Alison Watts

University of New Hampshire

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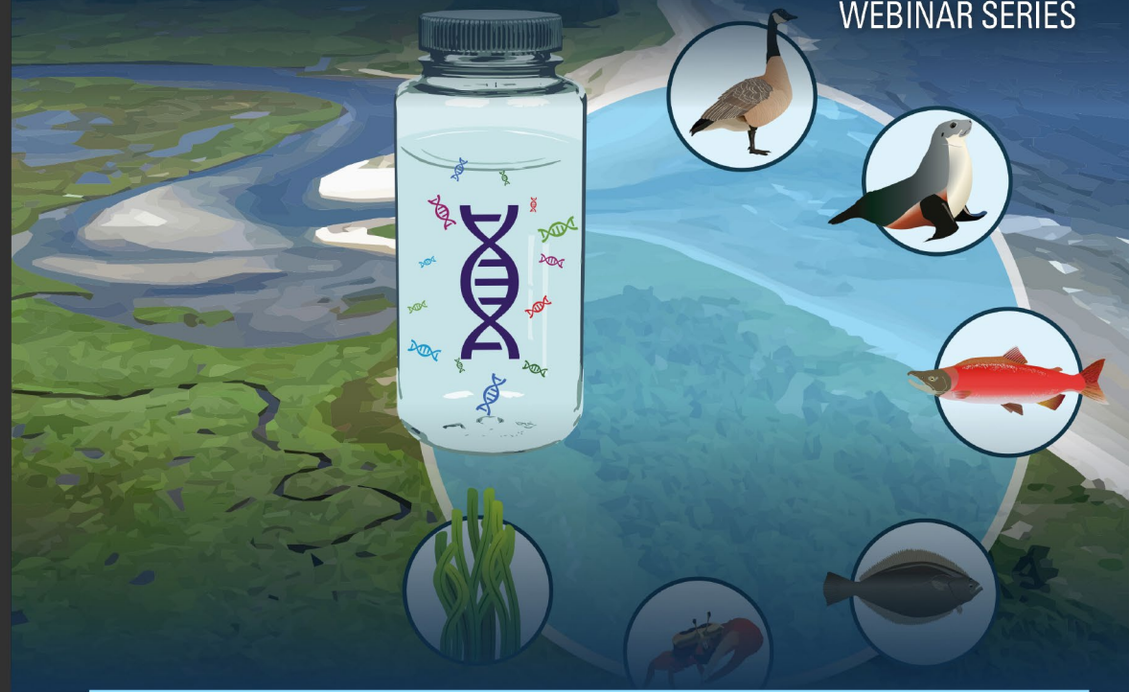
South Slough National Estuarine Research Reserve

Date: February 14th, 2019
Time: 3.00 (EST)

National Estuarine Research Reserve System Science Collaborative

COLLABORATIVE SCIENCE FOR ESTUARIES

WEBINAR SERIES



New technology for old problems: Exploring the use of eDNA in the reserve system

Summary Points:

Dr. Alison Watts has over 30 years of experience in water resource research and management. She has worked extensively with resource managers in the Great Bay region to reduce non-point source pollutants and to develop quantifiable metrics for ecological assessment. She is currently working on several projects incorporating molecular methods into environmental assessment of marine and freshwater ecosystems.

Dr. Bree Yednock has expertise in population genetics of estuarine organisms, molecular techniques, and bioinformatics. Her previous projects include a characterization of fish and invertebrate assemblages of the Coos estuary and an assessment of the local distribution and population structure of invasive European green crabs.

Bree will begin by providing some background on eDNA methodology, and then Alison will talk about the specifics of the project.

Poll Question

Which statement best describes your familiarity with eDNA?

Poll Question 1:
Which statement best describes your familiarity with eDNA?

- I've heard of eDNA (56.14%)
- I'm considering using it in research, monitoring, or engagement (19.30%)
- I'm using or have used eDNA approaches (21.05%)
- I have a lot of experience, and am willing to provide advice (3.51%)

Summary Points:

New Technology for Old Problems

Partnership:
University of New Hampshire
Alison Watts, Kelley Thomas

Nov 2017-2019

Wells, Maine

Jason Goldstein, Jake Aman

South Slough, Oregon

Bree Yednock, Shon Schooner, Alice Yeates

Great Bay, New Hampshire

Chris Peter, Steve Miller

Summary Points:

Traditional Biological Monitoring

Environment	Sample	Data Collection	Analyze
• Characterize sites, habitat	• Trap • Net • Direct observation	• Species ID • Count • Measure • Weigh	• Analysis and interpretation • Biodiversity • Species richness • Environmental trends

Summary Points:

The success of traditional biological monitoring methods relies heavily on expertise in taxonomy, and they are typically labor intensive in terms of sampling.

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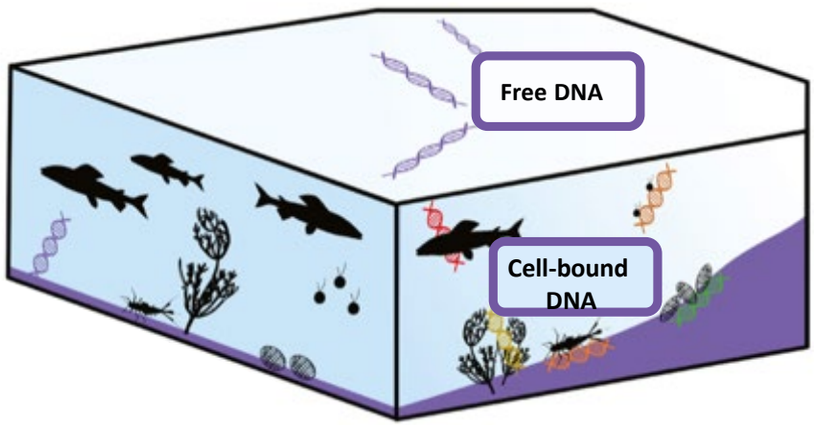
What is eDNA?

Environmental DNA (eDNA)

- All living things have and shed DNA
- eDNA is DNA released from an organism into the environment
- eDNA can come from:
  - Hair, scales, skin
  - Waste products
  - Reproductive cells

Summary Points:

eDNA sampling



- eDNA can be sampled from the water column and surface sediments
- Preserved and frozen samples are stable for months
- Excellent option for field-based analyses
- Multiple analytical options for eDNA

Summary Points:

Sampling of eDNA can be relatively straightforward.

Environmental DNA can be:

- **Free DNA**, which is freely-floating extracellular DNA; or
- **Cell-bound DNA**, which is inside cells that have sloughed off organisms.

Once samples are collected, if they are preserved correctly or frozen quickly, they can remain stable for several months. Long-term sample stability allows laboratory analysis to occur at the researchers' pace; this makes eDNA sampling an excellent option for field-based analyses.

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eDNA Methods

Single-species PCR

+ Simple, cheap, fast
- Only identifies one species

Metabarcoding

+ Identifies multiple species
- More complex, harder to interpret

Summary Points:

The two methods shown on the slide are currently being tested by Bree Yednock and her team.

Single-species polymerase chain reaction (PCR) allows researchers to determine whether a particular species of interest is in the target area. This method excels at identifying a single endangered, rare, or newly-arrived invasive species via a species-specific assay. The DNA probes used in this method only target a single species of interest.

Metabarcoding allows sequencing of all DNA from a sample, which can potentially identify all species present in an area. Results are complex, can be more challenging to interpret, and require vast computational power, but can offer an enhanced ability to examine biodiversity.

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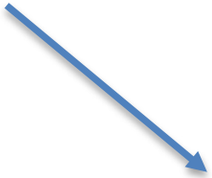
Species Identification

Goal: Match DNA sequences from eDNA sample with known sequences in a reference library

eDNA sample: AGGTGTGTAT



Species 1 : TGGTGAGTTT

Species 2 : TGGTGTGATT

Species 3 : TCGTGTGTTT

Species 4 : AGGTGTGTAT

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Summary Points:

eDNA Process

Sample

- Collect water and/or sediment samples

Extract DNA

- Water samples
  - filter
  - centrifuge
- Sediment samples
  - mix well
  - soils kit to collect

DNA Analysis

- **PCR:** Amplify DNA with species specific probes
- **Metabarcode:** Amplify and sequence DNA

Interpretation

- Analysis and interpretation (bioinformatics)

Summary Points:

Sampling is relatively straightforward and is not necessarily labor intensive.

Water samples are filtered, then DNA is extracted using blood and tissue kits (the team used kits from QIAGEN); sediment sample DNA is extracted using Powersoil kits.

DNA analysis requires DNA amplification prior to sequencing for both PCR and metabarcode methods.

- **DNA amplification**: Artificial replication, in a lab setting, of a particular DNA sequence to create millions of copies.

