

Developing Cost Effective Monitoring for Rainbow Smelt Using eDNA

Maine Outdoor Heritage Fund Grant #171-01-10

Final Report

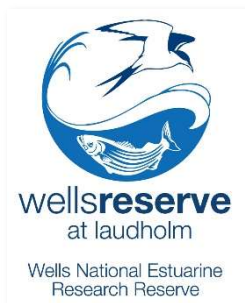
March 3, 2020

Jacob Aman, Wells National Estuarine Research Reserve

Dr. Michael T. Kinnison, University of Maine

Vaughn Holmes, University of Maine

Claire Gottsegen, Wells National Estuarine Research Reserve



We wish to thank the many colleagues that assisted with this study. In particular we appreciate planning and field support from Claire Enterline, Matt Craig, Jason Goldstein, and Michelle Furbeck, as well as sample processing and analysis by Geneva York, Mary Astumian, and Grace Fuchs.

This project was funded in part by the Maine Outdoor Heritage Fund, in which proceeds from the sale of a dedicated instant lottery ticket (currently “Lobster Loot”), are used to support outdoor recreation and natural resource conservation. For more information about MOHF, go to www.maine.gov/ifw/MOHF.html



Project funding was also provided by the Casco Bay Estuary Partnership under U.S. Environmental Protection Agency Cooperative Agreement #CE00A00063-0 with the University of Southern Maine, and by Maine Coastal Program, Wells National Estuarine Research Reserve, and the University of Maine.



Data disclaimer:

Data are preliminary and should not be cited without contacting the author.

Contents

| | |
|---|----|
| Summary | 1 |
| Management Challenges for Rainbow Smelt | 1 |
| eDNA for Effective Monitoring | 2 |
| 2017 Rainbow Smelt eDNA Pilot | 4 |
| 2018 Casco Bay Study | |
| Study Design | 6 |
| Results | 8 |
| Discussion and Lessons Learned | 11 |
| Communication | 13 |
| What's Next? | 13 |
| References | 14 |
| Appendices | 15 |

Summary

Environmental DNA (eDNA) tools developed at the University of Maine were successfully deployed in four coastal streams in Casco Bay, Maine in spring 2018 to detect the presence of anadromous rainbow smelt (*Osmerus mordax*), the first full application of this emerging technique. Field methods were refined and tested at sites with documented high (2) and low (2) spawning productivity. Samples were collected below known spawning areas immediately upstream of estuarine tidal influence 2-3 times each week during the spawning season. Three replicate samples were collected in the field at each site, as well as a contamination control, and all samples were filtered and preserved for laboratory analysis. Extracted eDNA samples and controls were run on three replicate qPCR assays.

Initial efforts to extract eDNA from samples were hampered by the presence of environmental inhibitors. Use of a Zymo OneStep PCR Inhibitor Removal Kits appears to have overcome this problem and field collected eDNA samples were amplified successfully using quantitative polymerase chain reaction (qPCR). In partnership with a qualified lab, these tools can now provide a low-cost, user-friendly, and reliable method for monitoring the presence of rainbow smelt.

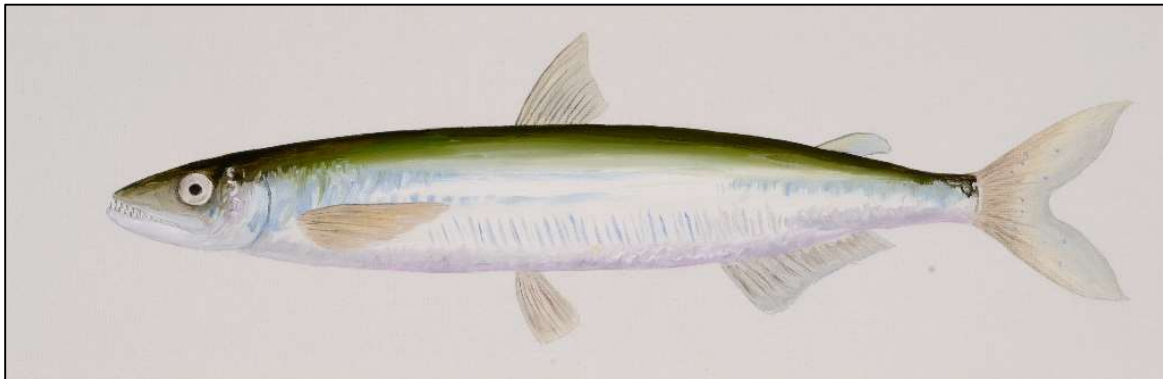


Figure 1. Rainbow smelt (*Osmerus mordax*), illustration by Jim Dochterman.

Management Challenges for Rainbow Smelt

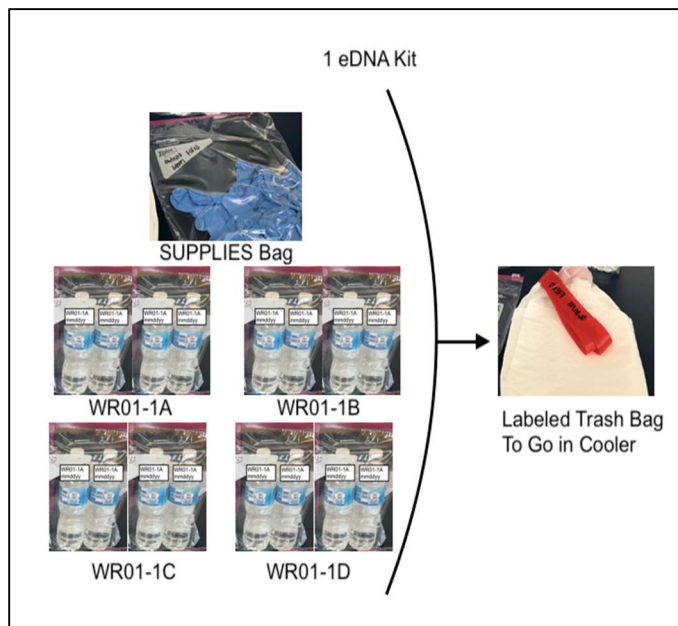
Rainbow smelt (*Osmerus mordax*) (Figure 1.) is an anadromous and landlocked fish species whose populations are in decline across its native range (Enterline et al. 2012). Sea-run smelt were listed as a *Species of Concern* by the National Marine Fisheries Service in 2004 and as a *Species of Greatest Conservation Need* in the 2015 Maine Wildlife Action Plan. Timely, strategic conservation actions are needed to prevent further decline or loss of imperiled smelt populations and to mitigate problematic ecological effects of illegal smelt introductions in inland waterbodies. These actions need to be guided by accurate, up-to-date, site specific information on the status of rainbow smelt populations.

In 2012, Maine, New Hampshire, and Massachusetts developed the “Regional Conservation Plan for Anadromous Rainbow Smelt.” This multi-agency plan identified key actions for conservation of rainbow smelt, including continued statewide monitoring (Enterline et al. 2012). Monitoring of anadromous rainbow smelt populations in Maine is carried out by the Department of Marine Resources (DMR) using traditional survey approaches, such as fyke nets, trawls, creel surveys and on foot surveys of spawning streams. DMR has documented 279 streams distributed along the Maine coast that either currently or

historically supported rainbow smelt, or have the potential to, based on modeled habitat and field observations (Enterline et al. 2012). The status of approximately 47% of these spawning runs is uncertain. Knowledge is similarly sparse across the species' freshwater range. Monitoring of so many locations presents an enormous challenge. This challenge will be difficult to overcome with traditional monitoring methods which are time and resource intensive, limiting surveys to only a few sites in any given year.

eDNA for Effective Monitoring

Methods for detection of environmental DNA (eDNA) offer an efficient and cost-effective technique to monitor rainbow smelt. Compared to conventional sampling methods eDNA sampling is less time and labor intensive for collecting presence/absence data. Additionally, eDNA sampling does not pose any risk to the smelt populations being monitored, for which disruptions in spawning activities could be detrimental. Smelt eDNA tools developed by Dr. Michael Kinnison's lab at the University of Maine (Evolutionary Applications Lab) have been shown to be extremely sensitive, with the ability to detect very low concentrations in stream water (Starting copy number .501 M.T.K, unpublished data). This makes eDNA very useful for detecting rainbow smelt in streams where they occur in low abundance and might otherwise be difficult to detect with conventional methods.



The use of eDNA can therefore provide critical information for conservation, restoration and fisheries enhancement by allowing organizations to focus efforts where they can have timely impact. The amount of field time required to collect water samples is significantly less than what is required to employ traditional sampling methods with traps and nets. This means that eDNA methods can facilitate a much more comprehensive and systematic approach to monitoring. Likewise, the low cost of eDNA sampling can preserve limited time and monetary resources for obtaining other critical data and for actual management interventions, such as enhancement or restoration.

Figure 2. eDNA sample kits include water bottles, sealable bags, gloves, and paper towels.

Environmental DNA has the potential to be an extremely accessible tool. The water sampling required for eDNA surveys can be carried out by organizations that do not have a high level of scientific sophistication, in partnership with a lab capable of conducting the genetic analysis. Dr. Kinnison has made this accessibility a priority, designing and validating the first-ever eDNA water sampling kit that uses materials from your local grocery store (Figure 2). Accessibility for agencies, NGOs and the public is also enhanced by the fact that eDNA is a very 'safe' survey method for both samplers and for target and non-target species.

Environmental DNA may afford an unprecedented capacity for volunteers and citizen groups to participate in local fisheries data collection and management (Biggs et al. 2015). Indeed, eDNA surveys could in principle be conducted by almost anyone, without risk of harm to protected species, without concern for legal harvest seasons, and without special licenses or permits.

Rainbow smelt eDNA primer-probe sets were developed and successfully lab tested by the Kinnison lab in 2016 (Figures 3 and 4.) Study design and methods for the use of eDNA for monitoring rainbow smelt have been outlined in an EPA approved Mini Quality Assurance Program Plan (QAPP) (Appendix A) developed in partnership with the Casco Bay Estuary Partnership. This document and its accompanying protocols will allow managers, conservation organizations, and citizen groups to undertake monitoring of rainbow smelt in coastal streams guided by best practices.

| Species | 5' Forward 3' | 5' Probe 3' | 5' Reverse 3' |
|---------|------------------------|-------------------------|------------------------|
| OSM | CACAACCATCCACCGCTCTCTC | TGGGCCAAGCTATCGCGAGCCA | TTGCTGCTGGGAGGTGAATAGC |
| ARC | TGCCGTCGTCCACCGATTAGCC | TAGGACAAACTATTGCCAGCCA | TGACTATGGGCAGGTAGTTGA |
| ATL | AGCCATTATCCACCGATTAGCC | TAGGACAAACCATGCCAGCCA | TGACCATAGGTAGGTGAGTTGA |
| BKT | TGCCATCGTACACCGATTGGCC | TAGGACAAACTATTGCCACCCA | TAACTATAGGGAGGTAGTTGA |
| LKT | TGCCGTCGTCCACCGATTAGCC | TAGGACAAACTATTGCCACCCA | TGACTATGGGCAGGTAGTTGA |
| RBT | CGCCATCATCCACCGATTGACC | TAGGACAAACCATCGCCAGCCA | TGACTATTAGGCAGGTGAGTTG |
| BNT | AACCATCATCCACCGATTGGCC | TGGGACAAACCATGCCAGCCA | TGACCATAGGCAGGTGAGTTGA |
| LWF | CGCCATTGTCCACCGACTAGCC | TGGTCAGGCATTGCCAGCCA | TAATTATGGGCAGATGGGTGGA |
| CP | ATCAATTATCCATCGGATAATT | TGGGTCAAATAATTGCTACCCA | TAATAAGGGGTAGATTAATTGA |
| NP | ATCAATTATCATCGCTTAACC | TCGGCCAAAATAATTGCCACCCA | TAATTATGGGAGGTGATGGA |
| LMB | AGGGGTTTCCACCGCCTCATG | TTGGCCAAGCAATTGCCAGCCA | AAGCTAAGGGGGTATTGAGGGA |
| SMB | CATAGTTTTCCACCGCCTTATG | TTGGCCAAGCAATTGCCAGTCA | AAACTAAAGGGGTGTTAAGGGA |
| BC | AATAATCACTCACCGCTTCATT | TAGGCCAGTCAATTGCTAGCCA | TAATTAAAGGGGTGTTGAGGGA |

Figure 3. Smelt TaqMan MGB-NFQ qPCR Primer-Probe Set develop by Kinnison Lab, University of Maine. Primers and probe regions of the smelt assay are aligned with the same gene regions in other common Maine freshwater fish species. Red shading indicates base pairs where smelt sequence differs from reference taxa. OSM = rainbow smelt (*O. mordax*), ARC = Arctic char, ATL = Atlantic salmon, BKT = brook trout, LKT = lake trout, RBT = rainbow trout, BNT = brown trout, LWF = lake whitefish, CP = chain pickerel, NP = northern pike, LMB = largemouth bass, SMB = smallmouth bass, BC = black crappie.

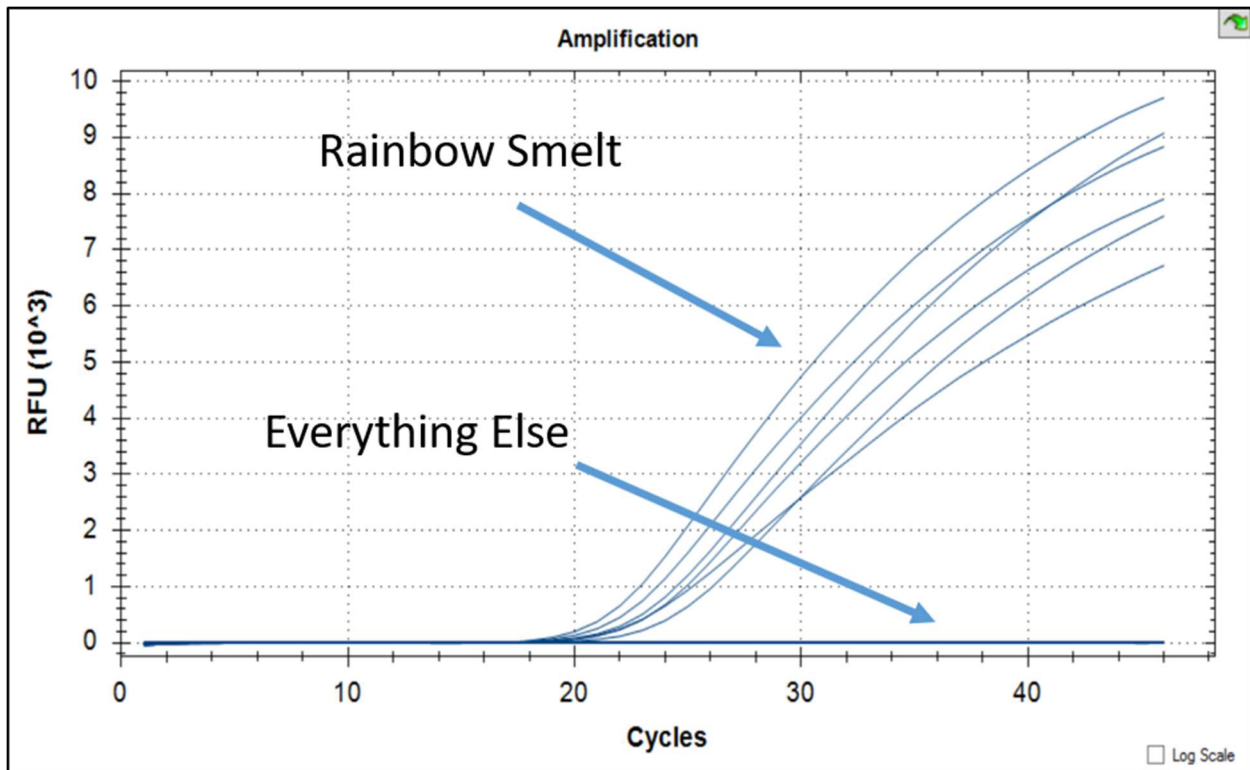


Figure 4. Quantitative PCR (qPCR) of smelt eDNA and eDNA of non-target species. Upward sweeping fluorescence (RFU) lines depict positive amplifications of smelt eDNA in laboratory trials. Flat lines show absence of amplification for non-target species. The cycle number where curves climb above baseline is indicative of initial eDNA concentrations, permitting eDNA quantification. Non-target species include: Atlantic salmon (*Salmo salar*), Arctic charr (*Salvelinus fontinalis*), brook trout (*Salvelinus fontinalis*), lak trout (*Salvelinus namaycush*), brown trout (*Salmo trutta*), largemouth bass (*Micropterus salmoides*), northern pike (*Esox lucius*), chain pickerel (*Esox niger*), and muskellunge (*Esox masquinongy*).

2017 Rainbow Smelt eDNA Pilot

The opportunity to field verify these tools arrived in spring 2017 during a Wells Reserve fyke net survey of anadromous fish species at two sites in York River estuary (Aman 2018). Water samples were collected upstream of net sites, prior to setting nets at low tide, and analyzed for smelt eDNA. This pilot allowed Wells Reserve staff to become familiar with eDNA sampling protocols and evaluate sample timing and location. We found that the highest concentrations of smelt DNA were detected approximately 10 to 18 days after peak catch of adult smelt in the fyke nets (Figure 5).

This period closely matches published egg incubation times for rainbow smelt (Chase 2006), and we surmised that the major source of smelt eDNA was likely associated with embryonic development rather than material shed by adults. This assumption is made more likely by the tendency for adult smelt to primarily spawn at night around high tide, while our sampling took place at daytime low tides, when shed DNA from adults was likely to have been washed downstream with the outgoing tide. These observations were valuable in developing our preliminary sampling and analysis plan for our 2018 study.

