October 21, 2022

Stanley Burgiel, Executive Director
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RE: Environmental DNA as a Tool for Invasive Species Detection and Management

Dear Mr. Burgiel:

Please accept and share the following comments and recommendations from the National Aquaculture Association[1] to the National Invasive Species Council (NISC) membership relative to the recent white paper: *Environmental DNA as a Tool for Invasive Species Detection and Management*.

We offer these comments predicated upon the almost unalloyed enthusiasm displayed for environmental DNA (eDNA) in the white paper, unlike the discerning analysis provided in Morisette *et al.* (2021). Unfortunately, the 2022 NISC white paper minimizes the substantial potential for false positives, which were described in Morisette *et al.*, a review that we assumed informed the NISC white paper.

We recommend the white paper be revisited and amended to:

- Recognize the uncertainties associated with inferring or concluding species presence through the sampling for deoxyribonucleic acid fragments in the environment. The compound is far more resilient to environmental degradation than has been recognized and is transported by a wide variety of biotic and abiotic means over significant spatial and temporal distances.
- Include standards and methodologies to reduce uncertainties in sample design, sampling, biotic and abiotic effects, stochastic environmental variables, and final analysis.
- Fix broken hot-links or provide alternative means to access the referenced information.

[1] The National Aquaculture Association (NAA) is a U.S. producer-based, non-profit trade association founded in 1991 that supports the establishment of governmental programs that further the common interest of our membership, both as individual producers and as members of the aquaculture community. For over 31 years NAA has been the united voice of the domestic aquaculture sector committed to the continued growth of our industry, working with state and federal governments to create a business climate conducive to our success, and fostering cost-effective environmental stewardship and sustainability.

We also strongly encourage NISC, as the federal interagency leader on invasive species, to thoughtfully address the issue of the use of eDNA for regulatory purposes in a revised white paper or as a second white paper. We believe NISC has a responsibility to identify and disseminate specific requirements that need to be in place before eDNA is used as a regulatory tool within the United States. Curiously, the 2022 NISC white paper ignores the 2012 ISAC white paper, which recommended assay validation and laboratory accreditation as critical steps necessary before the sampling for eDNA for regulatory purposes. From the viewpoint of aquaculture farming community, a regulated community, a false positive is any positive result other than a "true" positive resulting from the actual physical presence of the living target organism within a live haul truck or farm. We fully recognize there are a wide range of "false" positives, as described in the literature. However, for a regulated community, this specific definition is critical, as all false positives have the potential for significant economic and societal harm.

Uncertainty: eDNA origination, unpredictable degradation over time, abiotic and biotic transport, stochastic natural events

Current literature verifies numerous uncertainties with using, or relying on, sampling of deoxyribonucleic acid fragments. The uncertainties (e.g., origination, variable degradation over time, abiotic and biotic transport, stochastic natural events) have been discussed in the ecological literature more so than the scientific literature focused on eDNA to detect aquatic invasive species (Harrison *et al.* 2019; Stewart 2019; Jerde 2021; Jo and Minamoto 2020; Wang *et al*. 2021; Joseph *et al.* 2022). We recommend reviewing Stewart (2019) for the excellent analysis, Loeza-Quintana *et al.* (2020) and 11 associated papers for their argument supporting the need for improved eDNA validation, methods and standardization, and point specifically to Harrison *et al.* (2019) for their incisive thinking. They wrote:

> "…uncertainties persist surrounding the physical processes that influence eDNA persistence and its fate within the environment. Because these techniques use fragments of DNA recovered from environmental samples to infer species presence, uncertainties in the relationship between the source organism(s) and the physical DNA molecules in the environment can significantly limit inferences made from eDNA-based tools and preclude their widespread application."

Harrison *et al.* (2019) also provided five notable recommendations to reduce errors that generate uncertainty:

1) integrate hydrological modelling into eDNA sampling;
2) increased use of replicated, controlled experiments in naturalized systems when studying processes that affect eDNA and estimates of uncertainty, designed with an understanding of the potential mechanisms that impact these processes;
3) eDNA parametrization and conclusions drawn from eDNA studies should be considered as ecosystem-specific given the significant differences in transport and attenuation mechanisms between lentic, lotic and marine ecosystems;

4) collect and include environmental data when collecting eDNA samples so that environmentally driven variation can eventually be assessed; and,
5) develop a full model predicting the relationships between eDNA and the organisms being studied to elucidate the relative contribution of individual decay and transport processes in environment-specific contexts that contribute to patterns of bias and noise in varying environments.