Analysis and interpretation of eDNA results is not trivial; experience in bioinformatics is essential to ensure proper coding or use of analytical software.

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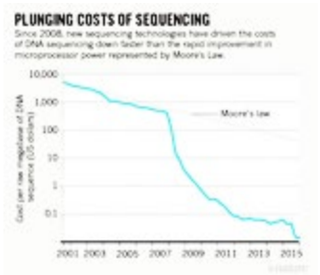
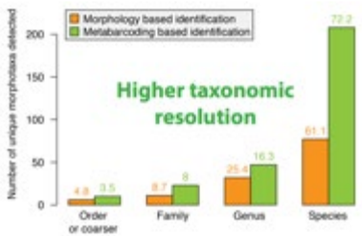
Key Advantages of eDNA

Cost & Speed



- Costs can be a fraction of morphological analyses
- DNA results within days/weeks, not months

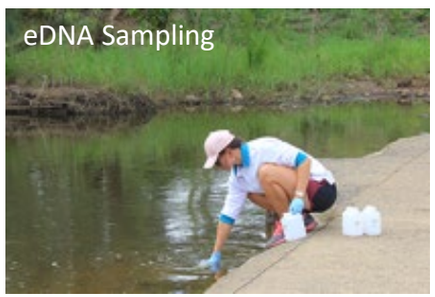
Sensitivity & Accuracy



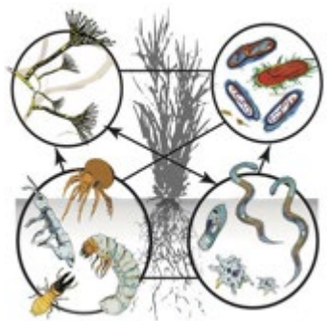
Elbrecht et al. 2017, Methods Ecol. Evol

- Able to resolve cryptic species complexes or ambiguous morphology
- Repeatability

Non-invasive sampling



Multi-trophic approach



- Targeting multiple phyla in single sequence runs
- Linking trophic networks

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Slide from Eric Stein, SCCWRP

Summary Points:

Some key advantages of eDNA are:

1. **Cost and Speed**: The cost of sequencing has declined over the past several years while speed has increased, which means samples can be sequenced within days or weeks instead of months.
2. **Sensitivity and accuracy**: DNA sequences unambiguously define species that are otherwise very challenging to differentiate, which also allows for higher repeatability in testing.
3. **Non-invasive sampling**: Non-invasive methods allow researchers to collect water samples without disturbing the habitat.
4. **Multi-trophic approach**: Sampling with nets or traps limits the catch to a subset of the organisms that are actually in the environment, while metabarcoding enables identification of multiple phyla in a single sequence run.

eDNA Reality Check… It's Not Magic

Summary Points:

eDNA can:

- Provide information on species presence
- Help target field sampling programs
- Reduce sampling effort
- Provide non-destructive, non-invasive sampling method

eDNA cannot:

- Confirm absolute absence
- Determine species abundance
- Determine life stage or condition
- Identify species without known DNA sequences

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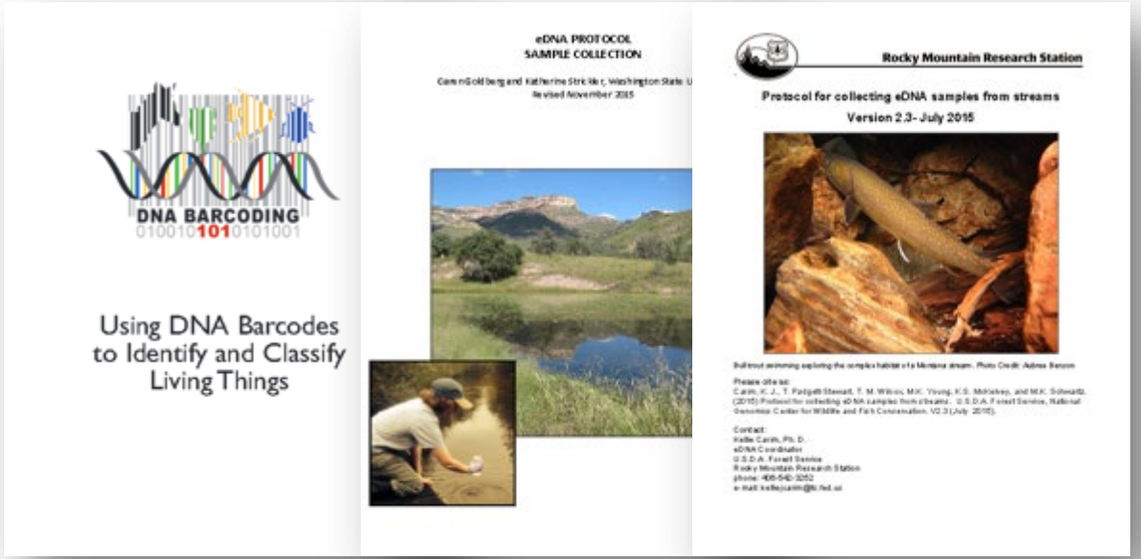
Obstacles to Implementation

Sample → Extract DNA → DNA Analysis → Interpretation

Which methods?

- sample volume
- number of samples
- storage
- processing speed



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Summary Points:

There is no standardized method accepted across all agencies and groups; investigators create their own protocols that work for them, which can act as an obstacle to determining the best methods.

Obstacles to Implementation

Which methods?

- kits
- modifications
- contamination prevention

Summary Points:

When extracting DNA, many different kinds of kits exist, and many modifications can be made with the kits available. There is also high potential for cross-contamination of samples.

Obstacles to Implementation

Which barcode?

COI – eukaryotes

12S – eukaryotes

16S – bacteria

CytB – fish

Which methods?

- sequencing
- PCR assays

Summary Points:

The slide shows four examples of barcode primers that work for the organism groups in question.

- **Primer**: A short single strand of RNA or DNA needed to start DNA synthesis.

In determining which method is most appropriate for analysis, investigators should evaluate the tools available to them before committing to an analytical approach.

Obstacles to Implementation

Sample → Extract DNA → DNA Analysis → Interpretation

Bioinformatic methods?

- custom or published
- QA/QC protocols
- Reference libraries

NCBI

BOLDDATA

international BARCODE OF LIFE

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Your DNA taxonomy is only as good as your DNA library

Summary Points:

Applying bioinformatic methods can be difficult, and handling errors is important, but there are a number of public scripts and programs available to assist researchers. Using the best available libraries for sequence comparisons is also vital; the ability to identify sequences is only as good as the reference library.

Overarching Project Goals

1. Design and implement a pilot environmental DNA (eDNA) monitoring program at several National Estuarine Research Reserve (NERR) sites.

2. Identify estuarine target species of concern, with a focus on invasive invertebrates and migratory fish.

3. Develop eDNA sample collection and analysis protocols, with training materials and recommendations for the appropriate use of eDNA in estuarine monitoring

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Summary Points:

Questions:

Comment: I imagine there are a lot of opportunities for false positives. Contamination from terrestrial waste, especially humans, boat bilge, and length of time the DNA persists after the species has departed.

Bree: Yes, you need to know your sites fairly well. We've been testing in South Slough where we have two years of monthly data for fish species collected through our seining program. As an example, we used eDNA sampling at our site as an initial test and ended up with a bunch of positives for species of fish that are not in our estuary, like albacore tuna and certain kinds of rockfish. We realized that our site is actually right next to the seafood processing plant in Charleston, and so those are actually fish that are being brought into that processing plant at that time of year; it's important to have some sense of what you expect to be there. Another concern we've been trying to deal with is how to deal with questionable positive hits.

Questions:

How does one see if an organism is in a Library?
Querying known databases for species of interest. The search results show what results are available for gene regions. Most fish have some information, but it needs to match the primer your using as well.

Is metadata collection becoming standardized? If so, how?
Bree: For the metadata that we collect in the field, we collect where we're sampling and the methods that we're using to sample

Alison: We collect some standard metadata around who did the extraction, where the data were collected, and so on. We keep those records with our information. The different databases have certain requirements for metadata. But that's really an important question because the reality is different people collect different metadata, and as we'll keep saying throughout this webinar, the way in which you collect and analyze data really influences results, and if you don't know exactly how someone collected their data then you don't know if you can do a direct comparison

Traditional Biological Monitoring Methods

New Hampshire - seining, coastal streams

Oregon - seining, crab trapping

Maine - plankton tows, crab trapping

How does eDNA compare?