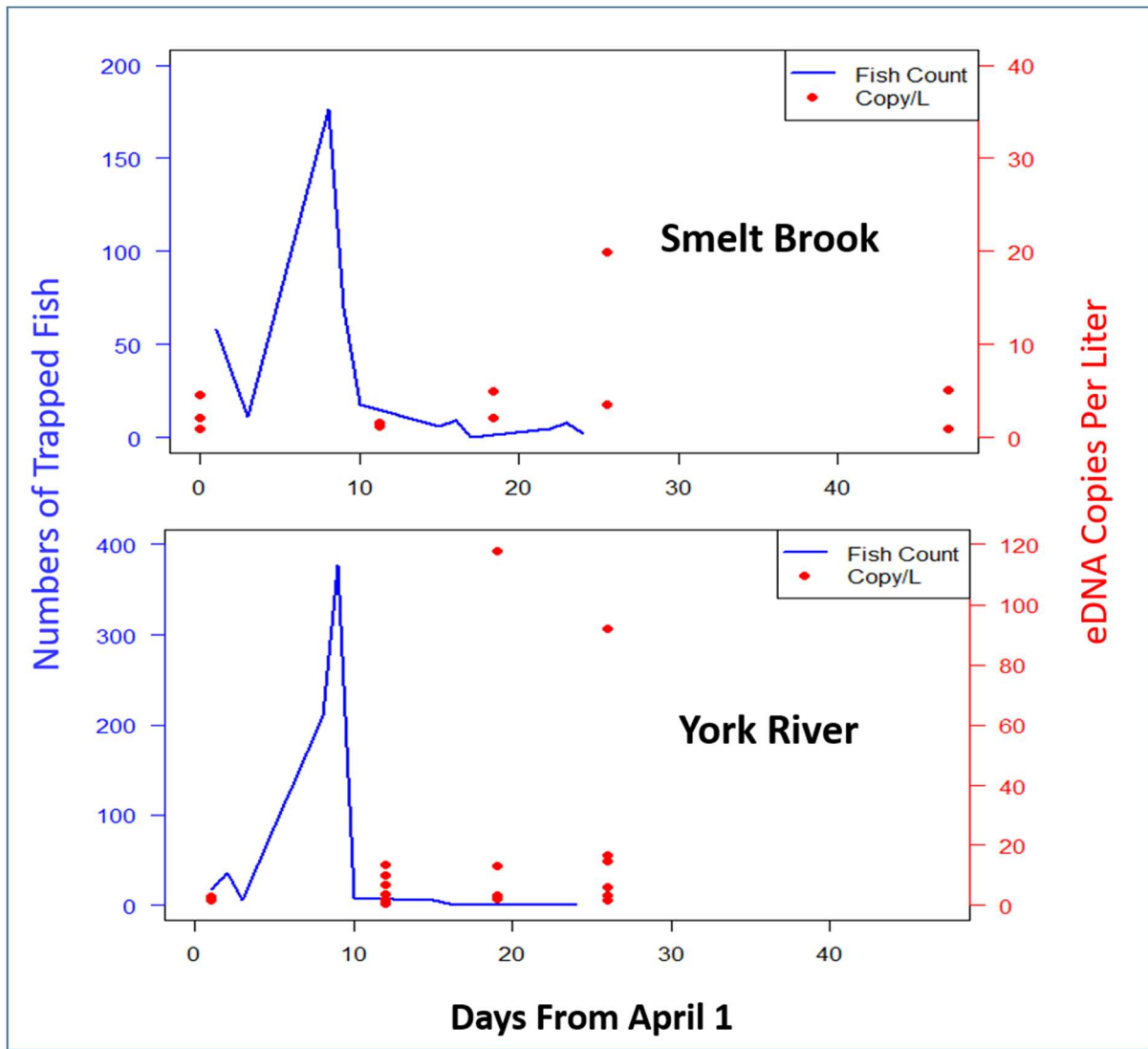


Figure 5. Captured adult smelt (total individuals) plotted with smelt eDNA concentration (copies/Liter) during April 2017. Positive eDNA assays occurred with samples collected after onset adults moving to spawning grounds.

2018 Casco Bay Study

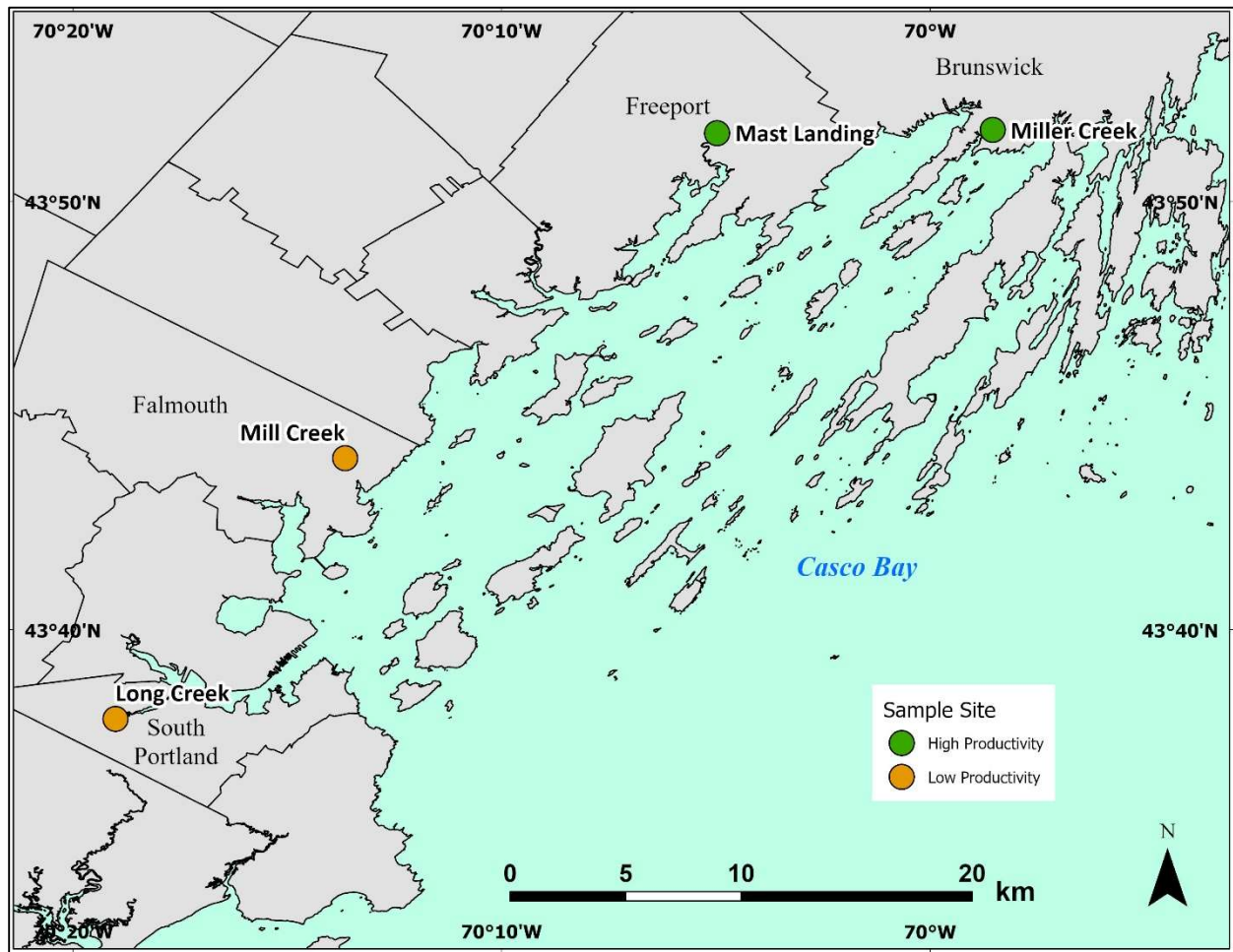


Figure 6. Casco Bay study sites at Long Creek (WR01), Mill Creek (WR02), Mast Landing (WR04), and Miller Creek (WR05). Samples were collected downstream of documented spawning areas.

Study Design

Our eDNA sampling protocols were field tested in four coastal streams in Casco Bay, Maine known to support spawning populations of rainbow smelt. Documented high (n=2) and low (n=2) productivity sites were selected based on DMR field observations from 2005-2009 (Figure 6). High productivity sites provided a positive control while low productivity sites tested the lower detection limits of the sampling protocol. Site visits were conducted in early March with Claire Enterline, Research Coordinator with the Maine Coastal Program, to identify spawning areas and discuss the sampling regime. Egg collection bricks were deployed at each spawning area to facilitate identification and possible collection for minimum detection trials. Bricks and surrounding substrate were checked for eggs after samples were collected on each sampling event and order of magnitude counts of eggs were recorded.

Each sample was comprised of 2000 ml of stream water collected in new (manufacturer sealed) drinking water bottles which are free of smelt eDNA. Samples were collected around daytime low tides at three

channel locations (A, B, C) downstream of known spawning habitat in each stream: river-right, river-center, and river-left. At site WR05 the channel was very narrow, and samples were collected moving upstream at approximately 2 m intervals. Spatially varied replicates were intended to provide better representation of in-stream eDNA concentrations. A contamination control (blank) was collected at each site as well, by opening and then closing the sample bottles, retaining the bottled water. Individual samples were isolated in Zip-loc freezer bags, grouped by site in trash bags, and transported on ice to the Wells Reserve lab where they were then transferred to freezers for short term storage.

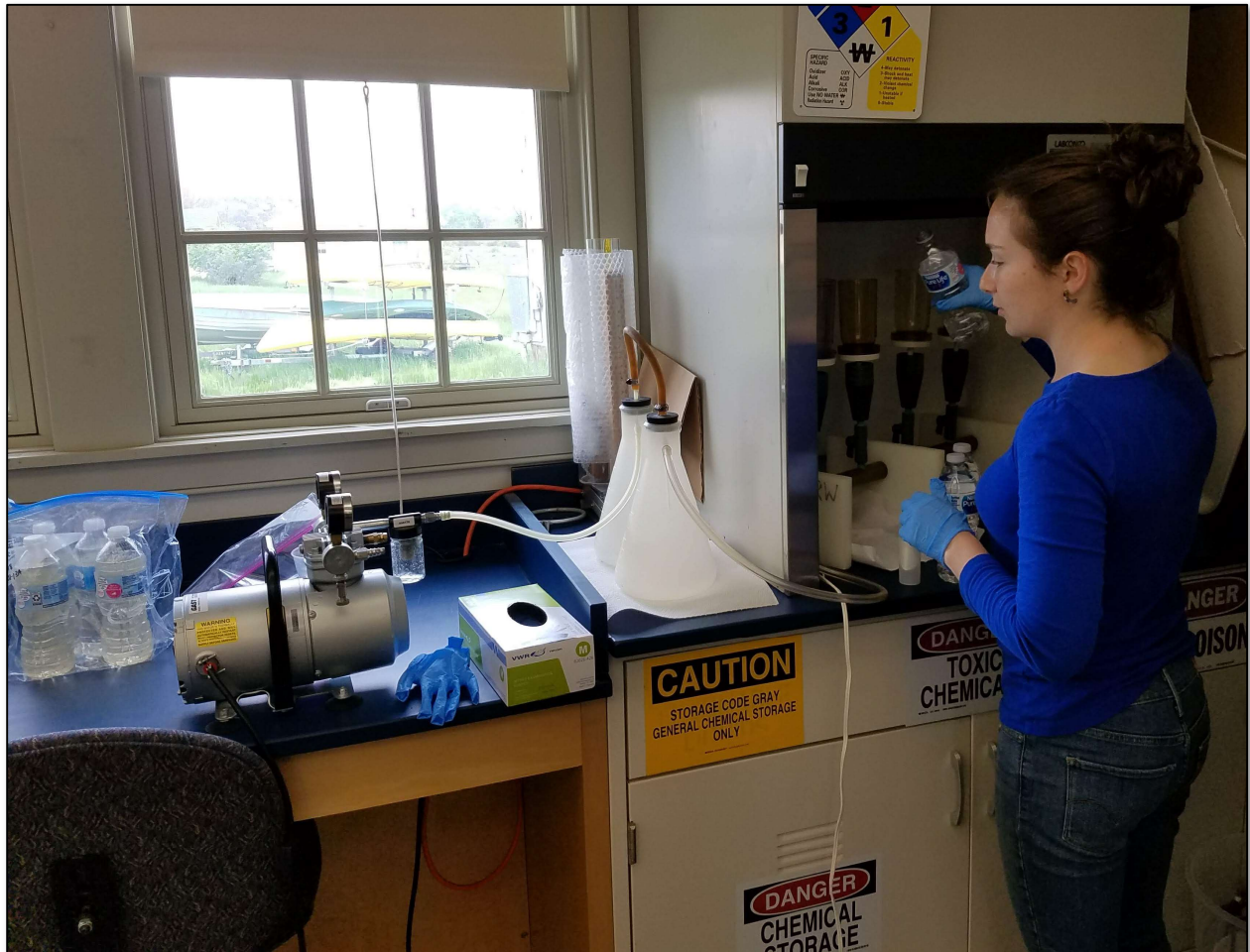
Samples were prepared in the lab first by thawing in a closed cooler and then filtering with a vacuum pump and Whatman GF 1.5-micron glass fiber filters. Filtration occurred as soon as possible after freezing to prevent degradation of eDNA, typically within 1-2 weeks. The filters retain genetic material and were placed in 1.5 ml centrifuge tubes after processing. A lab blank consisting of a new water bottle was filtered prior to every 10 samples as a filtering contamination control. The filtration area was decontaminated with UV light, and filtering equipment was bleached between samples. Processed samples were then stored in the freezer until they could be transferred to the University of Maine lab for DNA extraction and qPCR analysis. Extraction used Qiagen blood/tissue kit tissue kits followed by use of an inhibition clean-up kit. qPCR assays were based on the Taqman MGB-NFQ chemistry and run on a BioRad CFX96 Real-Time thermocycler. Environmental samples and negative controls were all plated in triplicate. We also ran positive control samples in the form of a dilution series of synthetic gene fragments (gBlocks) matching our targeted smelt amplicon and an Exogenous Internal Positive Control (TaqMan) to quantitatively assess the presence of PCR inhibition.

Initially, we had minimal success with eDNA amplification in field samples, even with water collected from a bucket with smelt eggs in it. Although use of Environmental Mastermix 2.0 can reduce the effects of some PCR inhibition (Jane et al. 2014, strong environmental inhibitors can still impair eDNA detection in field samples. However, subsequent inhibition clean-up of all samples using a Zymo One Step PCR Inhibitor Removal kit resolved these issues, revealing a high rate of eDNA detection in many of the field samples.



Smelt eggs adhere to the rough surface of a landscaping brick placed at the Mast Landing spawning site.

Because the goal of this project was to develop field-ready eDNA approaches for smelt surveys, we applied hierarchical occupancy modelling to our field data to estimate detection probabilities at each nested level of our sampling design. These models estimate the detection probability per date within site, per sample within date, and per technical replicate within sample. We in turn used these per date, per sample, and per replicate detection probabilities to provide a recommend number of dates, samples and replicate for a specified cumulative detection probability of $p=.95$.



Preparing to filter eDNA samples at the Wells Reserve.

Results

Order of magnitude estimates for egg abundance were tens of thousands at Mast Landing, and thousands at Miller Creek. Eggs were never observed at Long Creek or Mill Creek. Based on the visual observations we believe that we began sampling prior to the onset of spawning at all sites. Bricks were successfully used to collect smelt eggs. A minimum detection trial was attempted at Mast Landing by relocating bricks with eggs to a freshwater reach upstream of the spawning area, however a significant flow event on April 25th prevented sample collection for several days due to high water. Subsequently no eggs remained on relocated bricks or even much of the spawning area during our next site visit on April 27th. In Long Creek, bricks placed where riparian cover was sparse accumulated periphyton growth during the study, in some cases becoming completely covered. These conditions are not suitable for egg adhesion and these habitat conditions may have been reflected in the lower number of detections at this site.

In total, 177 samples were collected during 15 sampling events from March 29 to May 9, 2018. Rainbow smelt eggs were first observed at Mast Landing and Miller Creek sites on April 20th and were last

observed on May 7th at Miller Creek. Initially, a subset of samples was chosen for final qPCR analysis from the time period after eggs first appeared to when they were no longer observed at spawning sites, including seven samples from April 25th to May 9th. After having spent more project time on filtering and overcoming the environmental inhibition issues, sub-sampling allowed us to conserve funding for samples that were most likely to contain smelt eDNA and provide positive detections from qPCR. In late 2019, funding became available to analyze and additional 48 samples from April 13, April 20, and April 23, and these data will be included in a forthcoming Master’s thesis.

A total of 336 qPCR assays were carried out including 252 replicates from field samples and 84 replicates from contamination controls. The total number of positive qPCR replicates for each site was 52 from seven dates at Mast Landing (WR04), 31 from five dates at Mill Creek (WR02), 15 from three dates at Miller Creek (WR05), and nine from two dates at Long Creek (WR01) (Table 1). The field blank (contamination control) from the Miller Creek April 25th sample produced a single replicate, but none of the other 252 blanks produced a detection, indicating that contamination control was carried out properly.

Table 1. Positive eDNA detection from lab triplicate qPCR assays of samples collected at site replicates (A, B, and C) for our four study sites from April 25 to May 9.

| Date | Long Creek (WR01) | | | Mill Creek (WR02) | | | Mast Landing (WR04) | | | Miller Creek (WR05) | | |
|-----------|-------------------|---|---|-------------------|---|---|---------------------|---|---|---------------------|---|---|
| | A | B | C | A | B | C | A | B | C | A | B | C |
| 4/25/2018 | 0 | 0 | 0 | 3 | 3 | 0 | 3 | 0 | 0 | 0 | 0 | 0 |
| 4/27/2018 | 0 | 0 | 0 | 0 | 1 | 0 | 3 | 3 | 1 | 0 | 3 | 3 |
| 4/30/2018 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 3 | 0 | 0 | 0 | 0 |
| 5/2/2018 | 3 | 0 | 3 | 0 | 0 | 0 | 3 | 3 | 3 | 3 | 0 | 0 |
| 5/4/2018 | 0 | 1 | 1 | 3 | 0 | 3 | 3 | 3 | 3 | 3 | 0 | 3 |
| 5/7/2018 | 0 | 0 | 0 | 3 | 3 | 3 | 3 | 3 | 3 | 0 | 0 | 0 |
| 5/9/2018 | 0 | 0 | 0 | 2 | 3 | 3 | 3 | 3 | 3 | 0 | 0 | 0 |

Analysis of site-specific detection with hierarchical occupancy models indicated that the per date probability of detection ranged from 0.547 at Miller Creek to 0.908 at Mast Landing (Figure 7). The per sample detection probability ranged from 0.519 at Long Creek to 0.860 at Mast Landing (Figure 8). Finally, the per qPCR replicate detection probability ranged from 0.450 at Long Creek to 0.944 at Mast Landing (Figure 9). Based on the most conservative values from these estimates, we recommend that future surveys seeking to verify smelt presence during a given spawning season collect water on a minimum of 4 dates during the most likely 2 week spawning window, collect 5 water samples on each of those days, and run each sample for a total of 6 qPCR replicates to achieve a 95% probability of encountering smelt eDNA in a low abundance stream when it is present. This recommendation assumes that future sampling spaces dates 2-3 days apart as in the current study. Moreover, we suggest that odds of smelt detection may be somewhat improved by targeting sample dates more towards the latter part of the 2-week window.