The uncertainties related to the dynamic nature of aquatic ecosystems and species have resulted in a variety of authors recommending integrated, long term ecosystem wide studies to account for spatiotemporal change (Curtis *et al.* 2021; Burian *et al.* 2021; Mathieu *et al.* 2021; Troth *et al.* 2021). As an example, Mize *et al.* (2019) reported they:

> "…collected water samples from three different habitat types in the Upper Mississippi River when both Bighead Carp and Silver Carp were known to be present based on telemetry detections. Each habitat type (backwater, tributary, and impoundment) was sampled during April, May, and November. Detections of eDNA for both species varied across sites and months, but were generally low, 0–19.3% of samples were positive for eDNA. Overall, we found that eDNA-based sampling holds promise to be a powerful monitoring tool for resource managers; however, limitations of eDNA-based sampling include different biological and ecological characteristics of target species such as seasonal habitat usage patterns as well as aspects of different physical environments that impact the implementation of these methods such as water temperature."

The work by Troth *et al.* (2021) focused on the white-clawed crayfish (*Autropotamobius pallipes*) is particularly instructive. Their findings illuminate in a compelling manner how eDNA-based species detections can be substantially influenced by: 1) time scales of eDNA degradation, 2) seasonality in environmental conditions and species behavior, and 3) within habitat variation of eDNA concentrations. They reported:

> "…eDNA persisted for 14–21 days post species removal and that slow degradation processes may trigger false positive results in field surveys."
>
> "…eDNA persistence was much higher in the sediment than in the water column of our mesocosms. Hence, resuspension of sediment and historic eDNA after extinction or emigration of the target species represent a potential source of error for eDNA-based assessments."
>
> "…substantial changes in eDNA concentrations (Ct values), across the various months of the year and that these changes resulted in a 4-fold increase of detection probability between winter and summer seasons."
>
> "…striking differences in detection probabilities between sites that were less than 50 m[eters] apart. Sampling at some sites almost always failed to achieve positive detection, despite the presence of the target species in proximity of the sampling site."

In closing the authors noted:

> "…all three investigated factors can have considerable effects on detection probabilities of target species and may impact quantitative approaches to an even greater degree. However, drivers of variability can be substantially mitigated by implementing respective mitigation strategies controlling rates of false positive and false negative results (Table 1), at least to some degree. Yet, such mitigation strategies are most effective when site-specific environmental and ecological drivers are considered and consequently require adaptive applications instead of being followed like recipes in a cook book."

The mitigation strategies they recommended (i.e., Table 1) included:

> "Design sampling strategy to periods of highest species activity, and least weather extremes. If sampling in less reliable time periods or conditions, increase sample replication number.
>
> Combine eDNA-based methods with classical species presence/absence surveys. Repeat sample collection over time.
>
> Avoid sediment disruption during water sampling.
>
> Collect a representative sample for each habitat. Sample several sites in larger ecosystems."

Curtis *et al.* (2021) "…assessed how stream flow affects eDNA concentrations and detectability in situ using populations of an invasive freshwater mollusc, the Asian Clam (*Corbicula fluminea*). We used a longitudinal study to assess the role of stream flow, including high magnitude floods, on eDNA concentrations and detectability over an entire year at two stream sites, as well as a seasonal study (summer, autumn) to evaluate similar effects at eight stream sites over a gradient of low to high *C. fluminea* abundance." The authors concluded:

> "…high stream flows can dilute eDNA concentrations and produce false negatives, even in cases where study organisms are relatively abundant. We recommend that researchers and managers or practitioners avoid eDNA sampling during high stream flows or floods…if researchers must take eDNA samples during periods of high stream flows or floods, we recommend increased sample replication to improve detection probabilities."
>
> "…understanding the natural history of target species, and relationships to seasonal variability in abiotic and biotic conditions, should lead to improved eDNA sampling programmes. In our longitudinal study, apparent reproduction by *C. fluminea* in late spring and early summer resulted in high eDNA copy numbers at this time of the year, and monitoring programmes for this invasive species might seek to sample at temperatures associated with reproduction to improve detection probabilities. "

> "Researchers and managers should continue to apply occupancy estimation with detection probability frameworks to improve the design and implementation of eDNA sampling programmes for specific taxa and ecosystems, including for lotic ecosystems where variable stream flow and floods may strongly affect performance of this methodology."