Summary Points:

Most of the eDNA collection in this project is paired with traditional sampling to establish a baseline for interpreting results. It is important to understand that eDNA methods do not give the same information that traditional sampling methods do; comparing the two is somewhat akin to comparing apples to oranges, but drawing comparisons can help determine how the two methodologies relate without expecting results to be the same.

eDNA Monitoring and Analysis Methods

Derived from existing methods: EPA, US Fish and Wildlife, USGS, etc

Sample	Extract DNA	DNA Analysis	Interpretation
Water: Three 1-liter samples at each location, lab filtered through 1.5um glass fiber filter	Extracted with Qiagen DNeasy Blood & Tissue kit	Sequenced on Illumina HiSeq 12S MiFish CO1 18S	QIIME 2 Reality check results with local managers Adding to database

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Summary Points:

One area in which this project did not invest time was developing methods, because many different types exist. Instead, the team examined the literature, consulted with their advisory board, and spoke to experienced people, and then selected a set of methods that they believed were most likely to be appropriate. Most of these methods were developed for freshwater due to lack of data for estuaries. The team selected methods based on what appeared to be most effective, but also what was practical for resource managers.

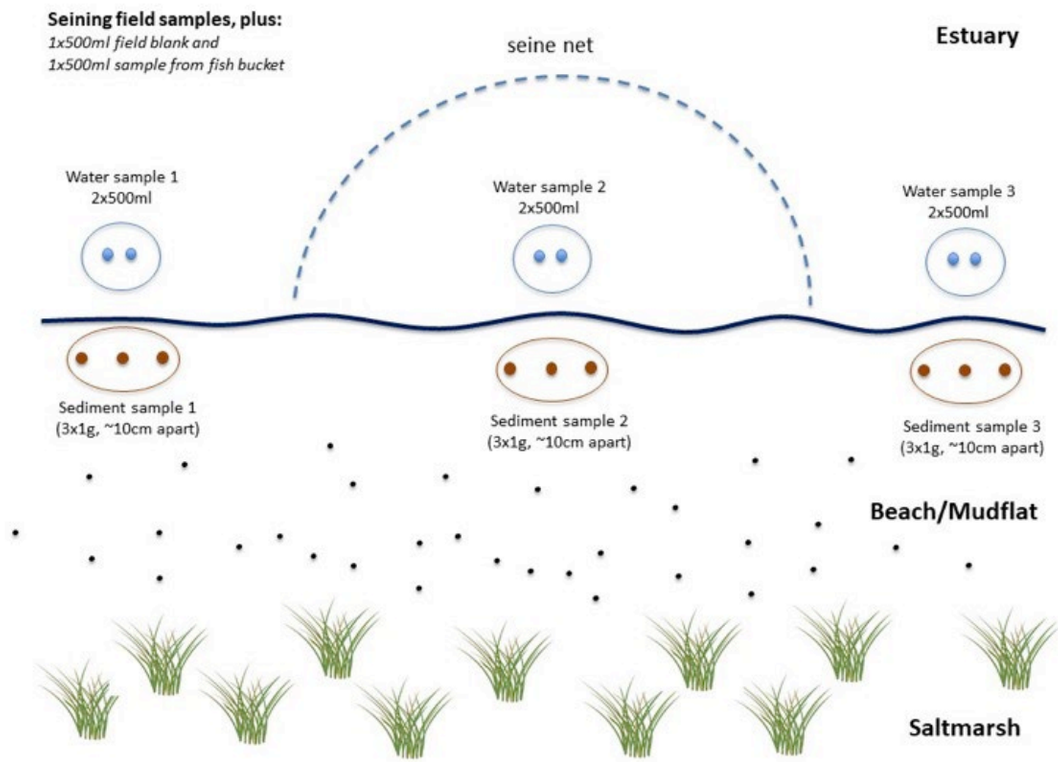
The team extracted DNA with a standardized kit to promote reproducibility of results for others, opting to use standard primers rather than developing custom ones in order to support proven methods that could be adopted by others.

The team then checked with local managers to ensure that results made sense in the context of particular estuaries.

Technical terms:

- **QIAGEN DNeasy blood & tissue kit**: A tool for purifying DNA from animal blood and tissues and from cells, yeast, bacteria, or viruses.
- **Illumina HiSeq**: Lab equipment used to sequence DNA.
- **QIIME 2**: Open-source bioinformatics software.

Seining sample collection



1-L water samples at 3 locations, 3 composite sediment samples at tideline

Summary Points:

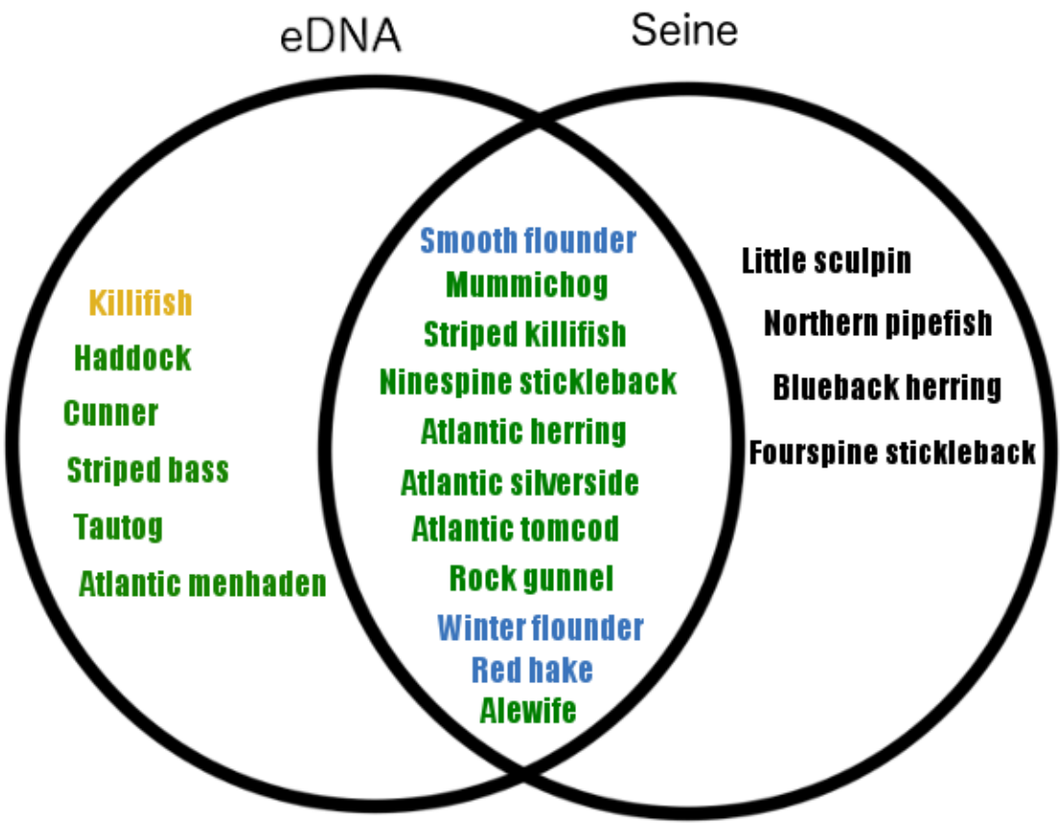
An overview of the seining process:

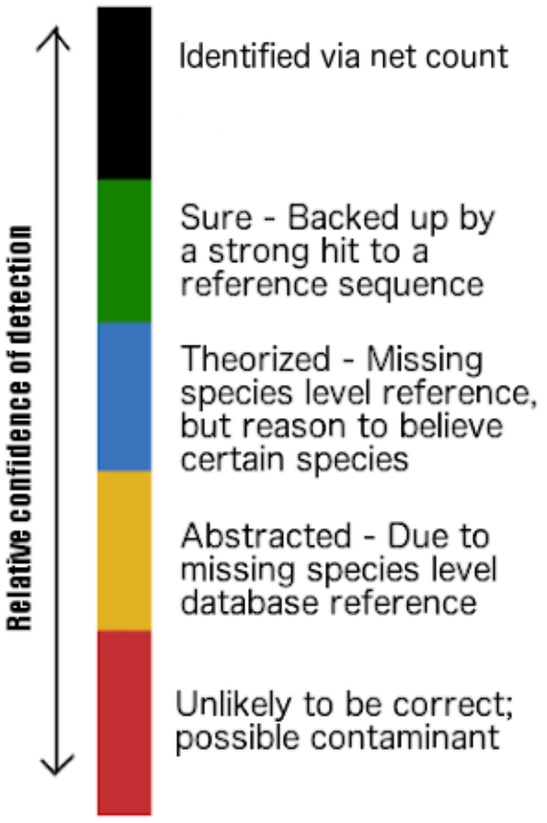
1. Pull in capture net, anchor one end, pull in the other end, and haul in species.
2. Sample water at three locations within the vicinity of the seine, and then take three sediment samples within the vicinity.
3. Examine water and sediment DNA.

DNA results support a direct comparison with the seine results.