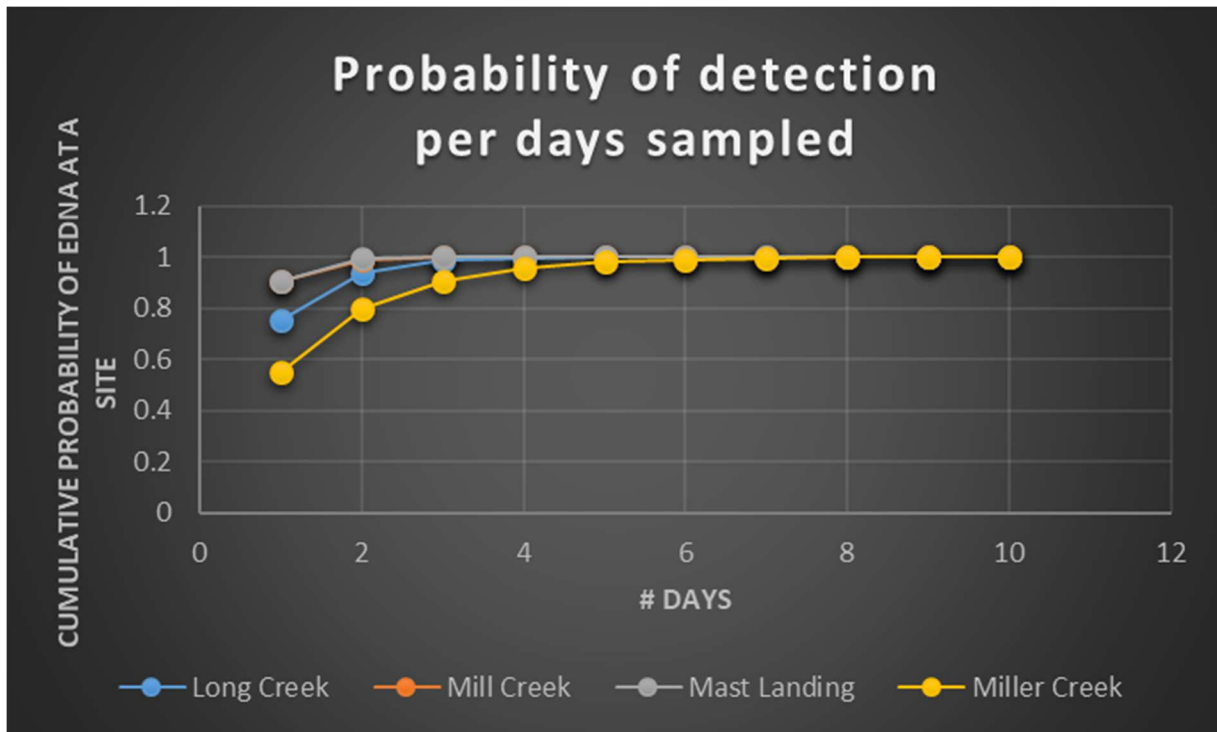


Figure 7. Results of the hierarchical occupancy model for detection per day sampled at each site.

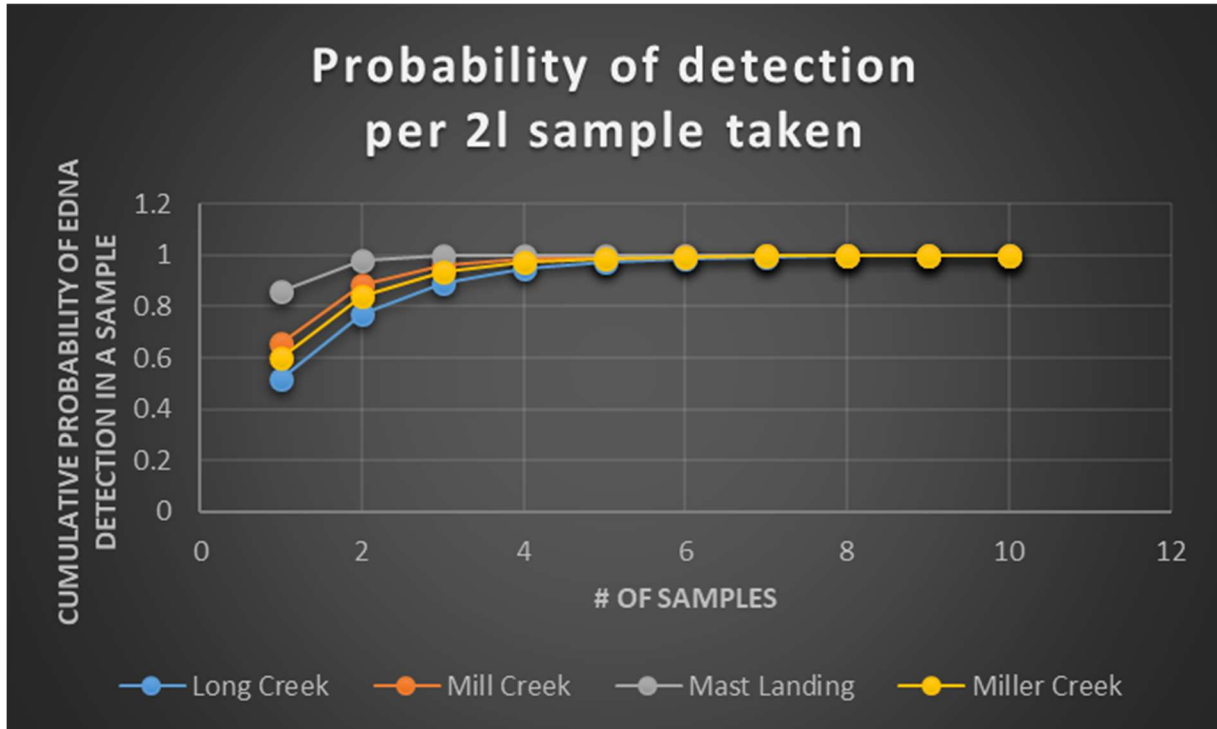


Figure 8. Results of the hierarchical occupancy model for detection per 2-liter sample taken at each site.

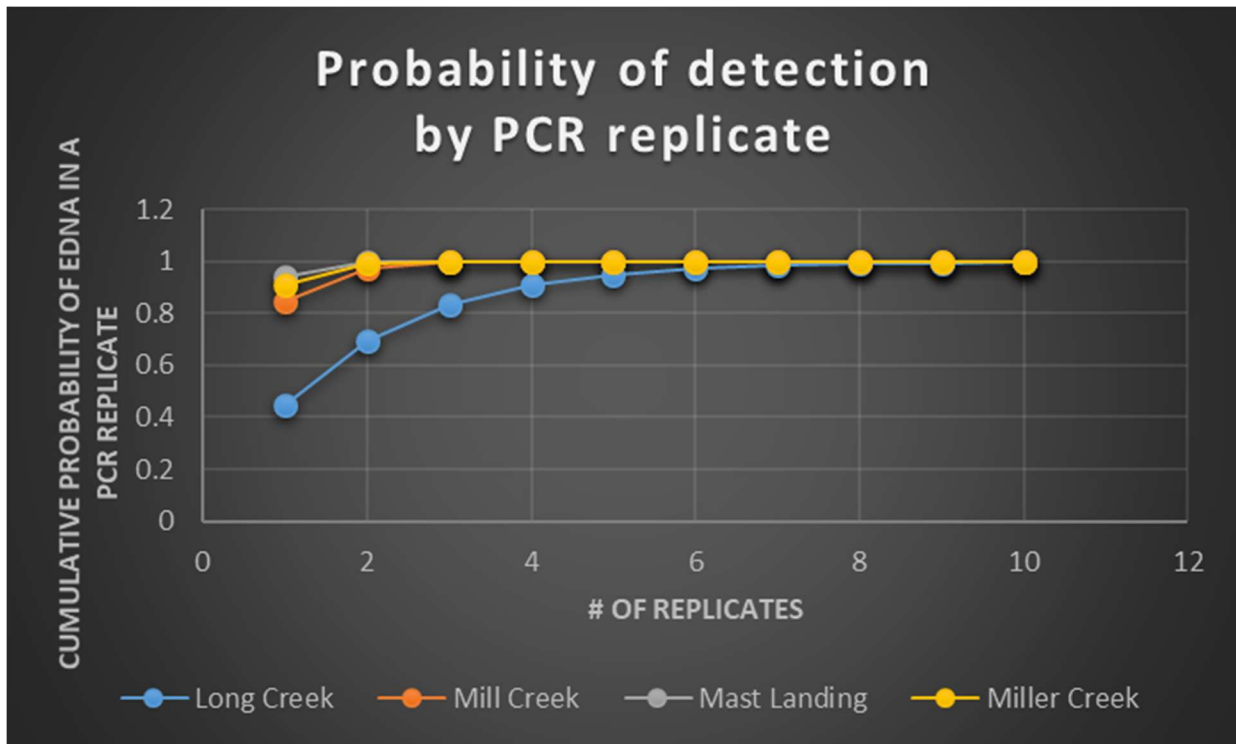


Figure 9. Results of the hierarchical occupancy model for detection per qPCR replicate for each site.

Discussion and Lessons Learned

The number of samples that produced positive replications was highest at Mast Landing where eggs were observed to be most abundant. Our other high abundance site at Miller Creek had a relatively small number of positive replications, almost half that of the low productivity site at Mill Creek. It should be noted that population information from MDMR is somewhat outdated, and it is likely that local populations have changed since last observed.

Though our visual surveys did not identify smelt eggs at spawning locations after May 7th, it appears from our qPCR results that spawning may have still been occurring at Mast Landing and Mill Creek. However, these late season detections may represent some combination of fish eDNA and eDNA shed from developing eggs deposited earlier in the season, consistent with detections well after spawning in our pilot study of populations sampled with fyke nets.

On most sampling dates with smelt detection, two or more samples tested positive for smelt eDNA. However, there were dates in three of the different streams where only one of three samples tested positive. This type of sample-to-sample variability is common in eDNA surveys and likely represents combined effects of 1) the actual spatial variation in eDNA availability and 2) the probabilistic nature of eDNA capture and amplification when it is at very low concentrations. The fact that single positive detections were still more often than not strong detections, in the sense of positive hits on three out of three technical replicates, suggests that spatial variability is the more important factor in these small streams. This may have to do with the observation that eDNA may require a breakout window before it is fully mixed in small streams (Wood et al. 2020). However, if maximum power is required, both of

these contributing causes for sample-to-sample detection variability are addressable through spatial sample replication in eDNA surveys. Increasing sample volumes may also improve detection rates, but this must be weighed against the challenges of transporting and filtering larger volumes. If larger volumes are collected, we still recommend the volume of a given sample be spread over a larger spatial area if possible.

However, it is certainly worth assessing whether sampling effort in these streams is best allocated to more survey dates or more samples per date. Based on our hierarchical occupancy models, the per date detection probability was much higher than the per sample detection probability, particularly in the stream with the lowest number of smelt detections. Indeed, it is reasonable to expect that for the lowest abundance smelt populations, most spawning might occur on just a few nights of the season, but because of the small size of such streams even a few individuals have decently high odds of eDNA detection in one or two samples. Because of this temporal constriction of spawning activity, more power can likely be obtained for any given eDNA budget by sampling more dates during the spawning window than by collecting more samples on fewer dates.

Given the detection probabilities from our occupancy modelling, we recommend that surveys of streams similar in size to this study target a minimum of 4 dates per spawning season per stream, and collection 5 samples per date. For larger streams it could be advisable to increase the number of samples per date to account for dilution effects and more spatial heterogeneity, but we would not recommend reducing the number of dates sampled, because the number of dates is again likely more determined by the temporally patchy spawning behavior of low abundance populations. The underlying assumption of course, is that sampling is conducted during the right time window and not too early or too late. This study cannot itself address when that timing be for a given stream. However, in many cases historical data and tracking of runs further south or regionally might provide a good basis for planning the start of a sampling window in a given season.

Samples collected in Casco Bay streams required significantly more time to filter than samples collected in the pilot York River sampling, likely owing to higher amounts of suspended material in the samples. This created a backlog of samples for UMaine and it became necessary to filter some samples at the Wells Reserve. Consequently, we developed a procedure for processing eDNA samples that can be utilized by organizations with basic facilities and equipment and increased organizational capacity for conducting eDNA studies. This procedure is described in the Wells NERR eDNA Manual (Appendix B).

Although eDNA sampling is accessible and powerful for smelt stream surveys, it does impose modest per sample costs that must be factored into survey work. However, there are adaptive sampling strategies that can help to limit these costs. First, if smelt or smelt eggs are visually observed at a site, then there is no need to subsequently confirm presence with eDNA. So, there is still value in samplers spending some time on quick visual surveys on any given date. Second, eDNA surveys will be most cost effective when analyzed samples come from a time window when spawning is most likely. As such, project funding for sites with suspected small runs can be most efficiently utilized by using visual observations of spawning at nearby known positive sites to help target sampling and prioritize processing of water samples to windows that may best overlap with probable active spawning and egg incubation times. In the absence of visual observations at nearby sites, all sites in a region might be surveyed over a wider range of dates and a subset of most probable sites analyzed for detection to prioritize focal dates for sample processing at other sites.

Savings can also be appreciated in cases where the only study goal might be detection. Detection is a largely binary outcome, and thus once smelt eDNA is substantively confirmed for a given site and date, further detections provide somewhat redundant information. While some confirmation is useful in



cases of initially weak detections (e.g., a single technical replicate or sample), in many cases detection on one or two sampling dates may be very conclusive. eDNA samples and controls are typically run in batches on 96 well plates. Although it might be intuitive to run samples from multiple dates at a given site together in a single processing batch, that strategy may result in many redundant detections for a given site. Alternatively, if samples are batched by dates, and the most probable dates are processed first, then many sites may initially test positive making it unnecessary to test samples from additional dates at those sites, reserving processing resources for sites that lacked initial detection and would benefit from more testing dates. A modification of this adaptive strategy could also be applied to save field labor if eDNA samples can be filtered and processed with a quick turnaround, such that early season samples are analyzed to determine detections that would obviate the need for further field work and lab processing of positive sites.

Collecting an eDNA water sample at Mill Creek.

Communication

Project results were presented to attendees at the Fall 2018 meeting of the New England Estuarine Research Society and to the 2019 Maine Water and Sustainability Conference. Project updates were made available on the Wells Reserve website. Final project materials were distributed to project partners and fisheries managers.

What's Next?

Now that we have optimized our smelt eDNA assay, established an effective clean-up method to remediate environmental PCR inhibition, demonstrated smelt detection in multiple streams, and used this data to develop recommended sampling approaches, we plan to more widely deploy our smelt assay in other coastal streams as well as freshwater systems where smelt present other management challenges.

As part of future work, we will seek to not only relate eDNA detection to other estimates of occurrence but begin to develop quantitative transfer functions between inferred eDNA concentrations in samples and the relative abundance of smelt.

To complement this field work we may conduct controlled detection experiments with caged smelt and eggs to provide high-confidence assessment of the specific effects of environmental factors like flow, temperature and tidal cycle on detection and quantification.

Given that sea-run and landlock smelt in this region have different mitochondrial haplotypes, we also have plans to explore modifications to our eDNA assay procedure that might distinguish these forms.

Finally, through wider-scale deployment we will plan to refine a citizen science accessible workplan and standard operating procedures for long-term smelt eDNA monitoring while building dialogs with resource agencies about how best to adapt citizen-science-based eDNA monitoring to current species assessment and policy.

In 2018, the University of Maine opened created a coordinated operating research entity (CORE) for eDNA. This lab will provide eDNA related services including development of probes, analysis of samples, training, and consultation for project development. The eDNA CORE provides an all-inclusive resource for organizations that wish to incorporate eDNA methods into their resource management activities.

References

- Aman, J.T. 2018. An Assessment of Spring Fish Communities in the York River. Report to the York River Study Committee. Wells National Estuarine Research Reserve. Wells, ME.
- Biggs, J., N. Ewald, A. Valentini, C. Gaboriaud, T. Dejean, R.A. Griffiths, and J. Foster. 2015. Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (*Triturus cristatus*). *Biological Conservation*. 183: 19-28.
- Chase, B. C. 2006. Rainbow smelt (*Osmerus mordax*) spawning habitat on the Gulf of Maine coast of Massachusetts. Mass. Div. of Mar. Fish., Tech. Report No. TR-30.
- Enterline, C.L., B.C. Chase, J.M. Carloni, and K.E. Mills. 2012. A regional conservation plan for anadromous Rainbow smelt in the U.S. Gulf of Maine. Report part of a multi-state collaborative to develop & implement a conservation program for three anadromous finfish species of concern in the Gulf of Maine (NOAA Species of Concern Grant Program Award #NA06NMF4720249A). 100 p.
- Jane, S.F., Wilcox, T.M., McKelvey, K.S., Young, M.K., Schwartz, M.K., Lowe, W.H., Letcher, B.H. and Whiteley, A.R. 2015. Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. *Mol Ecol Resour*, 15: 216-227. doi:10.1111/1755-0998.12285
- Wood, Z.T., E.B. Erdman, G. York, J.T. Trial, and M.T. Kinnison. 2020. Experimental assessment of optimal lotic eDNA sampling and assay multiplexing for a critically endangered fish. *Environmental DNA* (in press).

Appendix A. 2018 eDNA Mini-QAPP

Developing Cost Effective Monitoring for Rainbow Smelt Using eDNA

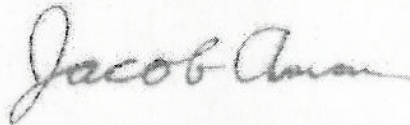
Mini-Quality Assurance Project Plan

Casco Bay Estuary Partnership

Year 1 - 2018

April 30, 2018

Partially funded by EPA grant number CE 00A00063-0 (FY 2016) to Casco Bay Estuary Partnership.



Jacob Aman, Wells National Estuarine Research Reserve

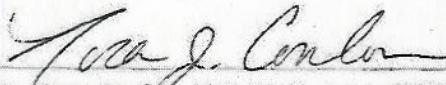
Date



5/7/18

Dr. Michael Kinnison, University of Maine

Date



5/11/2018

Dr. Nora Conlon, U.S. Environmental Protection Agency

Date



5/7/18

Claire Enterline, Maine Coastal Program/Maine Dept. of Marine Resources

Date



4/30/2018

Matthew Craig, Casco Bay Estuary Partnership

Date



5/11/18

Alicia Grimaldi, U.S. Environmental Protection Agency Project Officer

Date

1. Background

Rainbow smelt (*Osmerus mordax*) is an anadromous fish species native to Maine whose populations are in decline across its range in New England (Enterline *et al.* 2012). Smelt are listed as a Species of Concern by the National Marine Fisheries Service and as a Species of Greatest Conservation Need in the 2015 Maine Wildlife Action Plan. Action 1.2B of the Casco Bay Plan calls for CBEP to coordinate efforts to restore aquatic habitat connectivity, with an emphasis on restoration of access to spawning habitat for anadromous species (CBEP 2016). Timely, strategic conservation actions are needed to prevent further decline or loss of valued smelt populations. These actions need to be guided by accurate, up-to-date information on the local status of rainbow smelt populations.

