A Hard Look at Environmental DNA

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Living organisms constantly shed whole or fragmented deoxyribonucleic acid (DNA), in waste and reproductive products, in mucus, by touch, and even through the air. This 'loose' DNA is called environmental DNA (eDNA). Sampling aquatic environments for eDNA has gained considerable traction and focus since Ficetola *et al*. (2008) described their eDNA sampling of wetlands located in France to detect the nonnative American bullfrog (*Rana catesbeiana*).

FIGURE 1. *eDNA sampling. Courtesy Fingerlakes Partnership for Regional Invasive Species Management.*

As the costs of DNA analysis have decreased, scientists have increasingly used it for species identification, biomonitoring, disease pathogen detection (e.g., Bass *et al*. 2023), and for identifying whole community assemblages. Many publications by research scientists now call for resource managers to embrace and adopt eDNA tools to supplement or replace traditional monitoring methods. There is no doubt that eDNA is a phenomenal advancement in science with incredible potential. At the same time, the global aquaculture community should take a hard look at eDNA and fully understand the benefits and limitations, as there are considerable uncertainties associated with eDNA sampling and interpretation.

Regulatory Use of eDNA

It has long been obvious that eDNA will eventually be used for regulation. The question is, what safeguards will be in place to ensure that results of eDNA testing are accurate? 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.

Numerous uncertainties have been documented in regards to eDNA, concerns which are exacerbated regarding its potential use 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 rarely (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…)."

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 bio-aerosols 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).

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, 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 or under the shed for loading.

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

Current population biology and ecology literature verifies numerous uncertainties with using, or relying on, sampling of DNA 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). Stewart (2019) provides an excellent analysis and 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 write:

"…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) increase 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.

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."

Cristescu and Hebert (2018) 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…"

Recent work by Danziger and Frederich (2022) emphasizes the critical importance of primer specificity. They were 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."

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). 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. 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.

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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 and re-suspension) are critical to consider when developing regulatory sampling programs to avoid erroneously concluding species are present.

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 are not present.

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.

Ethical and Legal Issues

Using eDNA for marine turtle population studies, Whitmore *et al*. (2023) realized they were inadvertently collecting human genomic information that they termed human genetic bycatch (HGB). They noted, "…human DNA is rarely (if ever) the intended target of eDNA [wildlife population] studies, leaving the field with a lack of specific human-related regulatory guidelines or ethical approvals." Triggered by this epiphany, the authors conducted a series of samplings in environmental water from sites distant from and close to human habitation, from human footprints in beach sand and from occupied and unoccupied room air. The authors reported HGB was found in all field eDNA samples. "These samples had been collected primarily for the detection of non-human species, marine turtles, animal pathogens and metagenomics. With no human enrichment prior to shotgun sequencing and with sampling having been conducted in areas of relatively low human habitation densities, we nevertheless inadvertently captured a substantial amount of human genomic data."

The authors then discussed potential ethical and legal unintended consequences (lack of consent/breach of privacy, publicly accessible storage of eDNA samples, inadvertent individual tracking or genome harvesting). In sampling eDNA on aquaculture farms and facilities, it appears likely that the DNA of farm personnel will also be collected and stored, potentially for future uses, as long-term storage of eDNA samples has been advocated (Jarman et al. 2018; Zizka et al. 2022). Scientists working with eDNA have long known that human DNA could be found in samples; typically, this is simply excluded. In fact, human and domestic animal DNA can be found in negative control libraries and PCR mixes (Thaler et al. 2023). Only with the publication of Whitmore *et al*. (2023) did eDNA scientists and others come to the curiously belated realization that capturing human DNA raises significant ethical issues.

Summary

Environmental DNA methods and applications are advancing rapidly. There is great potential for useful applications in aquaculture, but also substantial risks. Our purpose in highlighting the issues of uncertainties, potential regulatory use, and human DNA bycatch, is to encourage the active participation of aquaculture scientists, farmers, and associated businesses in shaping legislation and regulations to ensure appropriate and ethical uses of eDNA. Until governments and institutions invoke restrictions or protections for human genetic bycatch, simple questions should be posed to the eDNA samplers: Do you obtain permission? What are your policies and practices to securely store or share samples?

Bruce *et al*. (2021) utilized a continent-wide approach to capture experienced eDNA user knowledge to inform an electronic handbook. We suggest the World Aquaculture Society could play a similar role, as exemplified by Bruce *et al*. (2021), to aggregate the rapidly evolving global knowledge and experience to produce an assessment that will thoroughly and objectively inform eDNA aficionados and novices, governmental 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.

Notes

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