Seining – New Hampshire

5 sites, 2 sampling events





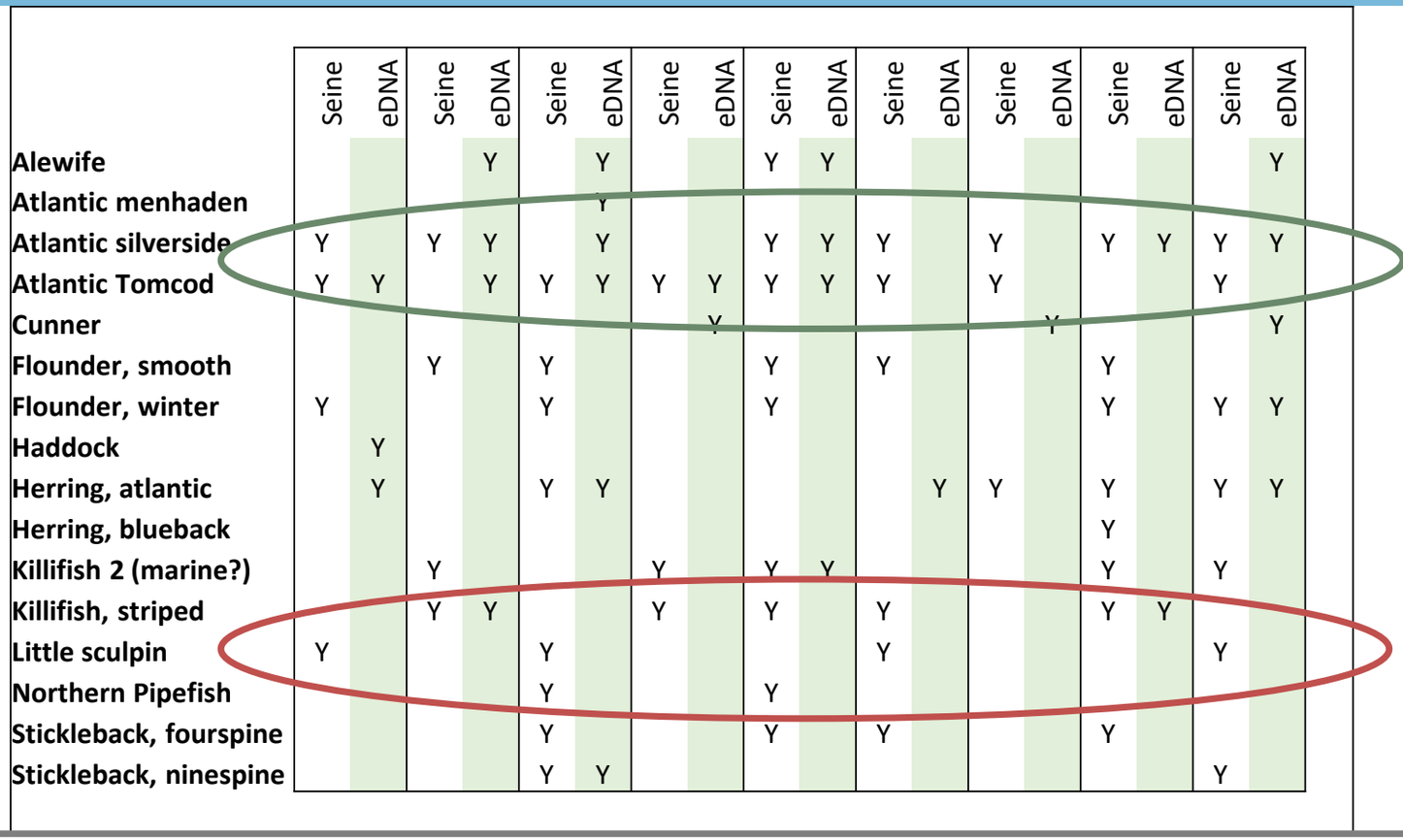
Summary Points:

The figure shows averages from a summer of sampling (30 samples); individual site correlation is not good. Species identification requires both database and reality checking.

There is a lot of overlap, but not complete overlap.

When seining, fish species can be identified based on knowledge of physical features; eDNA sampling generates a computer sequence, which makes species identification more challenging. Determining whether a given DNA sequence represents a particular species requires significant time investment, and helped convince the team to color-code results based on certainty.

Seining sample comparison



	Seine	eDNA	Seine	eDNA	Seine	eDNA	Seine	eDNA	Seine	eDNA	Seine	eDNA	Seine	eDNA	Seine	eDNA	Seine	eDNA
Alewife				Y		Y			Y	Y								Y
Atlantic menhaden						Y												
Atlantic silverside	Y		Y	Y		Y			Y	Y	Y		Y		Y	Y	Y	Y
Atlantic Tomcod	Y	Y		Y	Y	Y	Y	Y	Y	Y	Y		Y				Y	
Cunner								Y						Y				Y
Flounder, smooth			Y		Y				Y		Y				Y			
Flounder, winter	Y				Y				Y						Y		Y	Y
Haddock		Y																
Herring, atlantic		Y			Y	Y						Y	Y		Y		Y	Y
Herring, blueback															Y			
Killifish 2 (marine?)			Y				Y		Y	Y					Y		Y	
Killifish, striped			Y	Y			Y		Y		Y				Y	Y		
Little sculpin	Y				Y						Y						Y	
Northern Pipefish					Y				Y									
Stickleback, fourspine					Y				Y		Y				Y			
Stickleback, ninespine					Y	Y											Y	

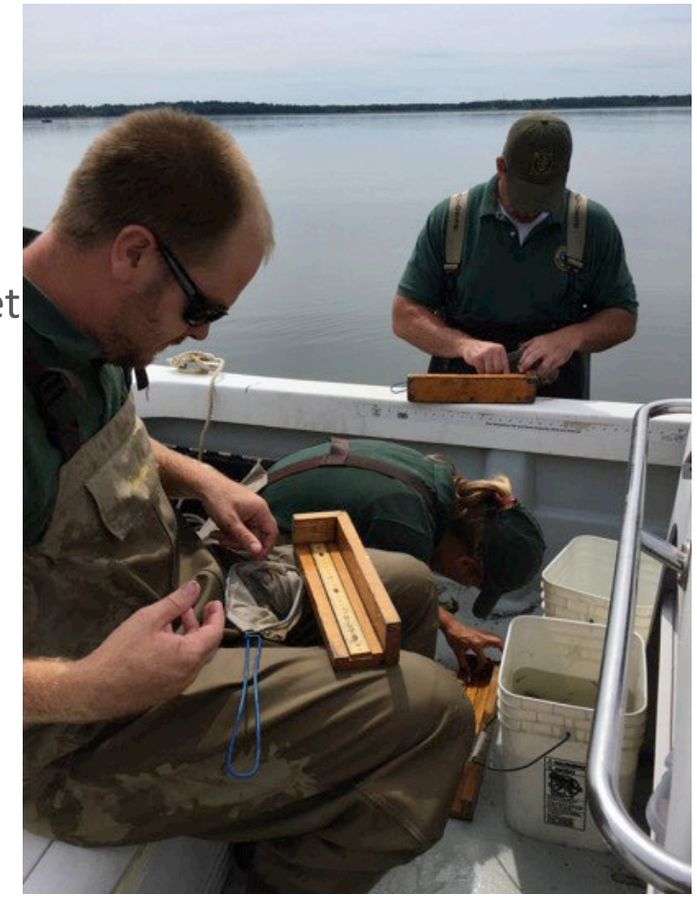
eDNA correlates well with abundant species (Silverside, Tomcod, Herring**), if enough samples are collected**

eDNA not detecting rarer species (Pipefish, sculpin)

Summary Points:

How good is good enough??

- General survey of fish in the region?
  - Yes – if species are in database
- Seine level survey of fish at a location?
  - Maybe, but takes a lot of samples, but also get fish that escape nets
- Number of fish, size, age, etc?
  - No



Summary Points:

Asking the question “how good is good enough” raises a second question: What is the goal of the research?

To reiterate, eDNA and traditional sampling methods are not the same; they have different strengths and weaknesses.