In 2012, Maine, New Hampshire, and Massachusetts developed the Regional Conservation Plan for Anadromous Rainbow Smelt, which identified continued statewide monitoring as a priority action (Enterline *et al.* 2012). Historically, monitoring of rainbow smelt populations in Maine has been carried out by the Maine Department of Marine Resources (DMR) and Maine Department of Inland Fisheries and Wildlife (IFW), using traditional survey approaches, such as fyke nets, trawls, creel surveys and on-foot surveys of spawning streams. Resource constraints require more efficient, decentralized, non-intrusive, and sensitive survey methods.

DNA is continually shed from fish and other organisms into their aquatic environments as part of natural cellular sloughing and decay (Thomsen *et al.* 2012). This DNA in the environment is known as environmental DNA, or 'eDNA', and it can be detected in water samples using modern genetic approaches similar to those used in forensics and genomics (Thomsen and Willerslev 2015). The use of eDNA methods have already been applied to early detection of invasive fish species in Maine by Dr. Michael Kinnison at the University of Maine, Orono (UMO). Dr. Kinnison has developed a suite of genetic markers for detecting eDNA of invasive centrarchids (bass and sunfishes) and esocids (pikes and pickerels), as well as native salmonids (York 2016). Dr. Kinnison has recently developed the necessary genetic markers for detection of rainbow smelt eDNA. The use of eDNA to detect rainbow smelt in locations where they may be present in low abundance could provide critical information for conservation, restoration and fisheries enhancement by allowing organizations to focus efforts where they can have timely impact. Environmental eDNA may afford an unprecedented capacity for volunteers and citizen groups to participate in local fisheries data collection and management (Biggs *et al.* 2015).

The next step in applying this technology to rainbow smelt conservation is to test and refine field survey protocols to ensure statistically meaningful results that can be accurately interpreted. If successful, WNER and CBEP intend to apply these methods to update habitat data for use in restoration planning, including targeted restoration activities for rainbow smelt such as removal of stream barriers at dams and road crossings. These methods will allow state partners to more accurately assess the status of rainbow smelt spawning in the majority small coastal streams, and detect early introductions of illegally stocked smelt in freshwater systems.

It is our hope that this project will facilitate earlier adoption of these methods in Maine and reduce the time and funds required to track the status of sea-run rainbow smelt spawning runs. The approaches and methodologies of using eDNA in fisheries science and conservation offer several advantages over traditional techniques including a high degree of sensitivity, and the development of standardized methods in a non-invasive manner (Thomsen and Willerslev 2015).

2. Purpose of the study

The purpose of the study is to test and refine sampling protocols for use of eDNA to detect the presence and distribution of anadromous rainbow smelt in Maine coastal stream habitats during spring spawning.

Project objectives include: 1) Conduct an intensive field survey for smelt eDNA in water samples using inexpensive and readily available collection kits. 2) Refine field collection protocols based on sampling results using a hierarchical occupancy model to establish the appropriate distribution of sampling effort (sample date, sample number, qPCR number) required to provide a high level of confidence of species detection. 3) Document and disseminate smelt eDNA survey guidelines to partner organizations.

These objectives will be met by: 1) WNERR staff receiving training from UMaine staff in eDNA sample collection and handling methods, and coordinated site selection and study design between project partners. 2) Collection of water samples in a hierarchically replicated fashion to permit estimation of detection probabilities at all levels of the survey process (sites, dates, sample locations). 3) Documentation and reporting of study methods and results, and implementation of the project communication plan (described below).

3. Organization

Jacob Aman is Project Manager at WNERR. Jacob conducts fisheries research and monitoring in southern Maine watersheds. Jacob is Principal Investigator responsible for coordination between partners; oversight of sample collection, custody and transfer; project documentation; reporting and outreach; and, administration of grant funds.

Dr. Michael Kinnison is Professor of Evolutionary Applications at UMO, with over 25 years of research and teaching experience. Dr. Kinnison will assist with study design, oversee qPCR analysis of samples, and assist with data interpretation, project documentation, outreach, and reporting.

Claire Enterline is a staff scientist with the Maine Coastal Program at DMR with expertise in smelt research, management, and monitoring. Claire provides assistance with study design, local habitat, and smelt life history, and assists with data interpretation and analysis.

Matthew Craig is Habitat Program Manager at CBEP. Matt provides assistance with QAPP preparation and site selection, project documentation, outreach, and reporting.

Dr. Nora Conlon is Quality Assurance Project Plan Coordinator with U.S. EPA Region 1. Dr. Conlon reviews study design and quality assurance protocols.

4. Sampling Plan

Sampling objective – minimum detection trials for detecting sea-run rainbow smelt. Data will be used to refine field sampling protocols for future use by partner organizations to detect smelt presence and assess smelt distribution in Maine coastal streams.

Four sample sites will be chosen from six streams where reliable data exist for the timing and health of past rainbow smelt spawning runs, and based on observation of early spawning activity in each stream. A high productivity stream will be used as a positive control. Final sites will consist of a mix of high productivity and lower productivity spawning runs. Study streams are listed in Table 1.

Table 1. eDNA Study Streams

| Site | Town | Stream | Status |
|------------------|----------------|------------------------|---------|
| Long Creek | South Portland | Long Creek | Decline |
| Mill Creek | Falmouth | Mill Creek | Limited |
| Mast Landing | Freeport | Mill Creek | Strong |
| Miller Creek | Brunswick | Miller Creek | Strong |
| Porter's Landing | Freeport | Porter's Landing Creek | Strong |
| Capisic Brook | Portland | Capisic Brook | Decline |

Each stream will have one sample site, and samples will be collected from three separate locations in close proximity at that site to better ensure sampling from a well-mixed area. Within each stream, sample sites were selected through: (a) expert knowledge of local habitat usage by DMR scientist Claire Enterline; (b) field verification and ground-truthing in March 2018; and, (c) safety/accessibility.

The start date of eDNA sampling will be determined from best professional judgement and observation of spawning activity in study streams. Sampling will take place over four weeks and start approximately one week prior to peak spawning activity. Three samples will be collected per week, per site, and samples will be collected at least one day apart. A total of 96 samples will be collected.

5. Methods and Protocols

Sample collection

Samples will be collected according to the Water Bottle Sampling for Environmental DNA Analysis (Appendix A; Kinnison 2018b).

Sampling kits will be prepared and stored in the lab on a weekly basis. Field collection equipment, including coolers, storage containers, and wading clothes and boots, will be decontaminated prior to each sampling event.

Samples will be collected by wading at a suitable location downstream of known spawning areas, and upstream of the extent of tidal influence if possible. If samples are collected from tidally influenced locations, collection will take place on an ebb low tide to allow for maximum flushing of tidal water. Three samples will be collected per site, one from each bank, and one from center of flow. Sampling will avoid pools and eddies where tide water could be held. Low productivity sites will be sampled before high productivity sites to reduce contamination risk.

Each sample will consist of 2 liters of collected stream water. One blank will be collected from each site per visit as a contamination control. Field decontamination procedures will be carried out between sample collections at each site.

Samples will be stored in marked bags for holding, placed in coolers with ice in the field to protect from heat and ultraviolet light, and then transferred to sterilized containers in dedicated lab freezers. Sample holding time will be 6-7 days. Frozen samples will be shipped to the University of Maine or transported by car in coolers packed with ice. If shipped, samples will be sent by 2-day delivery, and in the early part of the week to avoid weekend deliveries when no staff are present to receive them.

Field data sheet

Metadata will be recorded on field data sheets and will include: site name, waterbody name, date, field personnel, weather conditions, flow conditions (normal flow, storm flow). Field sheets will also include sample information: sample id, sample time, sample date. Field sheet copies will be kept with samples and used to track chain of custody including: date/time sample

transferred to freezer, date and time sample shipped, date and time sample received at lab. A copy of the data sheet is provided in Appendix B.

Sample analysis

Following delivery to the lab, frozen field samples will be thawed, filtered, extracted and prepared for replicate qPCR analysis according to methods detailed in York (2016). In addition to running the field blanks to account for sampling gear contamination, lab processing of samples will include use of filter-blanks and qPCR blanks to account for sources of potential lab contamination. qPCR analysis of field samples will be accompanied by qPCR of positive control samples of synthetic smelt eDNA in a dilution series. A subset of samples on each plate run will include an independent internal positive control (IPC) to detect potential PCR inhibition. Unused extracted DNA samples will be frozen for potential future analysis.

Results from qPCR analyses will be documented as the presence or absence of qPCR amplification in fewer than 45 cycles. For positive amplifications we will also estimate the qPCR cycle number (C_q value) at which fluorescence climbs above baseline (and estimator of initial eDNA concentration in the sample). Detection probabilities at the sample, site and date levels will be estimated from hierarchical occupancy models, as in York (2016) and de Souza *et al.* (2016).

Filtration and extraction protocols are described in detail in Appendix C (Kinnison 2018a).

5. Schedule

| Time Frame | Project Activity |
|----------------|--|
| Jan – Mar 2018 | Develop sampling plan and draft QAPP. |
| April 2018 | Implement sample and lab analysis plan during sea-run rainbow smelt migration. |
| May – Jul 2018 | Refine sampling protocols based on results of hierarchical occupancy model. |
| Jul – Aug 2018 | Document final sampling protocols. |
| Sep – Dec 2018 | Disseminate protocol and project results. |

6. Quality assurance and quality control

The following QA/QC measures for eDNA sampling are described in additional detail in Appendix A (Kinnison 2018b).

Whenever possible, supplies (Ziplock bags, gloves, etc.) will consist of new, unopened products to minimize risk of DNA contamination. Sampling kits will be compiled on sterilized surfaces. Sampling kits will be transported to the field inside sterilized containers.

At sampling, water bottles will not be opened until the moment samples are collected, and only when wearing clean gloves. Care will be taken to ensure that gloves, bottle surfaces, and bottle caps do not touch potentially contaminated surfaces.

Samples will be stored in marked bags for holding, placed in coolers with ice in the field to protect from heat and ultraviolet light, and then transferred to sterilized containers in dedicated lab freezers.

In the field, each sample site will include one blank for contamination control. These will be handled identically to field samples, including cap removal, but rather than collecting a field sample, the handler will retain the original contents rather than collecting a sample.

Field decontamination procedures will be carried out between sample collections at each site. Collected samples will be stored separately from sampling kits.

7. Corrective responses

After each sampling round, WNERR project manager Aman will review data sheets and interview the field technician to identify any deviations from the sampling protocols. As needed, the project manager will review protocols to ensure consistency of sampling in the field. Inconsistencies or modifications to the sampling protocols will be noted for consideration in results and analysis, and documented in the final report if necessary.

8. Data entry and validation

Datasheets will be stored at the WNERR. Metadata will be entered into a spreadsheet. Spreadsheets and scanned copies of the datasheets will be provided to project partners at the end of the study for back-up. Data sheets from each round of samples are reviewed by the project manager as they come in for errors in identification and/or data entry.

9. Data management and reporting

Results

A Bayesian hierarchical occupancy model will be used to analyze the data from each site to quantify detection probabilities associated with the date of sampling, number of dates sampled, number of samples per date and number of qPCR assays per sample.

Study Evaluation

Study success will be evaluated based on execution of the sampling plan, data analysis, data documentation, and analysis yielding high confidence in results. We will report this optimum survey design, along with all relevant field methods and quality assurance measures in a set of guidelines that will be disseminated to state and regional conservation partners to provide them with a field and laboratory validated tool for monitoring sea-run rainbow smelt with eDNA.

10. References

- Aman, J. 2016. Survey of Diadromous Fish Species of Greatest Conservation Need in Southern Maine: Report to the Nature Conservancy. Wells National Estuarine Research Reserve. Wells, ME.
- Biggs, J., N. Ewald, A. Valentini, C. Gaboriaud, T. Dejean, R.A. Griffiths, and J. Foster. 2015. Using eDNA to develop a national citizen-science-based monitoring programme for the great crested newt (*Triturus cristatus*). *Biological Conservation*. 183: 19-28.
- Casco Bay Estuary Partnership. 2016. *Casco Bay Plan 2016-2021*. Portland, ME.
www.cascobayestuary.org/wp-content/uploads/2015/10/casco-bay-plan-2016-2021.pdf.
- Collins, M., K. Lucey, B. Lambert, J. Kachmar, J. Turek, E. Hutchins, T. Purinton, and D. Neils. 2007. Stream Barrier Removal Monitoring Guide. Gulf of Maine Council on the Marine Environment. www.gulfofmaine.org/streambarrierremoval.
- de Souza, L.S., Goodwin, J.C. Renshaw, M.A., and E. Larson. 2016. Environmental DNA (eDNA) detection probability is influenced by seasonal activity of organisms. *PLoS One*.
<https://doi.org/10.1371/journal.pone.0165273>.
- Enterline, C.L., B.C. Chase, J.M. Carloni, and K.E. Mills. 2012. A regional conservation plan for anadromous Rainbow smelt in the U.S. Gulf of Maine. Report part of a multi-state collaborative to develop & implement a conservation program for three anadromous finfish species of concern in the Gulf of Maine (NOAA Species of Concern Grant Program Award #NA06NMF4720249A). 100 p.
- Kinnison, M. 2018a. Protocol for eDNA Filter Extraction Using the Qiagen DNeasy Kit. University of Maine.
- Kinnison, M. 2018b. Water Bottle Sampling for Environmental DNA Analysis. University of Maine.

Thomsen, P.F., J. Kielgast, L.L. Iversen, P.R. Moller, M. Rasmussen, and E. Willerslev. 2012. Detection of a diverse marine fish fauna using environmental DNA from seawater samples. PLoS ONE. 7: e41732.

Thomsen, P.F. and E. Willerslev. 2015. Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. Biological Conservation. 183: 4-18.

York, G. 2016. Environmental DNA Detection of Invasive Species. MS Thesis, University of Maine.

APPENDIX A

Water Bottle Sampling for Environmental DNA Analysis

University of Maine

This sampling protocol is intended for use by members of the general public, agencies or NGOs wanting to collect water samples for environmental DNA (eDNA) analysis to detect aquatic species. The protocol uses inexpensive supplies available at many supermarkets or general goods stores (e.g., Walmart) to collect water samples that will in turn be analyzed at the University of Maine. The lab assays we use are able to detect extremely small concentrations of eDNA. HENCE, CARE MUST BE TAKEN TO AVOID CONTAMINATION OF SAMPLES WITH TARGET SPECIES DNA ACCIDENTALLY TRANSFERRED FROM OTHER OBJECTS OR WATERBODIES. DNA contamination during field sampling can result in false detections and UMaine cannot be held responsible for contamination in samples collected by others. Hence, it is important that you be attentive of contamination risks and follow the procedures of this protocol.

DEFINITIONS FOR THIS PROTOCOL:

eDNA (Environmental DNA): Small fragments of DNA shed by organisms into their environment as part of their normal life and death processes

Target species: The organism you seek to detect with eDNA water samples

Waterbody: The lake, pond, stream, or river location where you require independent eDNA detection. This can be the whole waterway, or different important locations within a given waterway

Sampling Site: One location, often among several, where field samples will be collected within a waterbody to account for movements or local habitat use of organisms.

Sample Kit: A pre-assembled and labelled set of supplies used to collect a given field water sample

Field Sample: A 500 ml volume of water collected for eDNA analysis

Contamination Control: A 500 ml volume of pure water that is handled much like a field sample but contains no actual field sample water. Sometimes called a 'cooler blank'.

SUPPLIES SHOPPING LIST

The follow list provides enough materials for approximately 24 samples (field or control)

1. *Nestle Pure Life Water (24 Bottle Pack; half liter size = 500 ml = 16.9 fl. Oz.)
2. *Ziplock Slider All Purpose 1 Gallon Storage Bags (sliding zipper type is important in field)
3. *Disposable Exam Gloves (50 count box – Vinyl, Latex or Nitrile – no dish gloves)
4. *Paper towels (1-2 rolls)
5. Unscented Kitchen Trash Bags (about a dozen. *Bag should be new from box – white bags are preferred)
6. Indelible Ink Marker (Sharpie or similar waterproof)
7. Clorox Clean-Up Cleaner + Bleach Spray (1.84% Sodium Hypochlorite on label)

*IMPORTANT: Marked items must be bought new – do not reuse from home or office.

NOTE: THIS PROTOCOL DOES NOT OUTLINE A SPECIFIC SAMPLING DESIGN FOR WHERE,

WHEN OR HOW MUCH YOU SHOULD SAMPLE. Your sampling design is determined by the nature of your target species, the nature of the waterbody (e.g., size, flow), and your detection goals (e.g., presence-absence, relative abundance). If you are uncertain of an appropriate sampling design you should consult with the UMaine eDNA Lab (contact info is available at the end of this protocol).

PART 1: ASSEMBLING AND PREPARING SAMPLING KITS

The above shopping list provides resources for collecting up to 24 water samples that can be allocated to either actual field sampling or to field contamination controls. You will assemble these materials into kits that help to reduce the chances of contamination during field sampling. These kits should be assembled in a clean location before you head to the field for water sampling.