Using modeling, Erickson *et al.* (2019) estimated samples sizes of a 3-level occurrence model (occurrence, capture and detection) to suggest, "detecting eDNA in ≥1 sample at a site required ≤ 15 samples per site for common species…detecting eDNA when looking for rare species required 45 to 90 samples per site."

We recommend the white paper be revised to advise agencies to carefully review the Cristescu and Hebert (2018) analysis that described bioinformatics and taxonomic assignment challenges. Key to bioinformatics is designing primers to encompass the potential species encompassed by nationwide reporting. Relative to taxonomic identity, the authors noted, "Incomplete reference libraries and the presence of sequences derived from misidentified specimens mean that the species origin of many eDNA records remains uncertain" and "…users must ensure that reference databases are up-to-date and contain entries for species of interest. An accurate taxonomic assignment provides a robust way of linking genotype to phenotype…" These observations suggest primers specific to this nationwide effort must be developed.

Recent work by Danziger and Frederich (2022) emphasizes the critical importance of primer specificity. They have been focused on developing appropriate primers for the European green crab (*Carcinus maenas*) in the Pacific Northwest. They found species-specific eDNA primers for species distributed world-wide, may need to be tested carefully against related local species. In this instance primers developed for *C. maenas* found in Maine led to gene amplification, not only of Pacific Northwest *C. maenas*, but also the Asian shore crab, *Hemigrapsus sanguineus*, the Rock crab, *Cancer borealis*, and the Jonah crab, *Cancer irroratus*.

A concise examination by Lacoursière-Roussel and Deiner (2019) argued an integrated, multidisciplinary approach (i.e., life and physical sciences) is needed to create fundamental knowledge of what eDNA is and how it interacts with its surroundings. Until multidisciplinary analysis is accomplished, they noted, an accurate inference that a species was present in a place and time remains a challenge. As one of their several supporting examples, they reported:

> "…DNA in the environment has a fast degrading portion that is correlated with a species abundance, a portion that can remain detectable for weeks to months in water when the species is no longer present and a portion that can remain detectable for centuries in certain types of substrate such as lake sediments and permafrost."

Cristescu and Hebert (2018) spoke to the interaction of eDNA with the aquatic environment. Specific to one-off sampling for nonnative species, their comments reporting eDNA persistence in sediments is particularly problematical. They noted:

> "…eDNA in sediments can persist far longer and is often present at much higher concentration than is eDNA in the water column."
>
> "…eDNA extracts from river sediments generated sequences of resident freshwater species, marine and estuarine species unlikely to occur at the sampled site, and freshwater species unrecorded for more than a century."
>
> "Because aDNA [ancient DNA isolated from old specimens] from sediments may be resuspended, particularly in rapidly flowing rivers, DNA extracted from water may often contain eDNA that reflects historical deposits. Separating recent eDNA from aDNA is not straightforward. Moreover, discriminating between eDNA (particularly its cellular form) and genomic DNA from small organisms inadvertently captured during sampling is difficult."

Similarly, Joseph et al. (2022) summarized how biofilms, mineralogy, temperature, microorganisms, chemicals and DNA fragment length affect eDNA dynamics in aquatic environments. They concluded, "There is currently little research that concentrates on the parameters influencing the longevity of eDNA" and "further research on eDNA persistence is needed on a global scale."