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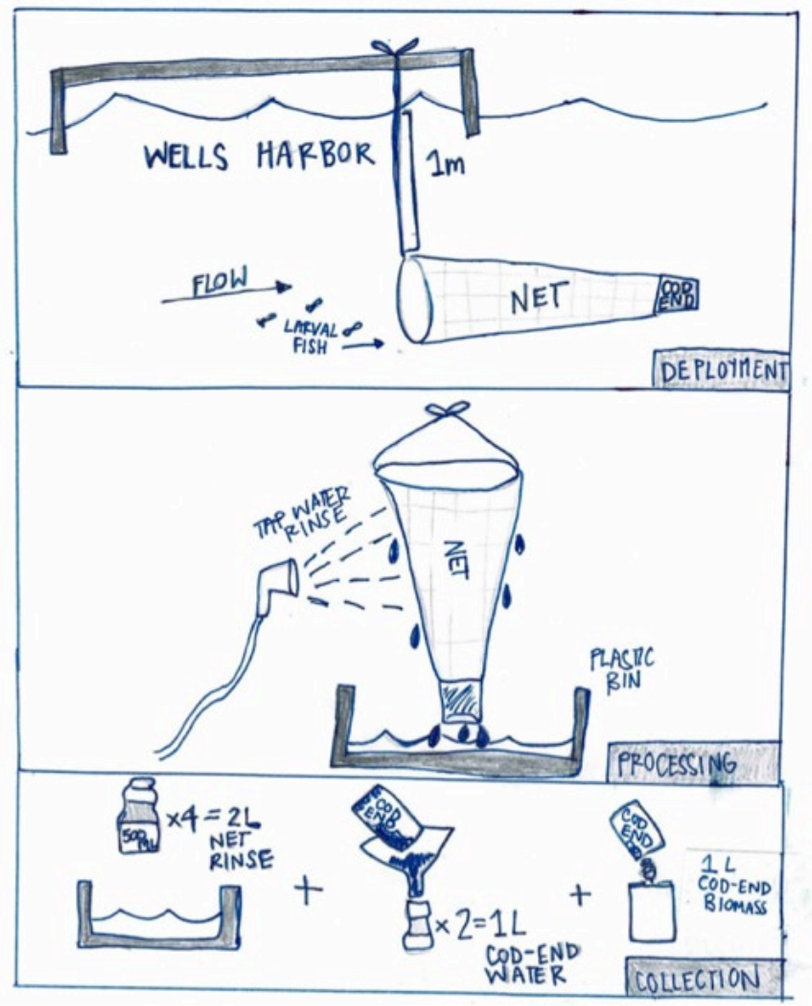
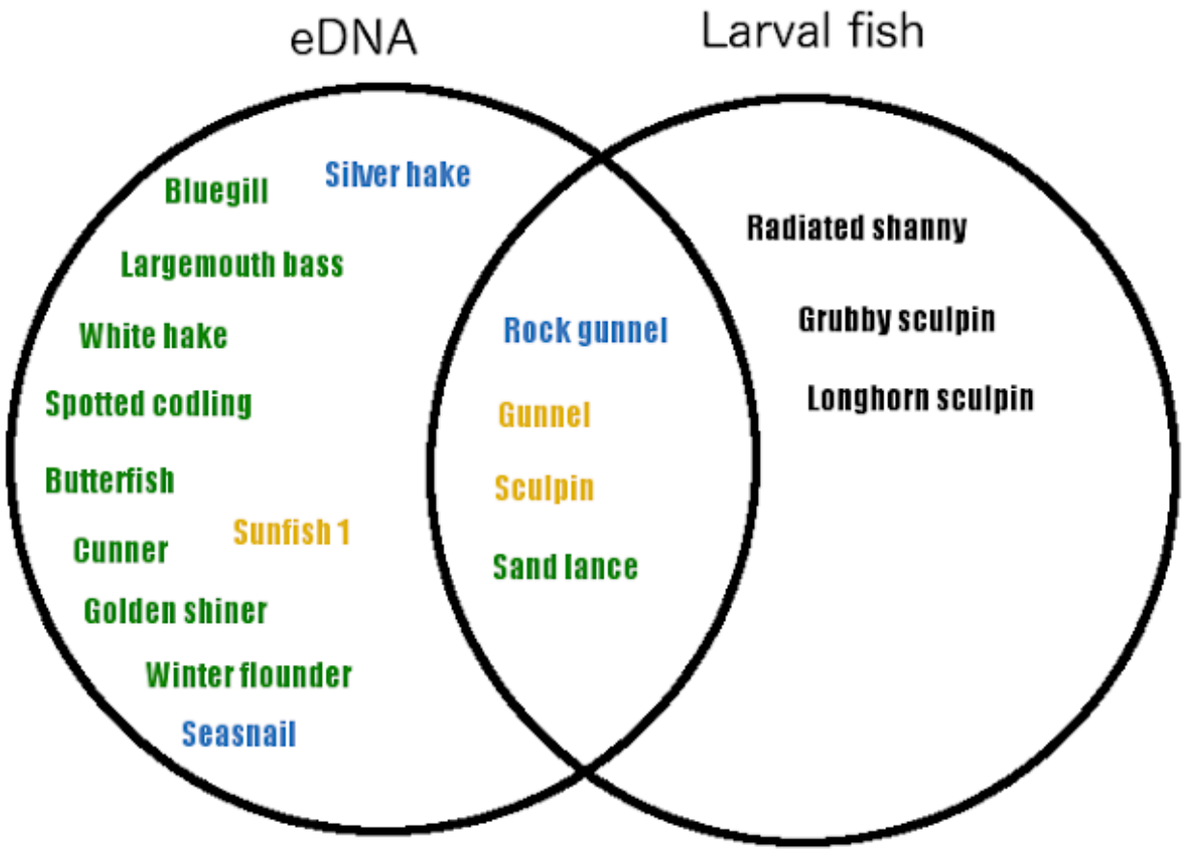
Larval Trawl (Wells)

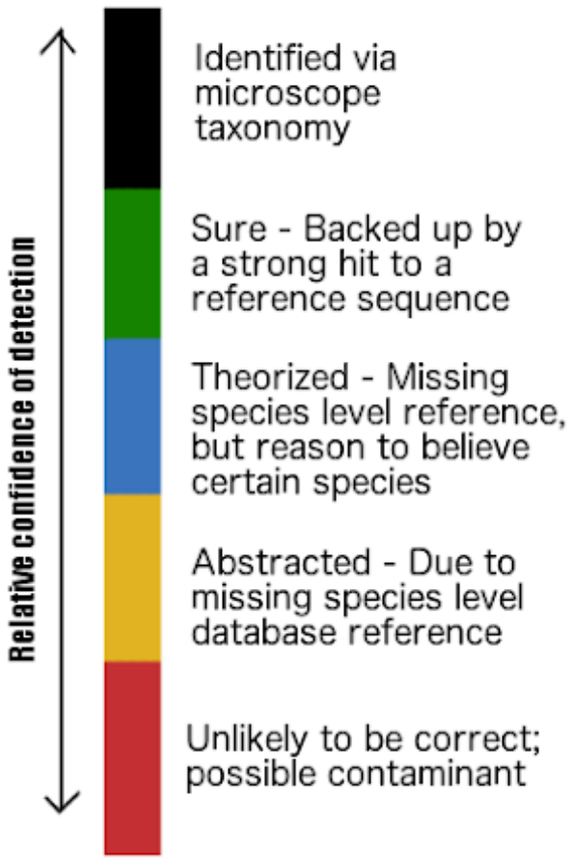


Summary Points:

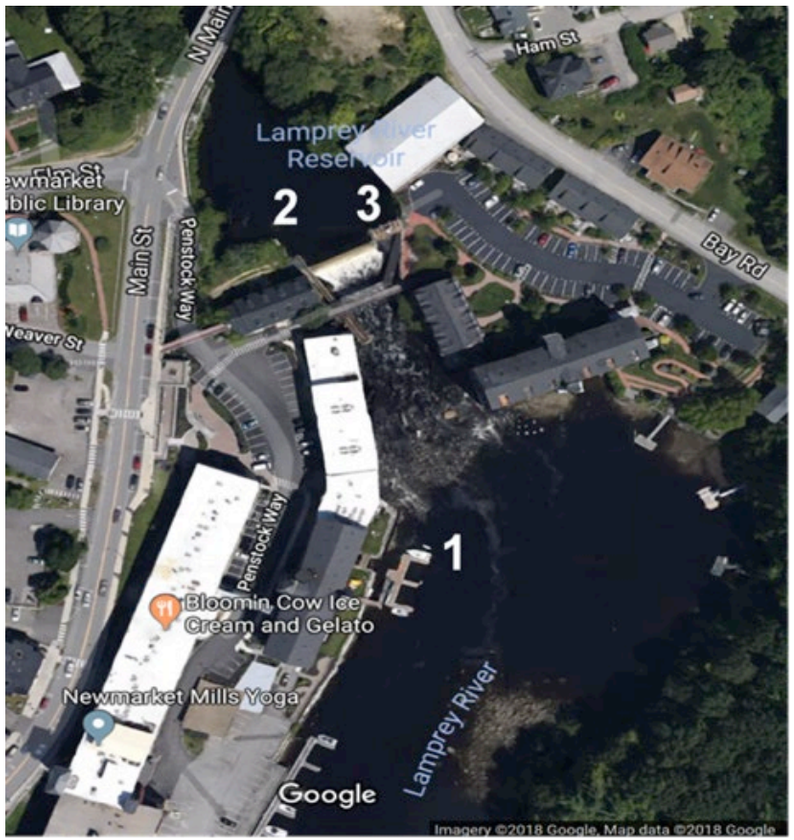
The Wells Reserve does periodic larval trawls, looking for fish and plankton. Jason Goldstein's team analyzes the larval mass in the net using DNA methods and microscope identification; this is an area where DNA methods can help streamline the speed of analyses.