1. Identify a clean and uncluttered work surface of about 2-foot-by-3-foot area to use for preparing your kits. Work surfaces might include a picnic table, a kitchen table, or even the tailgate of a truck. Avoid surfaces with high risk of DNA contamination (e.g., a countertop or table where the target species was recently prepared for eating).
2. Wash your hands with soap and tap water if available. Dry them with paper towels and put on a pair of the disposable gloves (leaving the rest in their original container).
3. If possible, spray down the work surface with the Chlorox bleach spray, let spray sit for 2-3 minutes and then wipe up with paper towels. (WARNING: follow safety precautions on bleach spray. Bleach can injure or irritate eyes, skin etc. It can also damage or discolor some fabrics and other surfaces)
4. Cover the 2-foot-by-3-foot work area with a layer of paper towels and place the rack of water bottles, the box of Ziplock bags, the remaining gloves, your kitchen trash bags, and your marker on this surface, leaving some space to write on bottles and bags.
5. This is your work area for all remaining steps. If you leave or handle objects outside this space (e.g., bathroom break, phone, etc.) you should remove and replace your gloves.
6. Label a Ziplock bag with either the intended waterbody, date of sampling, site etc., or with a sample ID number/code you can later record along with these details.
7. Remove a bottle of water from the store packaging and label the clear plastic portion of the bottle above the label with the same ID information and place it into your labelled Ziplock bag. We do not recommend that you write on the manufacturer label as those can be damaged or lost. If you plan to collect more than one water sample at a site you can repeat this process with several bottles placed in the same bag.
8. Add one paper towel per sample bottle to the bag before sealing it shut to create a 'sample kit'. It may be convenient to fold each paper towel in half one or two times to fit them neatly. The complete kit now includes one or more bottles and the same number of clean paper towels, all sealed up inside a single labelled Ziplock bag.
9. Repeat steps 8-11 for each of the remaining samples/sites you plan for a given body of water.
10. In most cases you will also want to create an label some kits to be used as 'Contamination Controls' (see below). You might also find it useful to make an extra sample kit as a back-up in case of any mishaps or sampling opportunities that arise while in the field.
11. While still wearing gloves, you should label and fill a 'glove bag' for each waterbody (or critical site) where you wish to have an independent assessment of eDNA presence or abundance. For example, if you will be sampling two lakes in a given day, but are not particularly concerned where you detect target eDNA within a given lake, you would require two glove bags (one labelled for each lake). Fill each glove bag with at least enough pairs of gloves to allow you to remove and replace your gloves whenever you move more than a short distance between sampling locations. We recommend you add a couple extra pairs per bag to accommodate torn or dropped gloves. Keep in mind, you should not reuse these glove bags among waterbodies

due to their potential field contamination. After creating the glove bags you can remove the gloves currently on your hands.

12. We recommend that you transport your sampling kits, and associated glove bags, to the field inside clean trash bags that you loosely knot or twist shut. This provides an extra layer of contamination protection and makes it less likely you will drop or lose a kit. If you have many kits to transport, it may be handy to further carry the trash bags of kits inside a 5-gallon bucket or a picnic cooler, but you should spray/wipe down those containers with Chlorox solution first.
13. Finally, you may want to place some extra kitchen trash bags, some extra Ziplock bags, a spare marker, paper towels, a pencil, and some note paper in a final Ziplock bag labelled with the waterbody name and "SUPPLIES". You may or may not need these materials while field sampling, but it is best to have them handy if you do. Unless you make a fresh supply bag for each waterbody, you should treat these supplies bags as a contaminated surface during later field sampling and not mix them with your sampling kits.

PART 2: PLANNING YOUR FIELD WORK

A SAMPLING TEAM: Field sampling of eDNA water samples involves two people. One person serves as the 'sampler' and the other person serves as a 'helper'. The helper can look up details in these instructions when needed, keep track of samples, handle objects that are contamination risks, serve as a second set of eyes for potential contamination, and ensure safety of the sampler in potentially hazardous field conditions.

A SAMPLING PLAN: You should plan where and in what order field samples will be collected before heading to the field so that you can decontaminate any associated field gear, collect your samples efficiently and legally, and minimize risks of sample contamination. Here are some tips:

- A positive control: consider including samples from a known positive waterbody when surveying sites with unknown species presence to confirm the effectiveness of your eDNA sampling (but see next tip).
- Reduce contamination by sampling any sites with suspected low odds of target species presence BEFORE sampling locations where the species is known or strongly suspected.
- You should always complete and store away samples from one waterbody before beginning sampling at another waterbody on the same day.
- If you will be sampling more than one waterbody, you will need to plan for time to wash your hands or wash down and Chlorox bleach spray your field gear (buckets, coolers, boots, boats, paddles etc.) between waterbodies. (WARNING: follow safety precautions on bleach spray. Bleach can injure or irritate eyes, skin etc. It can also damage or discolor some fabrics and other surfaces)

EXAMPLES OF CONTAMINATION SOURCES:

- Waders, hats, gloves, sunglasses or other fishing clothing
- Fishing gear (rods, nets, tackle boxes)
- Boat gunnels, paddles, cushions
- Vehicle upholstery, matts or carpeting
- Dogs that swim in other waterbodies
- Food preparation or serving areas

The shared feature in all of these cases is the risk that the surfaces of objects might carry fragments of the target organism's DNA that could be accidentally transferred by direct contact or your hands.

- You will almost always want to collect at more than one location in a given waterbody. We can provide recommendations to assist you in planning the number and location of sampling sites.
- You can greatly improve the odds of detecting species by sampling preferred habitats during various times of year. This includes tributaries, coves, or backwaters used by some species for feeding, migrations or spawning.
- You might improve odds of detecting species by collecting at natural or human-made constriction points that concentrate or mix DNA from larger areas, such as outlets of ponds or lakes, mouths of coves, mouths of tributaries, or below bends or rapids in rivers.

ACCESSING SAMPLING SITES: Part of your planning process involves a decision about how you will access waterbodies for sampling. There are pluses and minuses to different access methods.

Shore sampling: Shore access to water is often preferred for smaller waterbodies or detecting organisms that inhabit shoreline regions during the sampling period. Remember that water flow and wave action can mix DNA throughout a waterbody and so shore sampling can often even be effective for many species. Shore access presents less risk of introducing target DNA into the water from your waders or a boat, but shore sampling sites should still be picked carefully. Avoid sampling shore water that is very dirty from wave action. You may not want to sample shore areas close to busy boat launches or beaches where boats, swimmers or pets might introduce DNA. Docks are often convenient places to sample from shore, but boaters, swimmers, pets and birds can contaminate dock surfaces. This does not mean you cannot sample from docks, but you should take this into consideration. As with all sampling, avoid touching docks, boats or other surfaces with your gloved hands, the bottle, or the bottle cap during sampling. A clean trash bag can be laid on such surfaces to provide a non-contaminated work area if needed.

Wading: Entering the water does not necessarily provide better detection than shore sampling, but can help where water is too shallow, muddy or stagnant at the shoreline. An inexpensive pair of entirely rubber boots is often better, since these boots can and should be aggressively cleaned and sprayed down with Chlorox bleach solution before entering any new bodies of water (WARNING: follow safety precautions on bleach spray. Bleach can injure or irritate eyes, skin etc. It can also damage or discolor some fabrics and other surfaces). Your bare feet and legs are probably the next best option where conditions are safe, and it's not a bad idea to clean your legs and feet with soap and water before wading into a new waterbody for a sample. Waders or boots with felt soles or cloth surfaces that have been used in water bodies where the target species exists are not a good choice because DNA on such surfaces could persist for years under normal storage conditions and these surfaces are difficult to adequately treat with bleach spray or soaking. Whenever wading, try to avoid sampling where you have just walked through the water. Instead, try to collect your sample at an arms-length in an upstream or into-the-waves direction.

Boats: Boats are sometimes needed to sample larger bodies of water where shore access is difficult or when sampling for species suspected of residing in deeper water. However, boats can introduce eDNA contamination from other waterbodies. If you need to transport a boat for sampling, smaller hand-powered vessels like canoes and kayaks are often a good option because they are small enough to easily spray down the hull, paddles and contact surfaces (gunnels,

seats) with Chlorox bleach solution (WARNING: follow safety precautions on bleach spray. Bleach can injure or irritate eyes, skin etc. It can also damage or discolor some fabrics and other surfaces). Larger transported vessels can be used with special care. For example, you might wash down the hull and other surfaces at a car wash, spray down likely contact surfaces (e.g., gunnels, seats, motor handles) with Chlorox bleach solution and laydown a clean trash bag as a work surface. It is best to avoid vessels that have been used extensively for capture of the target species (e.g., bass boats to sample for bass). Regardless of the vessel it is a good idea if boat handling operations are managed by the helper and not the water sampler, particularly once the work area is prepared and gloves are put on. Coast Guard approved personal floatation devices are required by law when boating and should be worn, but they should also be considered contaminated if not brand new and should not be touched with gloves, sampling bottles, or bottle caps. Consider not sampling immediately upon launching the vessel, but instead travel for a few minutes in an indirect approach to your sampling site to 'rinse' the boat's hull. Finally, try to avoid sampling in the boat's wake or on the upwind side of drifting vessel.

NOTE: Remember to obtain landowner permissions if you will be accessing water over private property. You might also need permissions to sample waters within some public lands, including national and state parks or wildlife reserves.

TRANSPORTING SAMPLES IN THE FIELD:

Part of planning involves preparing for how you will manage and transport your samples in the field. Sampling kits do not require any special care prior to sampling. However, once samples are actually collected in the field they do require protection from contamination, heat and UV light. Collected samples should not be mixed in the same bag or container with unused kits. The best and most common option is to isolate samples by waterbody or site (usually in different trash bags) inside a picnic cooler filled with ice. That cooler should be decontaminated (inside and out) with Chlorox bleach spray before heading into the field (WARNING: follow safety precautions on bleach spray. Bleach can injure or irritate eyes, skin etc. It can also damage or discolor some fabrics and other surfaces). If you cannot place samples directly on ice, you should place them on ice or freeze them as soon as possible (preferably within a few hours). Ice is not required for field transport if ambient temperatures are below 45° F, but samples should still be kept out of direct sunlight, separated from unused kits and frozen for longer-term storage.

PART 3: COLLECTING WATER SAMPLES

With good planning, actual sample collection is a fairly straightforward process. The essential points to remember are:

- 1) NEVER OPEN A WATER BOTTLE UNTIL THE INSTANT YOU WILL TAKE THE SAMPLE
- 2) ONLY OPEN A BOTTLE WHILE WEARING CLEAN GLOVES

3) DON'T LET YOUR GLOVES, BOTTLE SURFACES OR BOTTLE CAPS TOUCH POTENTIALLY CONTAMINATED SURFACES (SEE EXAMPLES ABOVE)

Using commercially sealed water bottles for sampling greatly reduces risks of contaminating the interior of bottles and caps. Once the cap seal on a bottle is broken, it should be used immediately for eDNA sampling or be discarded. If you suspect a sample has been contaminated (e.g., the cap or open bottle is dropped onto the floor of a fishing boat) you may want to discard it or make a note concerning that sample.

Collecting Surface Water Samples:

1. Once you have assigned a sampler and a helper, the sampler should be the only person to ever place hands inside of a field sampling kit, and only while wearing exam gloves.
2. Do not open any field sampling kits until you reach the actual location where you plan to take a water sample.
3. By this point you should have determined if the sample you are about to take will be an actual field sample or a contamination control sample. The first sample at a waterbody is often a contamination control, however the following instructions are for an actual field sample because they involve more step. The procedures for contamination control are very similar (see below).
4. Once at the sampling location, the helper holds open the glove bag so the sampler can grab one glove at a time and put them on. If you tear a glove while putting it on, the helper can provide you with a replacement from the spare field supplies.
5. **Once gloves are on, the sampler should try to avoid touching any surfaces other than the bottle, bottle cap and field water (including clothing, backpacks, coolers etc.).** For example, the helper should be the one to reclose the glove bag.
6. The helper now unzips and holds open the sampling kit (without placing his or her hands inside).
7. The sampler is then able to reach into the kit bag with gloved hands and remove a sample bottle The sampler should NOT open the bottle until he or she is in place to collect the sample.
8. Once the sampler is in position, he or she opens the bottle and pours the drinking water out a few feet from where the actual water sample will be collected. Do not set down the bottle cap or put in a pocket. Keep it in a gloved hand. (Do not drink from the bottle)
9. To collect the sample, simply immerse the emptied bottle in the surface water of the lake, pond, stream or river (avoid sampling right where you poured). Unless you are collecting deep water with a sampler or pump, the water should be collected directly into the bottle and not be transferred from another container or hose. The bottle should be filled to about the same amount as the original unopened bottle. Some airspace (1/2 inch or so) reduces risk of bottle damage during later freezing.
10. The sampler then recaps the bottle using the original bottle cap. Recap the bottle tightly, but do not overtighten to the point of damaging the cap. If the cap requires force to tighten it is probably not threaded properly and will leak.
11. Once capped, give the bottle a gentle squeeze while holding the cap down to ensure that the cap is seated properly and the bottle does not leak. If it leaks, loosen the cap and reapply it.
12. If you have more than one bottle in your kit, you can set your completed sample aside or hand it to your helper, and proceed with the remaining samples (following steps 8-12).

13. Once all samples have been collected at that location, use the paper towels from the field sample kit to dry the bottle(s) and your gloves and place the completed samples back into their original labelled Ziplock sample kit bag.
14. Remove your gloves by grabbing them at the wrist cuffs and pulling toward your fingertips (turns the glove inside out). Dispose of the glove and paper towels in a kitchen trash bag or spare Ziplock bag designated for the purpose. Do not use that same trash bag at another waterbody.
15. You should store your completed samples away from unused kits, out of direct sunlight (e.g., in a cooler or trash bag).

Collecting Contamination Controls:

Contamination controls are not true field water samples, but are instead mock or mimic samples meant to help detect contamination in your field sampling supplies or field procedures. When target species DNA is detected in a contamination control that is strong indicator that other samples with positive detections could also be contaminated. If you are not sure of how many contamination control samples to collect, you should discuss your sampling needs with the UMaine eDNA lab. At a minimum you will want at least one contamination control per waterbody and sampling date, however, more are often useful.

For a contamination control to be meaningful it must be prepared and handled in all the same ways as your actual field samples, with the only exception being you won't collect actual field water.

1. Follow all of the above initial steps for field samples through step #7 (removing the sample bottle from the sampling kit bag).
2. OPEN THE CAP ON THE BOTTLE BUT DO NOT POUR OUT THE CONTENTS OR IMMERSE THE OPEN BOTTLE IN THE WATERBODY.
3. Leave the cap off the bottle for about 10 seconds and then recap it (roughly the same amount of time required to collect and cap an actual field sample)
4. Proceed with sealing the bottle, testing the seal, drying it, and placing it back into the sample bag (steps 10, 11, 12, 13).
5. After sampling, be sure to transport, freeze or ship your contamination control samples in the same bags, coolers etc. you use for their associated field samples.

PART 4: STORING AND SHIPPING SAMPLES

Environmental DNA in water samples can degrade due to physical, chemical and biological processes. The goal is to stabilize samples against such processes until they can be processed in the lab. Freezing is generally the easiest and most available way to accomplish this, assuming you have not made arrangements to process your samples in our lab within 24 hours (in which case they can remain on ice or be refrigerated).

Freezing: If you plan to freeze your eDNA samples this should occur as soon as possible following field collection. They can be stored frozen for several weeks, but it is preferable that they be shipped within a day or two of collection to avoid complications with modern frost-free freezers that

can subject samples to repeated freeze-thaw cycles that damage eDNA. As noted above, samples from different waterbodies and dates should not be mixed, but should instead be kept grouped by waterbody and date within separate kitchen trash bags before, during and after freezing. Do not reopen the Ziplock sample bags if you do not have to.

Packaging: eDNA samples can be shipped in picnic coolers or sturdy cardboard boxes. Coolers are preferable when shipping long distances that might take more than a day. Frozen eDNA samples are fairly durable, but the plastic bags and bottles can be cracked if the samples are subject to strong impacts while frozen. We recommend that samples (grouped by waterbody in kitchen trash bags) be packed securely with newspaper, bubble wrap, or packing peanuts. If you are shipping the samples in cardboard you should line the box with double trash bags to prevent damage to the cardboard from wet samples and secure those bags shut with a knot, twist tie, or cable tie. You can use the original bags holding the samples from a given water body for one of these layers, but adding an additional new bag can be helpful for containing leaks in case the original bags were damaged during field work or freezing.

Sample inventory: You should prepare a sample inventory with your name, contact information, the waterbody name, the sampling date(s), and a listing of all the individual samples by site/code (and date if more than one date is included). **This inventory should be sealed in its own Ziplock bag** and placed inside the shipping container with the samples. We also recommend that you e-mail a copy of the sample inventory to our lab directly.

Shipping: It is **NOT** necessary to ship samples in a fashion that would guarantee they arrive frozen (e.g., dry ice – which can be dangerous to ship and damage bottles). Rather, it is fine for samples to arrive partly or mostly thawed. Nonetheless, this will still generally require that you make use of shorter shipping times (e.g., 1 day in cardboard, 2 days in coolers). Be sure to notify the UMaine eDNA lab by phone or e-mail when you plan to ship the sample and include package tracking information if it is available so your samples will be anticipated and moved to cold storage or processing soon after arrival. Use the following shipping and contact information:

| | |
|-------------------------------|-----------------------------|
| Dr. Michael Kinnison | Phone: 207-581-2575 |
| School of Biology and Ecology | Fax: 207-581-2537 |
| University of Maine | E-mail: mkinnison@maine.edu |
| Orono, ME 04469-5751 | |

APPENDIX B

Developing Cost Effective Monitoring for Rainbow Smelt Using eDNA

Field Data Sheet

Date: _____ Field Personnel: _____

Site Name: _____ Flow Conditions: _____

Waterbody Name: _____ Tidal Stage: _____

Weather Conditions: _____

| Sample ID | Sample Time | Grab 1-3, blank? | Notes |
|-----------|-------------|------------------|-------|
| | | | |
| | | | |
| | | | |
| | | | |

Date & Time: Stored in Freezer _____
 Shipped to Lab _____
 Received by Lab _____

| Digital photo(s) taken? | No | Yes |
|--|------|------------------------------|
| Approximate EGG abundance per square foot (circle one) <small>single layer just touching = 60,000-70,000 eggs/ft2</small> | none | 100s 1,000s 10,000s millions |
| Upstream limit of egg bed - Latitude <small>Please specify units (e.g. 47° 39.521')</small> | | |
| Upstream limit of egg bed - Longitude <small>Please specify units (e.g. 69° 38.125')</small> | | |
| Downstream limit of egg bed - Latitude <small>Please specify units (e.g. 47° 39.234')</small> | | |
| Downstream limit of egg bed - Longitude <small>Please specify units (e.g. 69° 38.913')</small> | | |
| Approximate width (ft) of egg bed | | |
| Approximate length (ft) of egg bed | | |
| Water depth (ft) over egg bed time of survey | | |
| Algae on eggs? (circle one) <small>green slime on eggs?</small> | No | Yes |
| Additional Comments | | |
| | | |

Size and description for predominate stream bed and egg substrate (from Maine Road-Stream Crossing Survey – USF&W Gulf of Maine Program, 2007)

| Size Class | Millimeters | Inches | Approximate Relative Size |
|------------|-------------|--------------|---------------------------|
| Boulder | > 256 | > 10.1 | Bigger than a basketball |
| Cobble | 64 - 256 | 2.5 - 10.1 | Tennis ball to basketball |
| Gravel | 2 - 64 | 0.08 - 2.5 | Peppercorn to tennis ball |
| Sand | 0.06 - 2 | 0.002 - 0.08 | Salt to peppercorn |
| Silt-Clay | < 0.06 | < 0.002 | Finer than salt |

Egg data form based on that of Claire Enterline.