The potential long-distance transport of eDNA by birds, vessels and flowing waters and its persistence in sediments creates, through false positive inference, significant species location, eradication or control challenges. In addition, we note the evolving diversity of farmed aquatic species over time at any particular farm will deposit eDNA in sediments that will be re-suspended during typical farm operations (e.g., seine harvest) or storm events. Similarly, eDNA entrained in lotic waters near farms or the eDNA persisting in sediments in those flowing waters may be sampled. These observations are confirmed in a paper by Nevers *et al.* (2020). The authors conducted a series of field and controlled mesocosm experiments to examine the detection and accumulation of eDNA in sediment and water and the transport of eDNA in a small stream in the Lake Michigan watershed, using the invasive round goby (*Neogobius melanostomus*) as a DNA source. They reported round goby eDNA accumulated and decayed more slowly in sediment than water. In the stream, DNA shedding was markedly lower than calculated in the laboratory, but their modeling indicated eDNA could potentially travel long distances (up to 50 km) under certain circumstances. Collectively, these findings show that the interactive effects of ambient conditions (e.g., eDNA stability and decay, hydrology, settling-resuspension) are critical to consider when developing regulatory sampling programs to avoid erroneously concluding species are present.

Empirical research in lotic systems indicates fish eDNA can be detected 50 km (Laporte *et al.* 2020) to 130 km (Pont *et al.* 2018) from sources or 9 km from sources for crustaceans (Deiner and Altermatt 2014).

Biotic and abiotic eDNA transport is particularly problematic for aquatic environments visited by waterfowl or vessels. Merkes *et al.* (2014) reported the DNA of silver carp can be detected from multiple transmission pathways (barges, bald eagle [*Haliaeetus leucocephalus*] feces, and dead animals) independent of the presence of live fish, and this DNA can be detected for more than 28 days. This persistence suggests DNA could be transported long distances from its live fish source by processes unrelated to lentic or lotic hydrodynamics. They also reported DNA can be detected using markers less than 200 base pairs well after it has been deposited or shed or after the animal has died.

Guilfoyle and Schultz (2017) and Guilfoyle *et al.* (2017) demonstrated silver carp (*Hypophthalmichthys molitrix*) were a prey species for the double-crested cormorant (*Phalacrocorax auritus*) and then estimated silver carp eDNA loading to waters above the electric barriers on the Chicago Sanitary and Ship Canal via double-crested cormorant feces. Their study indicates piscivorous birds are potentially important sources of silver carp DNA when live fish were not present.

Through the repurposing of weather radar analysis, a multi-partner BirdCast partitions weather surveillance radar to gather information on the numbers, flight directions, speeds and altitudes of birds aloft in order to depict migratory bird movement. Please see: BirdCast - Bird migration forecasts in real-time. As an example, BirdCast estimated 7 million birds passed through the State of Mississippi during the night of October 12th with 19,239,700 birds in flight over the state. Much of the production for farmed catfish for the United States occurs in open ponds within this state: 176 farms and 39,561 water acres. Nationally, 2,475 farms and 253,498 water acres (USDA 2018). The potential for the deposition of eDNA to farm ponds by bird overflight cannot be ignored.

In addition, the biology and physiology of the target animal may influence detection. Adams *et al.* (2019) sampled four lentic ponds with different densities (0 kg/ha, 6 kg/ha, 9 kg/ha, and 13 kg/ha) of painted turtles (*Chrysemys picta*) over three months to detect differences in eDNA using a quantitative polymerase chain reaction assay amplifying the cytochrome oxidase I region of painted turtle mitochondrial DNA. Only one sample of the highest-density pond amplified eDNA for a positive detection.

Experienced practitioners strongly recommend adopting as standards of practice the *Minimum Information for Publication of Quantitative Real-Time PCR Experiments* (MIQE) guidelines (Bustin *et al.* 2009) and the survey design and validation reported by Thalinger *et al.* (2021). These papers reinforce a salient point of not depending upon published, on-line accessible animal DNA sequences, such as National Center for Biotechnology Information (nih.gov), for mitochondrial constructs. Through their prior experience they recommended, as has Thalinger *et al.*, to:

- Generate reference target sequence from target animal tissue collected from animals in the location or region of the study to avoid conspecific sequence matches.

- Collect tissue from local or regional conspecifics and cross match mitochondrial sequences to avoid false positives.

- Field trial the survey because trial lab assays may be confirmatory but field work reveals polymerase chain reaction (PCR) chemical inhibitory factors or false positive eDNA fragments or aggregates. PCR inhibition is a very real challenge in freshwater systems.