Larval trawl results(Wells)





Anadromous fish counts



Triplicate 1-liter water samples collected above dam and at base of fish ladder, 2-3 times/week May-June (Oyster and Lamprey Rivers, NH)

38 fish species: *Alewife, American eel, American shad, Atlantic herring, Atlantic menhaden, Atlantic silverside, Atlantic tomcod, Black crappie, Bluegill, Brook Trout, Butterfish, Chain pickerel, Chub, Common shiner, Cunner, Darter, Fourspine stickleback, Golden shiner, lake lamprey, Largemouth bass, Mud hake, Mummichog, North Pacific hake, Pumpkinseed, Rainbow trout, Redbreast Sunfish, Rock bass, Sand Lance*
Sculpin, Southern codling, Starry Flounder, Sunfish 1, white perch White sucker, yellow perch, Yellow perch

American beaver, Common Muskrat, Common Tern, Cow, Eastern Gray Squirrel, Eastern Newt, Pig, Mallard, Human

Summary Points:

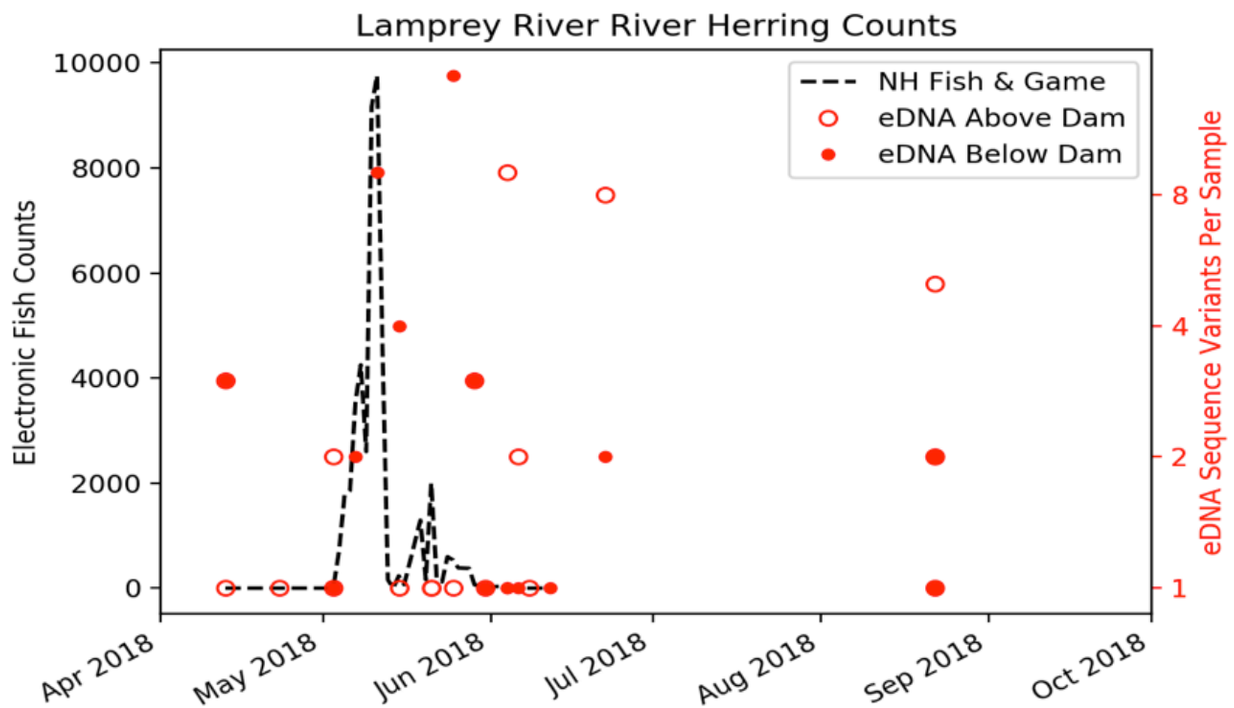
This work examines two tidal rivers in the Great Bay reserve, where the team takes samples from below and above a dam. The goal of the project is to identify the timing of fish return, focusing on river herring and American eel. Specifically, the team is exploring whether there is any kind of correlation between the DNA in the water and the number of fish in the river. The two rivers in question both have fish ladders with ongoing counts occurring every spring.

While not specifically looking for a variety of species, the project did identify 38 different fish species as a side benefit. The New Hampshire Fish and Game Department expressed that they were impressed by both the number of fish identified and the correlation with expected fish species.

Total samples of the target sites numbered around 40 or 50. Depending on the primer used, analyses can also yield mammals and other creatures, which could be identified by other primers.

Anadromous fish counts

DNA increases when fish return, but doesn't correlate with abundance



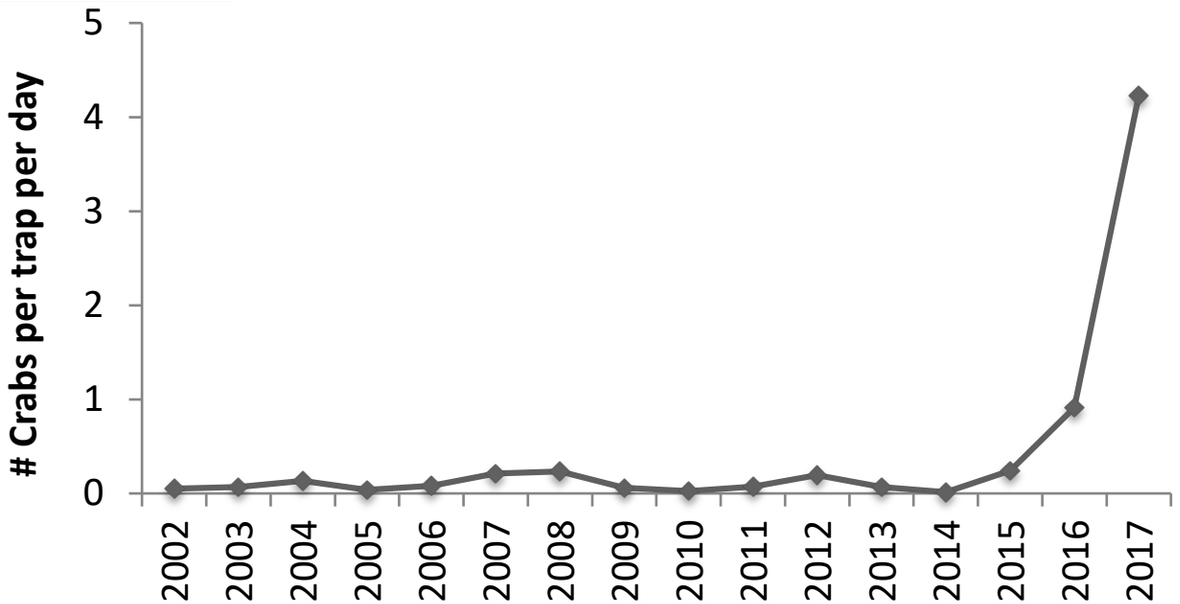
Summary Points:

Per the results, the number of eDNA sequence variants increases slightly before fish appear in the electronic fish count; this indicates that the fish are likely swimming in the river or preparing to move prior to a noticeable rise in the electronic count.

Another observation worth noting is that the amount of eDNA sequence variants is somewhat scattered across the plot. Even when many fish are detected, there are times when no DNA is detected because of high DNA variability in the sample.

Key takeaway: the team successfully detected fish return, but they were not confident in their ability to link eDNA to the quantity of fish.

Can we use eDNA to detect invasive species?