APPENDIX C

Protocol for eDNA Filter Extraction Using the Qiagen DNeasy Kit

This document is for reference only. You should consult Dr. Michael T. Kinnison, or another experienced employee or student, the first time you undertake this procedure. Do NOT proceed if you do not understand the procedure, or if you feel concerned for your safety. Refer to the MSDS notebook in room 317 for safety information (filed under Qiagen DNeasy Kit).

Safety: The chemicals noted below have safety issues noted by Qiagen:

Buffer AL/E (NFPA: H=2 F=1 R=0) Contains guanidinium chloride – harmful

Buffer AW1 (NFPA: H=2 F=0 R=0) Contains guanidinium chloride – harmful

Proteinase K (NFPA: H=1 F=0 R=0) Contains Proteinase, Tritirachium album serine - harmful

PPE: Use nitrile gloves

See protocol on reverse side

Protocol for eDNA Filter Extraction Using the Qiagen DNeasy Kit

Protocol: Before starting, make sure that ethanol has been added to buffers AW1 and AW2. Aliquot contents of kit to insure against contamination (kit is expensive). Preheat the incubator oven to 56 °C. Make sure all samples/chemicals are at room temperature. Redissolve any precipitates in buffers AL or ATL by incubating at 56 °C for at least 10 min.

- 1. Label two sets of 2.0 or 1.5 ml microcentrifuge tubes (MCT) for the extraction (one set for extraction, one set for final storage). Set storage tubes aside until final elution. Remove samples that have been designated to be extracted from freezer.
- 2. Prepare an **extraction negative control** by placing a new filter paper in a new sterile 1.5 ml MCT.
- 3. Add **370 µL of Buffer ATL** to each tube.
- 4. Add **30 µl Proteinase K** to each tube and mix immediately by pulse-vortexing for 15 s.
- 5. Incubate at 56 °C for 1 hour (TIMED).
- 6. Remove from incubator and centrifuge at $\geq 16,000 \times g$ for 5 minutes.
- 7. Transfer supernatant to a new 2 or 1.5 mL MCT**.
- 8. Add **400 µl Buffer AL** to each tube.
- 9. Add **400 µl 100% ETOH** to each tube, and vortex for 15 s.
- 10. Pipette **650 µl of the sample** into the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g for 1 min. Pour the filtrate into the discard beaker.
- 11. Repeat step 7 until the whole lysate is loaded. A maximum of 5 x 650 µl can be loaded onto the QIAamp Mini spin column.
- 12. Carefully open the QIAamp Mini spin column and add **500 µl Buffer AW1** without wetting the rim. Centrifuge at 6000 x g for 1 min.
- 13. Carefully open the QIAamp Mini spin column and add **500 µl Buffer AW2** without wetting the rim. Centrifuge at full speed (20,000 x g) for 3 min.
- 14. Place the QIAamp Mini spin column in a new 2 ml collection tube and discard the filtrate and old collection tube. Centrifuge at full speed for 1 min.
- 15. Place the QIAamp Mini spin column in a clean 2 ml collection tube and discard old collection tube.
- 16. Carefully open the QIAamp Mini spin column and add **100 µl TE Buffer** to the center of the column membrane. Incubate at room temperature for 2 min and then centrifuge at 6000 x g for 1 min.
- 17. Pipette extract from 2 ml collection tube into labeled 1.5 ml MCT. Discard spin column and empty 2 ml collection tube.
- 18. Carefully label storage box and double-check that tubes are correctly labeled. Combine multiple extractions for a single sample if necessary.
- 19. Store samples at -20 °C.

** If the filters absorb too much supernatant as you work through the sample batch, re-spin samples another 5 minutes as needed to ensure you can easily collect supernatant without too much effort.

Appendix B. Wells Reserve eDNA Manual

WNERR eDNA Manual

Claire Gottsegen, Spring 2018

| | |
|---|-----------|
| A NOTE ON EDNA | 2 |
| BEST PRACTICES | 3 |
| SPRING 2018 PROTOCOLS | 4 |
| RIVER EDNA SAMPLING PROTOCOL | 4 |
| LARVAL FISH EDNA SAMPLING PROTOCOL | 10 |
| GREEN CRAB EDNA SAMPLING PROTOCOL | 14 |
| EDNA WATER FILTRATION PROTOCOL | 20 |
| EDNA FILTER EXTRACTION PROTOCOL | 24 |
| UNH ONLINE EDNA DATABASE FOR NERRS | 27 |
| ADDITIONAL RESOURCES | 28 |

Wells National Estuarine Research Reserve

342 Laudholm Farm Road
Wells, Maine 04090

Phone: (207) 646-1555
www.wellsreserve.org
www.coast.noaa.gov

A Note on eDNA

Environmental DNA (eDNA) is a developing tool in conservation that allows scientists to establish the presence or absence of specific species in a system. This emerging technique relies on collaboration between ecologists, geneticists, and data scientists. One such group, the UNH-NERR Science Collaborative, explains its significance:

“Biological monitoring programs are essential foundations for effective management of estuaries and coasts, but they can be expensive, labor intensive, and intrusive on target species. Advancements in DNA methods now make it possible to identify organisms in an area by the DNA they leave behind. This residual or environmental DNA (eDNA) may be generated from feces, gametes, scales, bodily fluids, and cells that an organism sheds, and is easily collected from water and sediment samples. Rapid reductions in analytical costs now allow scientists to analyze eDNA in water samples and identify dozens of species without having to capture live animals or plants and reduce logistical challenges associated with traditional monitoring approaches.”

This manual will serve as a guide for eDNA practices at the Wells National Estuarine Research Reserve, and our methods will vary depending on the question we ask. In each case, DNA from many species will be present within one water grab. We might choose to target DNA sequences for all species under an umbrella taxon to obtain a snapshot of biodiversity at our site (**metabarcoding: Figure 1**). Alternatively, we might target the DNA sequence for one particular species to establish its presence or absence in the system (**qPCR or barcoding: Figure 2**).

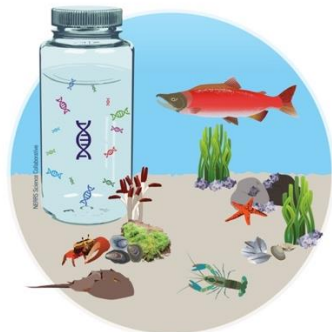


Figure 1: We may choose to isolate, amplify, and sequence the DNA for many species under an umbrella taxon to take a snapshot of biodiversity in the system. **Metabarcoding** allows us to sequence multiple species at once.

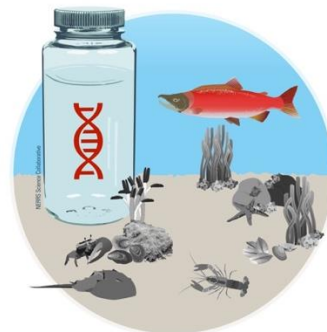


Figure 2: Alternatively, we may choose to target the DNA of a single species, which could be helpful for establishing the presence of an endangered or invasive species in a system. This can be achieved by **barcoding** or **qPCR**.

In general, the process will proceed in the following order:

- (1) collection fieldwork of water or sediment samples,
- (2) vacuum filtering for water samples,
- (3) DNA extraction using the Qiagen DNeasy kit,
- (4) Polymerase Chain Reaction (PCR) or quantitative PCR (qPCR) using primers for the desired taxa, and/or
- (5) Illumina sequencing to obtain genomic sequences for bioinformatic analysis.

Steps 1 and 2 will be performed at WNERR. While WNERR does have the ability to extract DNA, steps 3–5 will typically be outsourced to genomic centers such as the Hubbard Center for Genome Studies at UNH.

Best Practices

- Change gloves when the protocol requires **AND** when you suspect they may have become contaminated. This includes touching non-sterile surfaces, spilling a sample/reagents, and leaving the room. It may feel wasteful, but it is crucial to the integrity of the results. The little things matter!
- Aliquot your materials whenever possible, namely glassfiber filters, microcentrifuge tubes, and chemical reagents for DNA extraction. This decreases the chances of contaminating a bulk package – if a small aliquot is contaminated, you will always have more.
- Keep a lab notebook with detailed notes. This allows you and your collaborators to notice small details that may help to explain a hiccup in later lab processes or in the final results.
- Ask questions. Lots of little tricks are out there – reach out to collaborators at UMaine or UNH with any questions about materials or methods.

Spring 2018 Protocols

River eDNA Sampling Protocol



Protocol based on Water Bottle Sampling for Environmental DNA Analysis (Kinnison 2018)



Rainbow smelt (2001)



Dead rainbow smelt eggs on rock (2016)

Purpose: to collect water samples for use of eDNA to detect the presence and distribution of anadromous rainbow smelt in Maine coastal stream habitats during spring spawning.

Location: Four sites described below.

| Site ID | Stream | Town | Latitude | Longitude | Status |
|---------|--------------|----------------|------------|------------|---------|
| WR01 | Long Creek | South Portland | 43.633270 | -70.313263 | Decline |
| WR02 | Mill Creek | Falmouth | 43.731386 | -70.225159 | Limited |
| WR04 | Mast Landing | Freeport | 43.859627 | -70.083356 | Strong |
| WR05 | Miller Creek | Brunswick | 43.8611889 | -69.975642 | Strong |

Sampling frequency: 2-3x/week, March-May 2018

Equipment and materials for one eDNA kit (one site):

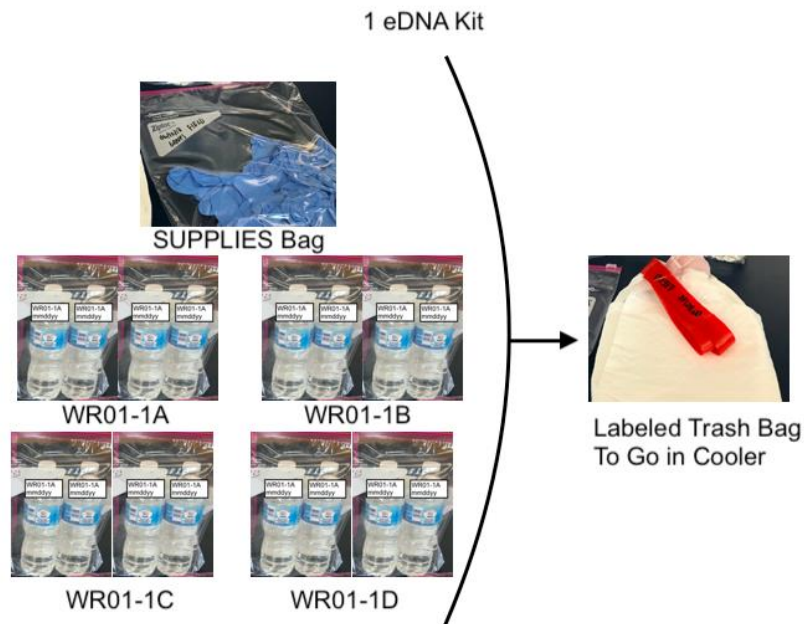
- 16 unopened Nestle Pure Life water bottles (500 mL size)
- 1 roll paper towels
- 5 pairs of disposable nitrile exam gloves + extra
- 9 Ziploc all-purpose 1-gallon slider bags
- 2 unscented kitchen trash bags
- Clorox bleach spray (1.84% NaClO)
- Spray bottle of tap water + scrubber brush
- Indelible ink marker
- Plastic tote to carry samples in the field (≥ 7.5 -gallon size)
- Waders/boots
- Coolers + ice packs to store samples after collection (one 15-gallon cooler fits 2 eDNA kits + ice)
- Field datasheet + writing utensil

Tips for Buying Bulk:

| | Amt. Needed per eDNA Kit | Amt. in Bulk Package | # eDNA Kits per Bulk Package |
|---|--------------------------|-----------------------|------------------------------|
| Nestle Pure Life water bottles (500 mL size) | 16 | 35 – Walmart (\$3.98) | 2 |
| Disposable nitrile exam gloves (pairs) | 5 | 50 – VWR | 10 |
| Ziploc all-purpose 1-gallon slider bags | 9 | 60 – Walmart (\$7.37) | 6 |
| Great Value Strong Flex kitchen trash bags | 2 | 45 – Walmart (6.98) | 22 |

eDNA Kit Preparation

1. Wearing disposable nitrile exam gloves, sterilize counter space with Clorox bleach spray and lay down paper towels for sterile prep workspace. Sterilize two coolers with Clorox bleach spray and set aside.
2. Prepare eDNA kit to include three replicate samples (A, B, C) and one blank (D). Each replicate sample as well as the blank consists of 2 L, or 4 water bottles. Thus One eDNA kit will contain 8 L, or 16 water bottles total.
 - a. Label each bottle with site ID, visit ID, sample ID, and date (SiteID-VisitIDSampleID Date).
Example: Sample **A** at **WR01** on the **first** sampling visit would be “**WR01- 1A** mm/dd/yyyy”.
 - b. Package like bottles in 1-gallon Ziploc sliders with 1 paper towel per bottle.
In example above, WR01-1A will consist of 4 water bottles packaged in 2 Ziploc sliders.
 - c. In one Ziploc slider, place 10 Nitrile exam gloves, 1 Ziploc bag designated for trash, and 1 extra trash bag for double-bagging collected samples. Label **SUPPLIES** + date.
 - d. Place set of bottles A, B, C, and D (16 bottles) + one **SUPPLIES** bag in a labeled trash bag. Place trash bag in sterile cooler with ice.



- e. Repeat **steps 2a–d** until you have assembled desired number of eDNA kits. Put the plastic tote and cooler/s with eDNA kits + ice into car.

NOTE: In car, keep extra gloves, paper towels, Clorox bleach spray, spray bottle of tap water, scrubber brush, field datasheets + writing utensil.

Sample Collection – for contamination control, protocol requires one Sampler and one Assistant

3. Before entering site, sterilize waders/boots with Clorox bleach spray, scrub vigorously with scrubber brush, and rinse with spray bottle of tap water. Spray plastic tote with Clorox and wipe down with paper towels.
4. Place appropriate eDNA kit into bleached plastic tote with field datasheet. Record data and notes in real time.
5. At the site, Assistant opens tote and retrieve the **SUPPLIES**. Both Assistant and Sampler put on gloves. NOTE: Throughout the sampling event, the Assistant will open appropriate Ziplocs for the Sampler. The Sampler will touch only the sample containers and sterile Ziploc interiors.
6. Take all samples A, B, C, and D.
 - a. Sampler changes gloves between each sample. Used gloves and other waste go into **TRASH** Ziploc.
 - b. For each replicate sample A, B, and C: Sampler opens the water bottle to break the seal, pours water out downstream of sample site, and submerges bottle ~1 inch under the surface. Bottle should retain ~1 inch of air to allow for expansion if freezing later. Sampler caps the sample, turns upside down, and squeeze to ensure a tight seal. Lastly, Sampler dries the bottle with paper towel and places bottle back in bag.
 - c. For the blank D: Sampler opens the water bottle to break the seal, holds it open for 15 seconds to expose to environmental conditions, and pours out some water so the bottle retains ~1 inch of air. Sampler caps the blank, turns upside down, and squeezes. Sampler dries bottle with paper towel and places bottle back in bag.
7. Following collection, Assistant will double-bag the samples with the extra trash bag and return to plastic tote.
8. Before returning to car, conduct **rainbow smelt egg survey** on field datasheet. Inspect the upstream spawning site and estimate egg abundance, extent, and any abnormalities.
9. At the car, transfer trash bag with collected samples from plastic tote to iced cooler for short-term storage.
10. Repeat steps #3–8 for all sites on your sampling schedule. Be sure to begin by sterilizing waders/boots and plastic tote between sites.

NOTE: Each set A, B, and C are replicate samples taken downstream of the spawning site in the same river. Based on bank accessibility and width of the stream, sets may refer to different locations within a stream. See table below for the descriptions of samples A, B, and C for each site this season. Sample D, unlisted, is always a blank.

| Site | A | B | C |
|-------------|-------------|---------------------|---------------------|
| WR01 | River Left | River Center | River Right |
| WR02 | River Right | River Center | River Left |
| WR04 | River Right | River Center | River Left |
| WR05 | Point A | Upstream of Point A | Upstream of Point B |

Sample Processing

11. At WNERR, store all samples immediately.

- a. For short-term storage, keep in refrigerator for up to a week and vacuum filter as soon as possible (see WNERR vacuum filter protocol). For long-term storage, keep in freezer.
- b. At the UMaine Kinnison Lab, DNA will be extracted from the filter products using the Qiagen DNeasy Blood & Tissue Kit. Polymerase Chain Reaction (PCR) will be run with appropriate primer in order to detect presence/absence of species in the system, and a hierarchical occupancy model will be employed to provide a high level of confidence of species detection.