Bruce *et al*. (2021) utilized a continent-wide approach to capture experienced eDNA user knowledge to inform an electronic handbook. We suggest NISC could play a similar role, as exemplified by Bruce *et al*. (2021), to reach beyond federal agency employees to aggregate the rapidly evolving knowledge and experience within North America to produce an assessment that will thoroughly and objectively inform eDNA aficionados and novices, agency leadership and program managers, and most importantly the public as to the practicalities and impracticalities of using eDNA to detect and manage invasive species.

Regulatory Use: False Positive/False Negative

The above section has outlined numerous uncertainties in regards to eDNA, concerns which are exacerbated regarding the potential use of eDNA for regulatory purposes, where false positives have the potential of causing significant harm. This concern is not unwarranted, given that natural resource conservation management often defaults to regulatory enforcement and litigation (Nie 2008). Although scientists involved in eDNA research are understandably enthusiastic about the repeatability and reproducibility of eDNA detections, one out of five laboratories that participated in a highly prescribed blind proficiency testing study recorded false findings, albeit rare (Sepulveda *et al*. 2020):

> "Rare instances of zebra or quagga mussel DNA amplification did occur in water bodies where one of the dreissenid mussel species is not known to occur, though only samples analyzed by Laboratory 4 amplified."

This amplifies concerns regarding the potential of false positives generated from samples collected and analyzed under less rigorous conditions. Among other sources, Farrell *et al*. (2021) describes benefits and uses of eDNA, and the potential for false positives:

> **"**Conversely, partly as a result of eDNA-based approaches being less likely to produce false negatives, they can be more prone to producing false positives (in comparison with eRNA-based studies and traditional studies) because of increased efficacy (detection of eDNA that does not come directly from a present or alive target species or pathogen…".

Before eDNA is used for testing in commercial aquaculture (either for monitoring or for regulatory purposes), every sampling protocol and test assay must be validated and standardized, participating laboratories must be nationally accredited, and each lab must participate in proficiency testing. This is no different than for other diagnostic tests. For details, see the 2012 ISAC white paper, *Validation of PCR-Based Assays and Laboratory Accreditation for Environmental Detection of Aquatic Invasive Species*.

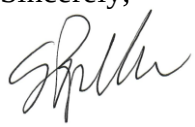
We suggest that at this time, there are simply too many unknowns for eDNA to be used for regulatory enforcement. The science of eDNA is evolving rapidly, and new findings provide intriguing results which have implications for the validity of potential regulatory applications.

For example, recent research has documented that eDNA can be airborne (Stokstad 2021; Clare *et al.* 2021; Clare *et al.* 2022), and DNA is found in bioaerosols in the air (Mainelis 2020; Gusareva *et al.* 2022) including eDNA for aquatic animals. Four species of fish fed to zoo animals were detected in the air (Lynggaard *et al.* 2022), as was the eDNA of many different marine fish species at a dockside sampling site (Klepke *et al*. 2022).

Given these findings, we are confident airborne eDNA from different species held in separate holding tanks, ponds or raceways on a farm will intermingle. As an example, farms producing baitfish, sportfish, triploid grass carp and other fish species, hold live fish before transport under open or closed sheds to protect them from weather, predators or theft. Fish are separated by species into different vats. A shed may contain a number of different species, one or more of which may not be legal for sale in other states. The water in each vat receives constant vigorous aeration from a low-pressure blower or surface aerator. The airborne eDNA as a bio-aerosol will circulate throughout the shed and adhere to other vats, dipnets, and even hauling tanks which are driven up close to the shed or under the shed for loading.

We welcome comments on our suggestions, request during your review the withdraw of the current white paper in favor of a revised white paper reflecting the uncertainties associated with eDNA sampling and the addition of standards of practice described in Bustin *et al.* (2009) and the survey design and validation described in Thalinger *et al.* (2021).

Sincerely,



Sebastian Belle
President

cc: Deborah Lee, Co-chair, Aquatic Nuisance Species Task Force
David Miko, Co-chair, Aquatic Nuisance Species Task Force

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