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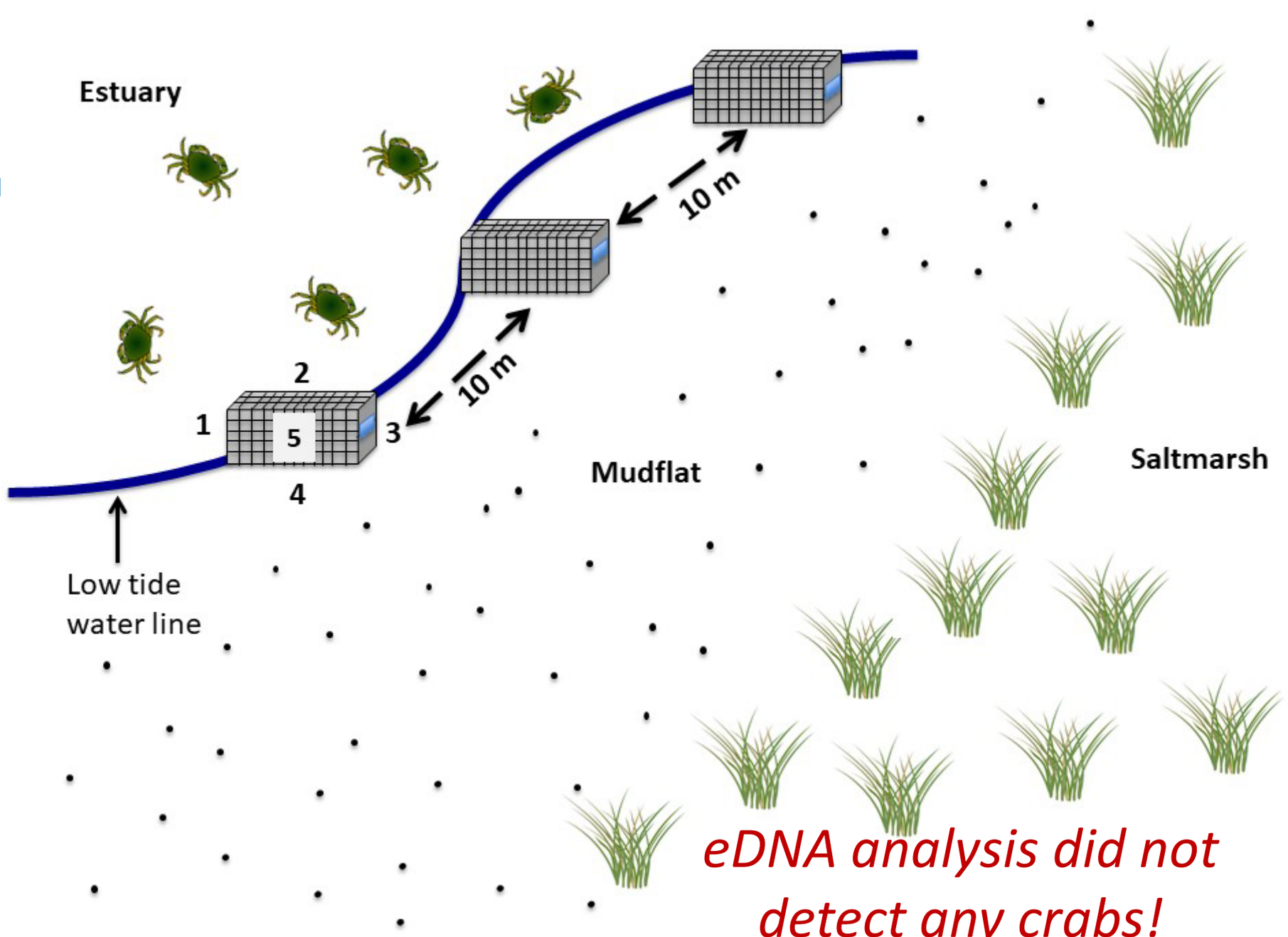
Summary Points:

Reserves have expressed interest in being able to detect invasive crabs early in order to mitigate effects or prepare for them as they move into new areas.

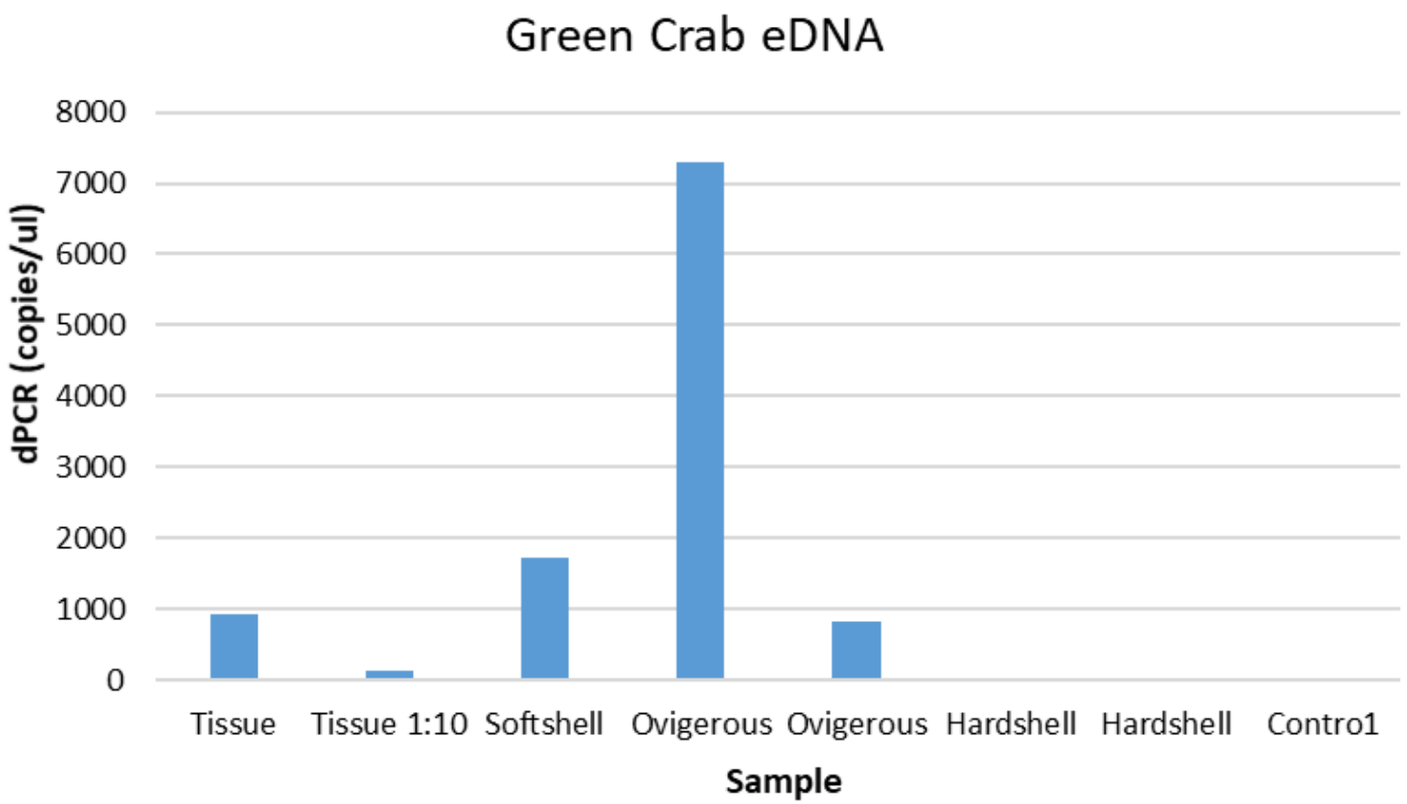


eDNA analysis did not detect any crabs!

Summary Points:

Sampling plan: Both Bree and Jason, at the Wells and South Slough reserves, respectively, deployed crab traps in the summer of 2018 and trapped a large quantity of crabs. They took samples of the sediment from within those traps, and did not detect crabs within the sediment. Baffled, they examined whether it was a fixable issue with the methods or primers.

Crab Experiment – Maine



Green crab DNA only detected from softshell and gravid crabs in tank experiment

Summary Points:

Later, Jason conducted a brief tank experiment at Wells reserve, in which he placed crabs in a series of tanks, left them in there for days, then pulled the water out and analyzed it for green crab DNA. For the graph shown, the tissue is a positive control to show the methodology worked as intended.