Developing Cost Effective Monitoring for Rainbow Smelt Using eDNA

Field Data Sheet

Date: _____ Field Personnel: _____
 Site Name: _____ Flow Conditions: _____
 Weather Conditions: _____

| Sample ID: | Sample Time | Notes by Grab | <u>General Notes:</u> |
|------------|-------------|---------------|-----------------------|
| A | | | |
| B | | | |
| C | | | |
| D | | | |

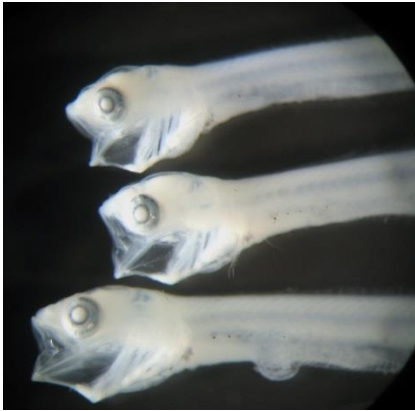
Date & Time: Stored in Freezer _____
 Shipped to Lab _____
 Received by Lab _____

| Digital photo(s) taken? | No | Yes |
|--|------|------------------------------|
| Approximate EGG abundance per square foot (circle one) <small>single layer just touching = 60,000-70,000 eggs/ft²</small> | none | 100s 1,000s 10,000s millions |
| Upstream limit of egg bed - Latitude <small>Please specify units (e.g. 47° 39.521')</small> | | |
| Upstream limit of egg bed - Longitude <small>Please specify units (e.g. 69° 38.125')</small> | | |
| Downstream limit of egg bed - Latitude <small>Please specify units (e.g. 47° 39.234')</small> | | |
| Downstream limit of egg bed - Longitude <small>Please specify units (e.g. 69° 38.913')</small> | | |
| Approximate width (ft) of egg bed | | |
| Approximate length (ft) of egg bed | | |
| Water depth (ft) over egg bed time of survey | | |
| Algae on eggs? (circle one) <small>green slime on eggs?</small> | No | Yes |
| Additional Comments | | |

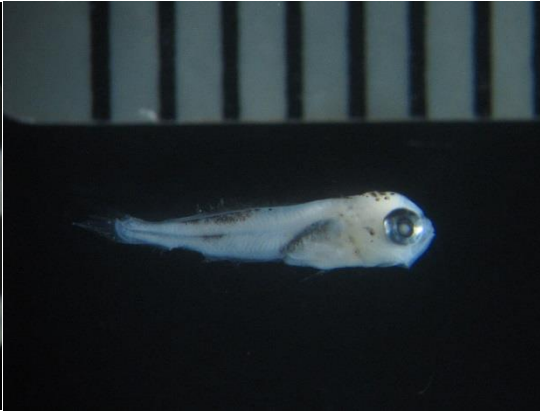
Larval Fish eDNA Sampling Protocol



Protocol based on “Water Bottle Sampling for Environmental DNA Analysis,” Kinnison (University of Maine); Wells NERR Larval Fish Sampling Protocol,” Miller, J. (WNERR)



Larval Atlantic herring (2009)



Larval red hake (2013)

Purpose: To detect Atlantic herring (*Clupea harengus*) in the Webhannet River Estuary by extracting eDNA from water and captured specimen biomass. This will be executed in coordination with our long-term larval fish and zooplankton monitoring initiative.

Location: Wells Harbor, off the main dock (site code: WHAR).

Sampling frequency: 60 minute deployments taken 1.5 hours before listed high tide, 4x/month.

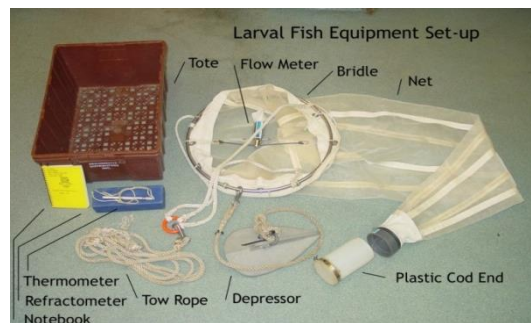
Equipment and materials for one collection visit:

eDNA Prep / Collection Materials

- Disposable exam gloves
- Indelible ink marker
- 6 Nestle Pure Life water bottles (500ml size)
- Two 1.5 or 2.0 μ L microcentrifuge tubes
- 6 Ziploc all-purpose 1-gal slider bags
- 2 unscented kitchen trash bags
- 1 roll paper towels
- Clorox bleach spray (1.84% NaClO)
- Concentrated bleach solution
- 1 large plastic bin with lid
- “Grabber” tool
- 1 cooler + ice pack

Larval Tow Equipment

- 0.5 m diameter plankton net (500 μ l mesh)
- Flow meter (attached to net bridle)
- Cod-end bottle
- Tow rope (attached to net)
- Depressor (anchor)
- Notebook with pen/pencil



Preparing eDNA Kits

1. Wearing gloves, wipe down a surface with Clorox spray and cover with paper towels. Sterilize 2 coolers with Clorox bleach spray.
2. On sterile surface, prepare kits in 1-gal Ziploc sliders to include water bottles (1 for blanks, 2 for water samples) and 1 paper towel. Label:
 1. **WHAR B1 date:** Blank #1, tap water run through sterile net pre-deployment. 500 mL
 2. **WHAR B2 date:** Blank #2, water bottle to be opened and closed at harbor. 500 mL
 3. **WHAR W1 date:** Water #1, net rinse water grab sample post-deployment. 1 L
 4. **WHAR W2 date:** Water #2, harbor grab. 1 L
 5. **WHAR LAR date:** larval biomass blend, 4 µL
3. Place 4 pairs gloves in a Ziploc labeled LAB GLOVES and 4 pairs gloves in a Ziploc labeled FIELD GLOVES
4. Place **WHAR B1, W1, LAR** and LAB gloves in trash bag marked LAB. Set aside.



5. Place **WHAR B2, W2,** and FIELD gloves in trash bag marked FIELD. Set in bleached cooler with ice.



Preparing Tow Net

1. Prep flow meter by filling with water, replace the screw, and attach meter to net bridle with cable tie as back-up. Attach the anchor, tow rope, and cod-end bottle to net.
2. Wearing gloves, fill large plastic bin with 10% bleach solution. Fully immerse for 15 minutes: net, flow meter, anchor, tow rope, cod-end bottle and food processor cup, blade, and top.



3. Rinse all bleached materials with tap water, including the plastic bin. Set food processor items inside for later use.
4. Place sterile bin under sterile net. Rinse net again with tap water so it collects in the bin. Retrieve LAB trash bag to collect first blank.
 - a. Collect sample **WHAR B1 date:** Empty water from bottle and submerge it in the sterile rinse water. Store immediately in fridge if vacuum filtering within 7 days, or in freezer if storing long-term.
5. Empty tap water from bin, place net with attachments in sterile bin, and close the lid.



Materials for the harbor: bin with net and attached accessories, FIELD bag in cooler, “grabber” tool.

Deployment & FIELD Sample Collection

1. Arrive at the harbor 1.5 hours before high tide.
2. Retrieve FIELD bag for sample collection.
 - a. Wear gloves. Have Assistant open bags and Sampler collect the samples.
 - b. Collect sample **WHAR B2 date** by opening the bottle (NOT pouring out water), waiting a few seconds, and closing it.
 - c. Collect sample **WHAR W2 date**: pour out water, grasp bottle with extender claw, submerge bottle face-down 1m, and flip to fill face-up at 1m below the surface.



Store immediately in FIELD cooler with ice.

3. Change gloves and open plastic bin. Record the number on the flow meter.

4. Making sure the net does not touch the dock and the ropes are untangled, tie the longer rope to the dock cleat, and lower the “stabilizer” weight and net into the water 1 meter deep (rope is marked with duct tape). Make sure the open end of the net is facing the incoming current. Record time.
5. Monitor net during deployment: make sure the open end stays facing the incoming current and stays deployed at 1 meter, watch for incoming boats and be prepared to move the net to another cleat if need be, keep equipment in a safe location and out of walking path.



6. After 60 minutes, put on a new pair of gloves and retrieve the net. While holding the bridle firmly, gently dip the net in and out of the water to flush sample material down through the cod end. Bring the entire net out of the water and **place directly into plastic tote** without touching anything else.
7. Record number on flow meter and time when net was retrieved. Fasten the lid onto the bin.

Post-Deployment & LAB Sample Collection

1. Back at the lab, put on new gloves and open the plastic bin to detach flow meter and anchor. Set aside.
2. Remove the net from the bin and hang it on an outdoor hook. Place the plastic bin directly underneath to collect rinse water.
3. Rinse the net thoroughly with tap water from a hose to lead all biomass into the cod-end. Rinse water will collect in the plastic bin.



4. Retrieve LAB bag for sample collection. Collect sample **WHAR W1 date**: empty bottle and submerge in rinse-water bucket.
5. Screw off the cod-end bottle and transfer contents into the bleached food processor cup. Inside, cover and “puree” to obtain homogenous mixture. Collect sample **WHAR LAR date**: one after another, dip the microcentrifuge tubes into larval mixture.

6. Transfer all samples in FIELD and LAB bags to fridge if vacuum filtering within 7 days, or in freezer if storing long-term.
7. Clean and store all equipment.

Green Crab eDNA Sampling Protocol

Protocol based on "Water Bottle Sampling for Environmental DNA Analysis," Kinnison (University of Maine)



Purpose: To detect green crabs (*Carcinus maenus*) in the Little River Estuary by extracting eDNA from sediment. Samples will be collected within and around baited crab traps to validate detection methods.

Location: Little River Estuary, WNERR Research Marsh (site code: LRIV).

Sampling frequency: Every 2-3 weeks (or later as needed) at low tide. Sediment collected during trap deployment and trap retrieval forty-eight hours later

Equipment and materials for one full collection event:

Part I. Trap Deployment

- 3 crab traps (62 cm x 31 cm x 27 cm) with buoy and rebar
- 3 bait bags filled with oily fish (e.g. herring or shad preferred)
- 3 trash bags
- Waders / boots
- Clorox bleach spray (household concentration, 1.84% NaClO)
- Spray bottle filled with tap water
- Small cooler + ice pack
- Clipboard with datasheet and pencil
- eDNA kit for sediment collection (see table)

Part II. Trap Retrieval

- 1+ pair heavy work gloves
- Three 1-gallon buckets with lids
- Waders / boots
- Clorox bleach spray (1.84% NaClO)
- Spray bottle filled with tap water
- Small cooler + ice pack
- Clipboard with datasheet and pencil
- eDNA kit for sediment collection

eDNA Kit Materials for Sample Collection

- 7 Ziploc sandwich bags
- 7 tongue depressors
- Six 1-gal Ziploc slider bags
- 2 trash bags
- Aquarium sand
- 1 unopened Nestle Pure Life water bottle, size 500 mL
- Indelible marker (Sharpie)
- 4 pairs disposable Nitrile exam gloves

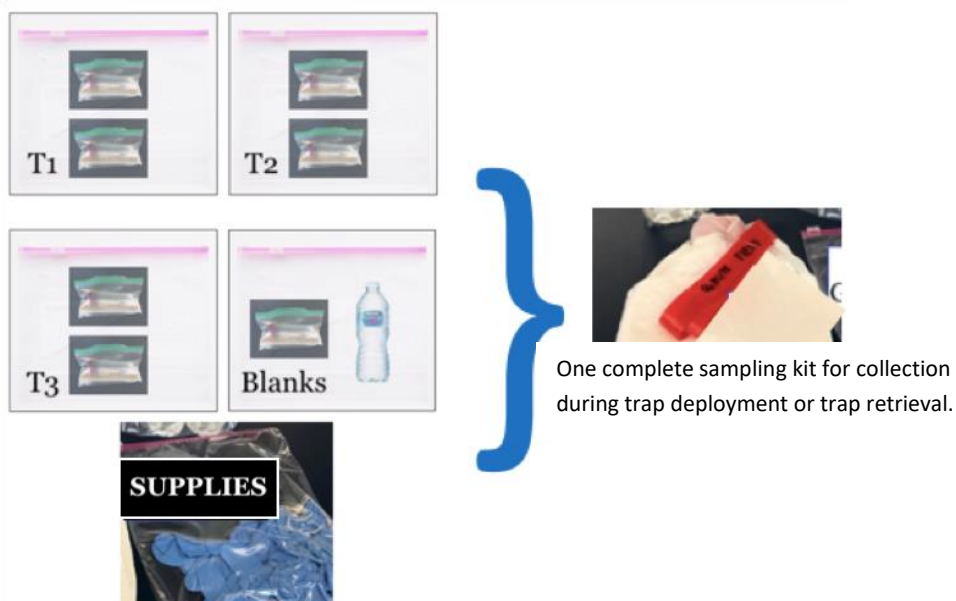
Preparing eDNA Kits

1. Wearing gloves, wipe down a surface with Clorox spray and cover with paper towels. Bleach small cooler.
2. On a sterile surface, label seven Ziploc sandwich bags as listed in table below. Place a new tongue depressor in each bag. **One is the sediment blank: fill with ~5g aquarium sand.**
3. For the water blank, label one unopened Nestle Pure Life water bottle (500 mL size) as listed in table below.

| Label ID | Trap (T) # | Relative to Trap |
|---------------|----------------------------|------------------|
| LRIVmddyT1IN | T1 | Inside |
| LRIVmddyT1OUT | T1 | Outside |
| LRIVmddyT2IN | T2 | Inside |
| LRIVmddyT2OUT | T2 | Outside |
| LRIVmddyT3IN | T3 | Inside |
| LRIVmddyT3OUT | T3 | Outside |
| LRIVmddyB1 | (Blank - sed) | - |
| LRIVmddyB2 | (Blank - H ₂ O) | - |

You should now have 7 sediment sampling units (2 per trap + 1 sediment blank) and 1 water blank.

4. Place each trap's "IN" and "OUT" bags in the 1-gal Ziploc slider bag labeled with that trap number. Example: LRIVmddyT1IN and LRIVmddyT1OUT go in a 1-gal bag labeled **T1**. Place blanks in slider bag "**Blanks**."
5. Place 4 pairs disposable Nitrile exam gloves, a rolled up 1-gal Ziploc slider bag, and one trash bag into another 1-gal Ziploc slider bag labeled "Supplies."
6. You should now have 5 full Ziploc slider bags: T1, T2, T3, Blanks, and "Supplies." Place these in a trash bag labeled with site name and date. Store in bleached cooler with ice pack.

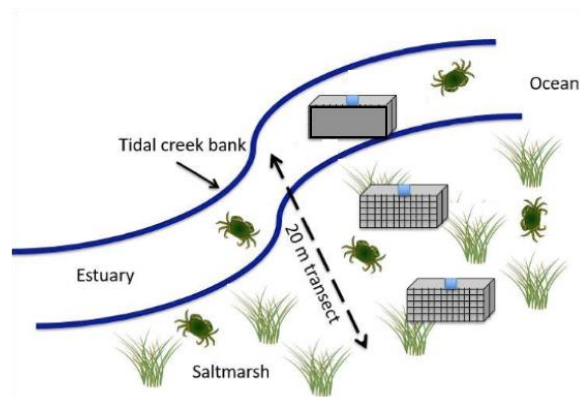


Part I. Trap Deployment

1. Outside, sterilize three crab traps by spraying Clorox bleach and scrubbing vigorously. Rinse with tap water.
2. Fill three clean bait bags with oily fish (1-2 fish/bait bag is usually plenty). Place one in each crab trap.
3. After sterilizing, store the crab traps in trash bags until ready to set them at sampling site.

To bring to the site:

- Crab traps in trash bags
 - Clorox spray & bottle of tap water
 - Cooler with eDNA collection kit + ice pack
 - Clipboard with datasheet and pencil
4. Arrive at the site at low tide. Sterilize your boots with bleach spray and rinse with spray bottle of tap water. Record metadata on field datasheet in real time.
 5. Uncover crab traps and set in designated locations along the Little River (see picture below; transect distance may vary along marsh gradient). Secure with rope and rebar if necessary.



Sample Collection: best practice requires one Sampler and one Assistant. As an anti-contamination measure, Assistant should open/transfer all bags, while Sampler should touch only the sampling materials.

6. Open eDNA collection kit in cooler.
 - a. Take out the "Gloves" Ziploc. Both Sampler and Assistant will put on a pair of gloves.
 - b. Set aside the extra rolled-up Ziploc slider to store trash (used gloves, tongue depressors).
 - c. Set aside the extra rolled-up trash bag to collect samples post-collection.
7. Open "Blanks" Ziploc.
 - a. Take the water blank: open the bottle, wait 15 seconds, then close the bottle. If planning to freeze the sample before filtering, make sure to pour out some water to allow for expansion.
 - b. Take the sediment blank: remove the tongue depressor from plastic packaging and place briefly in the aquarium sand. Discard the tongue depressor. Store samples in the extra trash bag for collection .

8. Proceed to take “OUT” and “IN” sediment samples at the traps. Sampler should change gloves between traps. Store samples in the extra trash bag for collection.
 - a. For each trap, collect the “OUT” sample first. Using a clean tongue depressor, take sediment grabs ½ m from the trap on each of the four sides of the trap. These four grabs = 1 composite “OUT” sample.
 - b. Afterwards, collect the “IN” sample. Using a clean tongue depressor, lift the trap and take 2 sediment grabs. These 2 grabs = 1 composite “IN” grab.
9. After collecting all samples and placing them in the extra trash bag, double-bag with your original trash bag.
10. Back at the lab, store sediment samples in freezer. Store water blank in fridge if filtering within 7 days, or store in freezer for long-term.

Part II. Trap Retrieval

1. Prepare a second eDNA kit as described in the section “Preparing eDNA Kits.” Make sure to label everything with the retrieval date, not the deployment date.

Plan to retrieve the crab traps at low tide ~48 hours after deployment.

To bring to the site:

- 1+ pair heavy work gloves
- Three 1-gallon buckets with lids
- Clorox spray & bottle of tap water
- Cooler with eDNA collection kit
- Clipboard with datasheet and pencil



2. Arrive at the site at low tide. Sterilize your boots with bleach spray and rinse with spray bottle of tap water. Record metadata on field datasheet in real time.
3. Wearing heavy work gloves, collect crabs from the traps and store in buckets **by trap number**. Make sure these buckets are marked in some way.
4. Retrieve the cooler with the eDNA sampling kit and repeat **steps #6–9** from Part I, “Trap Deployment.”



5. Bring the crab traps back to the lab. Dispose of remaining bait, rinse thoroughly, and store for next use.
6. Transfer crabs into Ziploc bags labeled by trap number and store in freezer. Rinse and store buckets.

Part III. Post-Processing

1. The next day—or whenever the crabs are good and frozen—record crabs' sex, carapace width, wet weight, and any additional notes.
2. Coordinate with UNH to transfer samples to the Hubbard Center for Genome Studies for DNA extraction.

New Technology for Old Problems – Using DNA Methods to Monitor Invasive Species and Biodiversity in Estuarine Systems

WNERR Project: Crab eDNA Collection in the Little River Estuary

DEPLOYMENT Date: _____ Time: _____ Field Personnel: _____
 Low Tide: _____ Weather Conditions: _____

SEDIMENT COLLECTION (CHECK OFF)

SOIL BLANK ____ WATER BLANK ____

| | T1 | T2 | T3 |
|-----|----|----|----|
| IN | | | |
| OUT | | | |

NOTES:

RETRIEVAL Date: _____ Time: _____ Field Personnel: _____
 Low Tide: _____ Weather Conditions: _____

SEDIMENT COLLECTION (CHECK OFF)

SOIL BLANK ____ WATER BLANK ____

| | T1 | T2 | T3 |
|-----|----|----|----|
| IN | | | |
| OUT | | | |

NOTES:

CRAB COLLECTION

See excel for size/sex data (R DRIVE: eDNA 2018 > UNH eDNA > Data Collection > Crab eDNA > Crab eDNA Master Datasheet)

| | T1 | T2 | T3 | Total |
|------------|----|----|----|-------|
| Crab Count | | | | |

NOTES:

WNERR Protocol based on UMaine Kinnison Lab Protocol (2018).

