Developing Cost Effective Monitoring for Rainbow Smelt Using eDNA

Mini-Quality Assurance Project Plan

Casco Bay Estuary Partnership

Year 1 - 2018

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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, WNERR 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 (Cq 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

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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 natureof 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:

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.

- 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)

- 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^{o} 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.

<u>For a contamination control to be meaningful it must be prepared and handled in all the same ways as your actual field samples,</u> 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. <u>OPEN THE CAP ON THE BOTTLE BUT DO NOT POUR OUT THE CONTENTS OR IMMERSE THE OPEN BOTTLE IN THE WATERBODY</u>.
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).

<u>Freezing</u>: 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
School of Biology and Ecology
University of Maine
Orono, ME 04469-5751

Phone: 207-581-2575
Fax: 207-581-2537
E-mail: mkinnison@maine.edu

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) single layer just touching = 60,000-70,000 eggs/ft2)	none 100s 1,000s 10,000s millions
Upstream limit of egg bed - Latitude Please specify units (e.g. 47º 39.521')	
Upstream limit of egg bed - Longitude Please specify units (e.g. 69º 38.125')	
Downstream limit of egg bed - Latitude Please specify units (e.g. 47º 39.234')	
Downstream limit of egg bed - Longitude Please specify units (e.g. 69º 38.913')	
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) green slime on eggs?	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	0.0.6 - 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) Containes guanidinium chloride – harmful

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

Proteinase K (NFPA: H=1 F=0 R=0) Containes 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 ≥ 16,000 x 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.