequencing, significantly reducing the time to process samples. Finally, a qPCR approach can detect lower concentrations of amplification products.

The processing time for eDNA is based upon the initial assay, a validation assay, sequence validation, and then running equipment controls. Presently, it takes about 10 days to complete this process to get a confirmed positive result. Only confirmed results are reported to the MRRWG. The ECALS study has an objective to increase this throughput (incorporating qPCR mentioned above) that will decrease this processing time from 10 days to 5 days. Quantifying the amount of Asian carp eDNA present in monitoring and other uncontrolled samples may not tell us much, as different types of eDNA release (e.g. fecal matter, sloughing of slime coat, sloughing of scales, tissue disruption by predators or propeller other disturbances.) could result in highly variable amounts of eDNA in the water. However, qPCR would be of considerable use in ECALS where DNA release and degradation rates are studied. Furthermore, increasing sample throughput via qPCR would be highly beneficial for the MRRP and associated response actions.

8. IEPR Comment - Medium Significance: The production, movement, and degradation of eDNA in the system should be evaluated.

USACE Response: Adopted.

Action Taken: These are highly relevant issues. USACE has incorporated these requirements into the interagency eDNA calibration study. USACE has a great deal of expertise in modeling the movement of particles in water systems, and should be able to model the movement of DNA-containing particles (e.g. scales, feces) in the Chicago Area Waterways System and elsewhere. USACE also has expertise in modeling fish behavioral patterns, which could be used, in conjunction with particle flow models to develop targeted sampling schemes. Specific knowledge of eDNA movement in the environment would facilitate the development of accurate statistically based sampling methodologies.

The recommendations for resolution called for a methodology to include:

1. The production rates of various sources of eDNA (e.g., feces, mucus, urine). If the methodology can be used to quantify the amount of DNA in a sample, the production rate could be determined from captive fish. Because the production rate likely depends on the size of fish, water temperature, and possibly the diet of fish (among other factors), some type of factorial design would be a useful approach.
2. Degradation rates of DNA from direct DNA quantification measures or qPCR using an exponential model of decay. Degradation rates likely depend on a variety of factors, such as water temperature, UV light exposure, and water chemistry; as above, a factorial design would be a useful first step. Even if quantitative measures cannot be implemented, the amount of time that eDNA persists at a detectable level could still be determined. An expansion of the UND laboratory experiment reported above should be carried out upon bighead and silver carp DNA detections using the primers developed for these species under laboratory and "natural" conditions (i.e., with the complex microbiotic community present in CAWS water samples).
3. General maps of water flow and velocity within the CAWS to broadly scale the area likely contributing to positive eDNA samples (i.e., is the eDNA likely to have come from less than 100 meters in distance, 500 to 1,000 meters in distance, or farther?). Feces, mucus, and urine sample dilution should be modeled under these water conditions.

Recommendation 2 would be the most useful of these for application in Asian carp monitoring in the Chicago Area Waterways System. Cause and rate of eDNA degradation would appear to be a critical component of being able to determine the link to the question "does eDNA come from a live fish", and also would assist the development of the statistical aspects of future sampling protocols. Again, any information learned from studies that investigate the environmental factors on eDNA production, movement, and degradation (all objectives of the eDNA Calibration study) will be immediately applied to the current sampling strategy for eDNA as an early detection surveillance tool in the Chicago Area Waterways System. Recommendations 1 and 3 could also be performed if resources exist to perform this work in the future.

Literature Cited:

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

Gu, W. & Swihart, R.K. 2004. Absent or undetected? Effects of non-detection of species occurrence on wildlife-habitat models. Biological Conservation. 116: 195-203.

Hedmark, E., and H. Ellegren (2006). A test of the multiplex pre-amplification approach in microsatellite genotyping of wolverine faecal DNA. Conservation Genetics 7:289-293.

Ibrahim, K.M., R.A. Nichols, and G.M. Hewitt (1996). Spatial patterns of genetic variation generated by different forms of dispersal during range expansion. Heredity 77:282-291.

Jerde, CL, MA Barnes, J McNulty, AR Mahon, WL Chadderton, and DM Lodge. 2010. Final Report: Aquatic Invasive Species Risk Assessment for the Chicago Sanitary and Ship Canal. Report to the United Sates Army Corps of Engineers, Environmental Laboratories, Cooperative Environmental Studies Unit, Vicksburg, Mississippi. CESU agreement #W912HZ-08-2-0014, modification P00001.

Jerde, CL, AR Mahon, WL Chadderton, and DM Lodge. 2011. 'Sight-unseen' detection of rare aquatic species using environmental DNA. Conservation Letters. doi: 10.1111/j.1755-263X.2010.000158.x Online 5 January 2011.

Pielaat, A., M.A. Lewis, S.R. Lele, and T. de-Camino-Beck. 2006. Ecological modeling. Sequential sampling designs for catching the tail of dispersal kernels. Ecological Modelling. 190: 205-222.

Piggott, M.P., E. Bellemain, P. Taberlet, and A.C. Taylor (2004). A multiplex pre-amplification method that significantly improves microsatellite amplification and error rates for faecal DNA in limiting conditions. Conservation Genetics 5:417-420.

Thompson, W.L. 2004. Introduction to sampling rare or elusive species. In: Sampling Rare or Elusive Species, Editor, Thompson, W.L.. Island Press, New York. Pages 1-7.