Equipment needed

- Gloves, nitrile
- 3 plastic bins for 50% bleach bath, water bath, and ice bath
- Magnetic filter cups & stoppers
- Bleached table top
- 2 Erlenmeyer filtering flasks, 1-2L, along with tubing and plugs for spout
- Vacuum pump
- Power strip with on/off flip switch to control pump by stepping on it (avoid hand contamination)
- glassfiber filters, 47 mm diameter
- Filter tweezers
- Paper towels
- Field samples bottles, new water bottles for negative control
- Prelabeled 1.5 ml conical microcentrifuge tubes
- Indelible markers
- Bag for recycling used bottles hung onto shelf where convenient to use

1) Wear Gloves

2) Bleach necessary surfaces

3) Prepare bleach bath (50% bleach), water bath, and ice bath (*Image A*)

4) Bleach magnetic filter cups, stoppers, and filter tweezers: **soak in bleach bath 1 min, rinse with tap water, air dried on bench, and change gloves after cleaning items.**

5) Assemble the filtering equipment. Set up pump and Erlenmeyer filtering flasks on bleached table to left of fume hood. Connect: (1) pump to first Erlenmeyer flask with the shorter opaque tubing; 2) rubber black stopper to mouths of both Erlenmeyer flasks; (3) filtering stand to second Erlenmeyer flask with the longer clear tubing. Plug vacuum pump into power strip with switch that you can step on to turn on/off. (*Image B*)

6) Prepare for UV. Put four bleached stoppers into the filtering stand under the hood. Put 4 bleached filter cups beneath for UV. Cover the fume hood with cardboard and plug in germicidal UV light. Leave for 15 minutes. NOTE: place aliquots of filters and tubes in hood at start of the day **OR** per every 10 samples, whichever comes first. (*Image C*)

7) In the meantime, Pre-label the 1.5 ml centrifuge tubes and set up ice bath for storage later.

8) After UV: Unplug light. With bleached tweezers, place clean filter onto the stopper and attach magnetic cup. (*Image D*)

- 9) Turn on pump by stepping on power switch (no hands!). Pour water bottle into cup.
 - a. Filter lab blank first (a store-bought water bottle that hasn't left the lab). Run at the start of each day **OR** per every ten samples, whichever comes first.
 - b. Filter field samples, including field blank.
 - i. Change gloves when pouring separate samples.
 - ii. If water is high in particulates or flow is slow, use an additional filter (max 2 filters/sample)
 - iii. Run pump a bit longer than it takes for the water to run through to "dry" the filter.
- 10) Roll up filter. Use clean filter tweezers to roll filter up and clean left gloved pinky finger to help if needed. Use tweezers to place rolled filter into microcentrifuge tube. Place tube/s in labeled Ziploc bag.
Store tubes of filters on ice bath until moving to freezer. (Image E)
NOTE: Clean tweezers in bleach/water bath between each sample.
- 11) When finished with a round of samples, place filter cup, stopper, and tweezers in bleach bath and then proceed to change gloves, grab a clean filter cup stopper/cup for next sample.
- 12) Repeat beginning with step 6 until finished filtering for the day.
- 13) Disconnect tubing from vacuum pump and run the pump for 10 minutes to ensure it stays dry.
- 14) Store samples in -80 freezer if not extracting for DNA soon.
- 15) Put equipment away and wipe down surfaces.

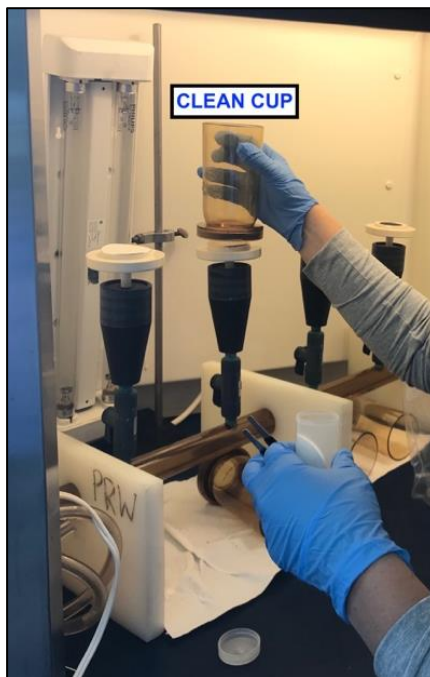
Reference Images:



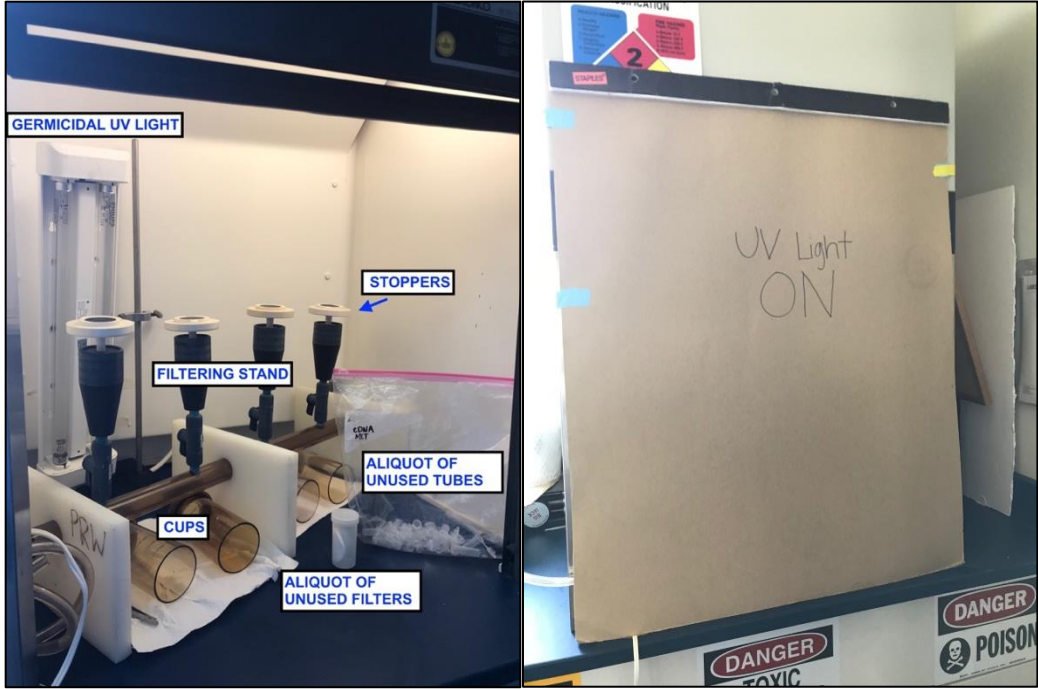
A.



B.



C.



D.



E.

Qiagen DNeasy Blood & Tissue Kit

UNH Protocol modified from Watts 2018, Kinnison 2017, & Qiagen DNeasy Manufacturers Instructions 2017

Equipment needed:

- Gloves, nitrile
- Clorox bleach spray + paper towels
- 56 °C water bath + 2.0-mL centrifuge tube rack
- 97-100% EtOH
- Mini-vortex
- Centrifuge with rotor for 1.5 or 2.0-mL microcentrifuge tubes, reaches 15-20,000 x g
- 1.5 or 2.0-mL Eppendorf Safe-Lock microcentrifuge tubes (MCT) \$55/500 MCT
- 1000 µL pipette, 200 µL pipette + disposable Eppendorf pipette filter tips \$200/960 filter tips
- Qiagen DNeasy Blood & Tissue Kit \$163.00/50 extractions or \$700.00/250 extractions)

Before starting:

- Make sure that ethanol has been added to buffers AW1 and AW2.
- Aliquot contents of kit to insure against contamination (kit is expensive).
- Preheat the incubator oven to 56 °C.
- Make sure all samples/chemicals are at room temperature. Re-dissolve any precipitates in buffers AL or ATL by incubating at 56 °C for at least 10 min.

Label one set of spin columns for extraction and one set of 2.0 or 1.5 ml microcentrifuge tubes (MCT) for final storage. Remove samples that have been designated to be extracted from freezer, and make sure the tubes they are in are properly labeled.

1. Prepare an **extraction negative control** by placing a new filter paper in a new sterile 1.5 ml MCT.
2. Add **370 µL of Buffer ATL** (37 on P1000 pipette) to each tube.
3. Add **30 µL Proteinase K** (30 on P200) to each tube and mix immediately by pulse-vortexing for 15 s.
4. Incubate at 56 °C for 1 hour (TIMED). Remove and vortex 15s approx every 15 min.
5. Remove from incubator and centrifuge at $\geq 16,000$ x g for 3 minutes.
6. "Squeegee" filter and remove from the 1.5 mL MCT tube, leaving only supernatant.
7. Add **400 µL Buffer AL** (40 on P1000) to each tube.
8. Add **400 µL 100% ETOH** to each tube, and vortex for 15 s.
Note: for very "dirty" samples, centrifuge briefly to condense particles into a pellet. In the next step, transfer supernatant while avoiding the pellet.
9. Pipette **650 µL of the sample** into the labeled QIAamp Mini spin column (in a 2-ml collection tube) without wetting the rim. Close and centrifuge at **6000 x g for 1 min**. Pour the filtrate into the discard beaker.
10. Repeat until the whole lysate is loaded. A maximum of 5 x 650 µL can be loaded onto the QIAamp Mini spin column.
Note: here you can combine any samples that initially required two filters.

11. Carefully open the QIAamp Mini spin column, discard the fluid in the lower chamber, and add **500 µl Buffer AW1** without wetting the rim. Centrifuge at 6000 x g for 1 min.
12. Carefully open the QIAamp Mini spin column, discard the fluid in the lower chamber, and add **500 µl Buffer AW2** without wetting the rim. Centrifuge at full speed (15,000-20,000 x g) for 3 min.
13. Discard the filtrate and place the QIAamp Mini spin column into labeled MCT for final elution.
14. Carefully open the QIAamp Mini spin column and add **100 µl Elution Buffer** (100 on P200) to the center of the column membrane. Incubate at room temperature for 2 min and then centrifuge at 6000 x g for 1 min. DO NOT DISCARD FILTRATE.
15. Discard spin column and keep the labeled 2.0mL MCT (or 1.5 mL storage tube) with extracted DNA.
16. Carefully label storage box and double-check that tubes are correctly labeled.
17. Store samples at -20 °C

** If the filters absorb too much supernatant as you work through the sample batch, re-spin samples another 5 minutes as needed to ensure you can easily collect supernatant without too much effort.

Safety: The chemicals noted below have safety issues noted by Qiagen:

Buffer AL/E (NFPA: H=2 F=1 R=0) Contains guanidinium chloride – harmful

Buffer AW1 (NFPA: H=2 F=0 R=0) Contains guanidinium chloride – harmful

Proteinase K (NFPA: H=1 F=0 R=0) Contains Proteinase, Tritirachium album serine - harmful

PPE: Use nitrile gloves

Qubit DNA Concentration Measurement

Label a Qubit tube for each sample. Prepare a 10ml tube for the buffer mixture.

1. Make a dye/buffer solution of 1:199 for each sample, plus one extra (n+1). Add **199 μ l x (n+1) buffer** and **1 μ l x (n+1) dye** to the 30 ml tube. Vortex for 15 s.
2. Add **199 μ l dye mixture** to each Qubit tube.
3. Add **1 μ l dye mixture** to each Qubit tube.
4. Vortex.
5. For each sample: place vial in Qubit reader. Set to high sensitivity, read next sample, calculate concentration, select ng/ml.
6. For each sample, record the amount of DNA present.

RPM to RCF Conversion for Hermle Z320 Centrifuge

RCF = relative centrifugal force or **g force**, the acceleration applied to the sample.

RCF is relative to the force of Earth's gravity and depends on **revolutions per minute (RPM)** and radius of the rotor.

Conversion chart for rotor with 92 mm radius, which fits twenty-four 1.5-2.0 mL MCT:

| RPM | RCF (g) |
|--------------|---------|
| 14,000 (MAX) | 20,000 |
| 12,000 | 15,000 |
| 11,000 | 12,000 |
| 8,500 | 7,500 |
| 7,500 | 6,000 |

Safety: do not exceed the maximum RPM. When protocol calls for 20,000 x g, even 15,000 x g will suffice.

UNH Online eDNA Database for NERRs

WNERR samples collected using the Larval Fish eDNA Sampling Protocol and the Green Crab eDNA Sampling Protocol are typically vacuum filtered at WNERR and sent to UNH for DNA extraction and further processing. WNERR is able to extract samples in the Reserve laboratory if time allows for the research staff. Records of collection events, sample processing, and results are kept on the **eDNA–NSC** Google Drive.

In addition to serving as a database for participating NERRs, the **eDNA–NSC** Google Drive provides resources such as study methods, contact lists, and reference literature.

You'll record data and view results in the **Results & Data** folder.

To record sampling events: Navigate to *Sample Tracking* → *Production* → [2018 Sample Tracking](#).

- For Larval Fish eDNA samples
 - **Sample site – official** = WHAR (stands for Wells Harbor)
 - **Sample type** = Larval Fish Catch
 - **Sample counts** = 2 water samples, 1 biomass sample
- For Green Crab eDNA samples
 - **Sample site – official** = LRIV (stands for Little River)
 - **Sample type** = Sediment
 - **Sample counts** = 2 composite replicate samples

To record filtering: Navigate to *Sample Tracking* → *Production* → [To Filter](#)

- If you filled out the 2018 Sample Tracking survey, you should see your sample IDs already copied here. Once filtered, mark as complete.

To record metadata: Navigate to *Sample Tracking* → *Wells*

- For Larval Fish eDNA samples: → *Larval Fish Metadata*
 - Here you'll record (1) [the visual taxonomic IDs](#), (2) [tow water volumes](#)
- For Green Crab eDNA samples: → *Green Crab Metadata*
 - Here you'll record [all crab sample data](#) including catch weights and measurements.

To view results: find the file name of the appropriate primer. This will be MiFish for larval fish or LoBo for crabs. Once you have the **barplots.qzv** file (name may vary), upload file onto [Qiime2View](#) to visualize initial results online.

- For Larval Fish eDNA samples: Navigate to *MiFish* → *Larval Fish*.
- For Green Crab eDNA samples: database formatting in progress.

For Green Contact Dr. Alison Watts (alison.watts@unh.edu) for access to this database. If you have technical questions or would like to suggest formatting changes, contact **Devin Thomas (devin.w.thomas@unh.edu)**.

Additional Resources

Helpful Papers

eDNA Overview

Laramie, M. B., Pilliod, D. S., Goldberg, C. S., & Strickler, K. M. (2015). *Environmental DNA sampling protocol — filtering water to capture DNA from aquatic organisms* (No. 2-A13). US Geological Survey.

Schmelzle, M. C., & Kinziger, A. P. (2016). Using occupancy modelling to compare environmental DNA to traditional field methods for regional-scale monitoring of an endangered aquatic species. *Molecular ecology resources*, 16(4), 895-908.

Thomsen, P. F., & Willerslev, E. (2015). *Environmental DNA—An emerging tool in conservation for monitoring past and present biodiversity*. *Biological Conservation*, 183, 4-18.

Larval Fish eDNA Studies

Bréchon, A. L., Coombs, S. H., Sims, D. W., & Griffiths, A. M. (2012). Development of a rapid genetic technique for the identification of clupeid larvae in the Western English Channel and investigation of mislabelling in processed fish products. *ICES Journal of Marine Science*, 70(2), 399-407.

Loh, W. K. W., Bond, P., Ashton, K. J., Roberts, D. T., & Tibbetts, I. R. (2014). DNA barcoding of freshwater fishes and the development of a quantitative qPCR assay for the species-specific detection and quantification of fish larvae from plankton samples. *Journal of Fish Biology*, 85(2), 307-328.

Project Contacts for UNH-NERR Science Collaborative

| Name | Affiliation | Preferred Phone | Preferred Email | Brief Summary Of Role |
|---------------------------|-------------|-----------------|-----------------------------------|--|
| Alison Watts | UNH | 6033127654 | Alison.watts@unh.edu | Project Manager @ Unh |
| Dylan Trueblood | UNH | 6032054376 | Dylan.trueblood9513@gmail.com | Research Assistant @ Unh |
| Jessica Haskins | UNH | 4135526521 | Jlh1020@wildcats.unh.edu | Research Assistant Under Related Sea Grant Project @ Unh |
| Devin Thomas | UNH | 6037673908 | Devin.w.thomas@gmail.com | Bioinformatics @ Unh |
| Krystalynne Morris | UNH | | Krystalynne.morris@unh.edu | Research Supervisor @ Unh HCGS |
| Kelley Thomas | UNH | | Kelley.thomas@unh.edu | Director Of Hubbard Center For Genomics @ Unh |
| Jess Waters | Great Bay | 6104174007 | Waters.summer@gmail.com | Project Facilitator Through Great Bay |
| Chris Peter | Great Bay | (603) 305-8693 | Christopher.peter@wildlife.nh.gov | Research Director @ Great Bay |

| | | | | |
|-------------------------|----------------|---------------------------------|----------------------------------|---|
| Steve Miller | Great Bay | 6038282954 Cell 2940146 Work | Steve.miller@wildlife.nh.gov | Collaborative Lead Ctpc @ Gbnerr |
| Cory Riley | Great Bay | 603 868 1095 | Cory.riley@wildlife.nh.gov | Reserve Manager @ Great Bay |
| Michelle Furbeck | Wells | (978) 660-9343 | Mfurbeck@une.edu | Research Assistant @ Wells |
| Claire Gottsegen | Wells | 7138289061 | Claire.gottsegen@yale.edu | Seasonal Research Assistant @ Wells (March-July 2018) |
| Jason Goldstein | Wells | | Jgoldstein@wellsnerr.org | Research Director @ Wells |
| Jacob Aman | Wells | | Jacobaman@wellsnerr.org | Project Manager @ Wells |
| Bree Yeednock | South Slough | 541-888-8270 X302 Work | Bree.yednock@state.or.us | Reserve Manager @ South Slough |
| Paul Stacey | Advisory Board | | Footprintsinthewater@outlook.com | Advisory Board Member, Formerly With Great Bay |
| Erik Pilgrim | Advisory Board | | Pilgrim.erik@epa.gov | Advisory Board Member, Epa In Cincinnati |
| Michael Kinnison | Advisory Board | | Mkinnison@maine.edu | Advisory Board Member, UMaine |
| Jaime Anthony | Advisory Board | | Jamie.anthony@oregonstate.edu | Advisory Board Member, Oregon Fish & Wildlife |