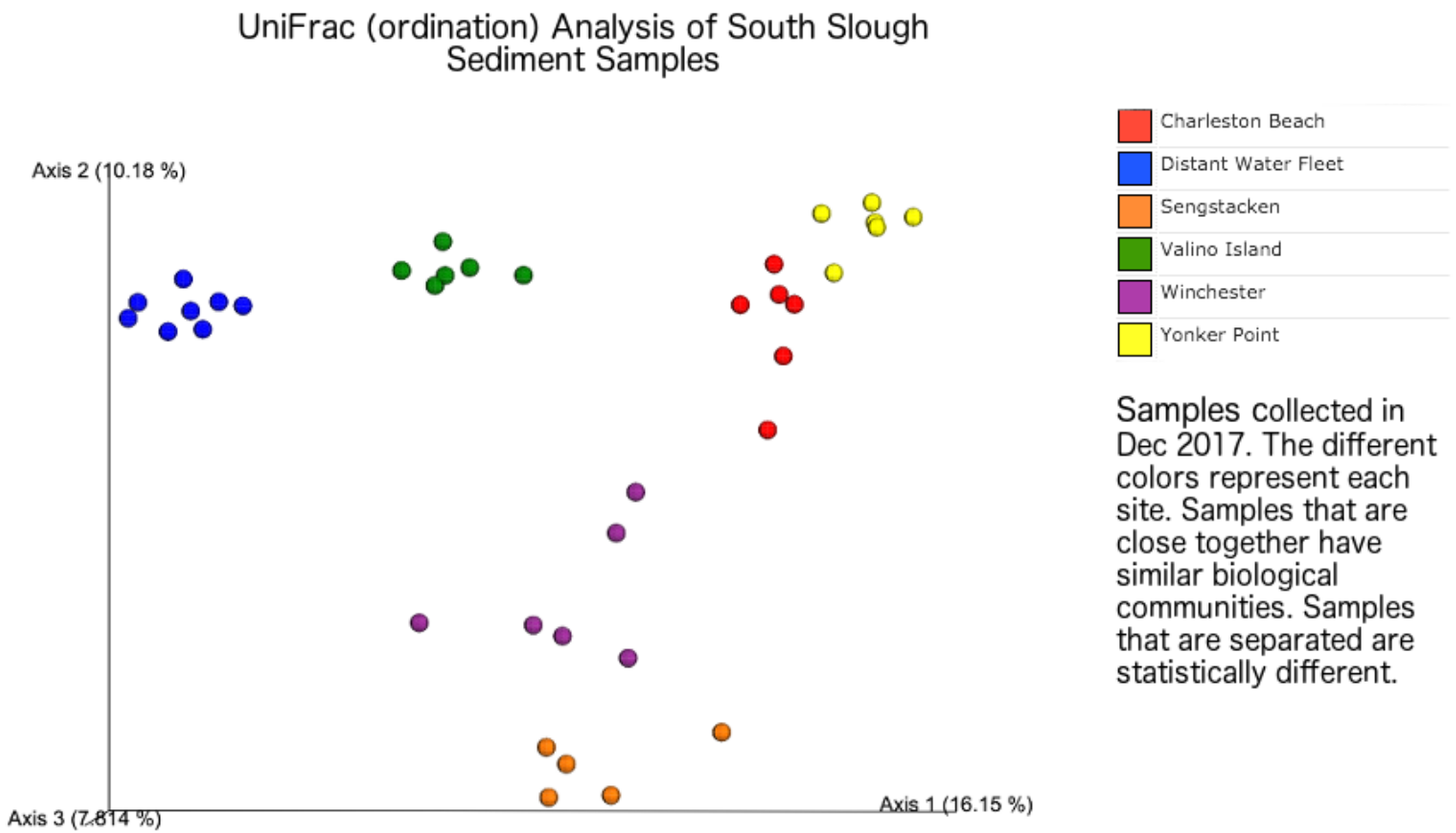
According to the results, the method was able to pick up DNA for softshell and ovigerous -- but not hardshell -- crabs.

These results raise an important reminder; namely, if an organism is not shedding DNA, DNA will not be detected.

The team would like to reproduce these results in continued experiments using larger sample sizes to determine whether there is a more appropriate time in their life cycle that green crab can be detected.

Biodiversity

Several hundred eukaryote species in each sample



Summary Points:

Shown here are the results from an 18S (broad eukaryote primer) analysis. These data are from South Slough; the researchers took the samples at the same time and each color represents a location. The analyses detected hundreds of species in each sample; community analysis shows that each location seems to have a distinct community.

Analyzing the biodiversity of these data over time could also allow the team to determine whether any correlation exists between different locations or stressors and biodiversity and site characteristics. This is an area that the team would like to explore in future work using frozen samples.

Lessons so far:

- Methods matter! How samples are collected, processed, interpreted will effect results.
- Contamination (primarily lab) is challenging.
- This is very interdisciplinary – biology, water quality, computer science, resource managers, communication.

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Next steps (summer 2019)

Still deciding, but possibly:

- Developing recommendations for fish surveys in estuaries – how many samples, volume, analysis
- More larval fish surveys – comparison between microscope, DNA from tow sample and DNA from water samples
- Find the #%* crabs!

estuaries eDNA

Poll Question

Which potential applications for eDNA seem most useful to you?

Poll Question 2: Which potential applications for eDNA seem most useful to you?

- To monitor adult or larval fish communities (20.37%)
- To study non-fish species in estuaries (3.70%)
- To assess biodiversity (44.44%)
- To track invasive species (18.52%)
- To assess populations of rare species (12.96%)

Questions?

Questions:

Is it possible to detect the planktonic stages of green crab larvae?
Yes, we haven't done this but there are some papers that do demonstrate success. Since the larvae are extracted directly, there wouldn't be the same problem with organisms not shedding DNA, however, because most crustacean larvae contain thin exoskeletons, it may be easier to detect eDNA from this life-stage compared with adults. Something we should think about testing.

Have you tried detecting plant life using eDNA methods?
Yes, the 18S primer that we mentioned detects a wide range of plants, although it doesn't do a great job of identifying different species. More specific primers can be developed to identify a group of plants of interest.

Here's a link to a study on pondweeds, but the analysis would be similar: https://news.mongabay.com/wildtech/2018/08/researchers-weed-out-a-way-to-identify-plants-using-environmental-dna/.

Check out the Barcode of Life website for potential primer pairs that allow species level resolution in plants:

- rbcL (RuBisCO large subunit, a plant gene) and matK (Maturase K, a plastid plant gene).
- trnH-psbA (non-coding spacer) and the internal transcribed spacer (ITS) region of nuclear ribosomal DNA.

eDNA Questions and Answers

- **How specific can eDNA be? Can it go up to subspecies?**
  - Yes, if there is a section of the DNA that can be isolated for a primer. People are also just starting to develop ways to start to look at population dynamics with eDNA as in this study on porpoises: https://royalsocietypublishing.org/doi/full/10.1098/rsos.180537
- **Have you determined a distance or radius of confidence from the point of sampling that would encapsulate the area of habitat that is being used? In other words, how could you get to a finer resolution for habitat association?**
  - No, we have not and that's a really important point. It probably varies with the tidal and transport characteristics at each site.
- **How does one see if an organism is in a Library?**
  - For MiFish, our database of reference sequences is made by mining the NCBI nucleotide database for Mitochondrial genomes and 12S sequences. Some of the 12S sequences may not contain the full region amplified by the MiFish primer though so, to be sure, we align the primers to the prospective reference in silico and make sure the MiFish region is actually present. Once we have the database built, it is a simple query to ask if a species is in the database or not. You can have a good idea just by searching for 12S or Mitochondrion on the nucleotide database website and seeing if there are any sequences.
- **With a positive hit on eDNA from a water column sample, do we have any idea how long tissues / cells / free-floating DNA may have been floating around in the water body?**
  - Most of the work we've seen suggests a day or so, with warm water and sunlight increasing degradation rates. There are some great studies in freshwater, but much less in marine systems.

Questions:

https://royalsocietypublishing.org/doi/full/10.1098/rsos.180537

estuaries eDNA

University of New Hampshire

National Estuarine Research Reserve System Science Collaborative

NOAA National Oceanic and Atmospheric Administration U.S. Department of Commerce

eDNA Questions and Answers

Questions:

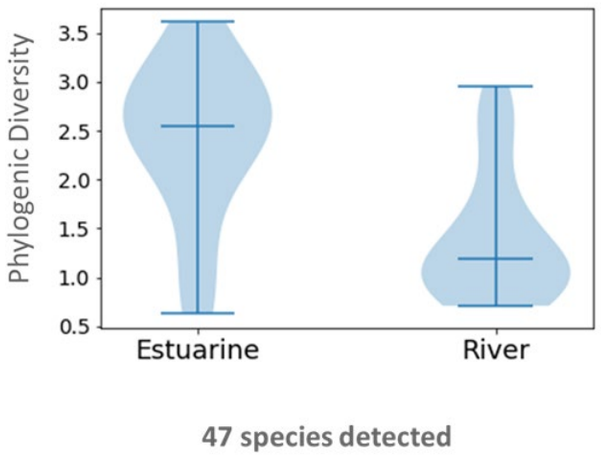
- **If you have an invasive species of zooplankton *Ctenophora*, could you find their presence using this method?**
  - There are 4 reference sequences for Ctenophora in the Silva database, so it is likely that you could detect it with 18S. You could also design a PCR probe that would detect it in a sample, but would need to develop a sampling plan (maybe plankton tows?) that would have a reasonable chance of intersecting the species.
- **For sampling below and above dam, when did you sample -- spring, summer?**
  - Mostly in the spring, as we were trying to identify the fish returns. So we collected samples 2-3 times a week May into June (more often would have been better, but we didn't have the capacity to handle a lot of samples at that point).
- **When you do the larval tows, how do you know you are not picking up adults?**
  - Great question! We did pick up some DNA from freshwater species, which may be from water upstream, or could have been contamination. We'll have a better idea as we process more samples. With the eDNA we presumably are picking up adults in the water samples, but we would expect most of the DNA to be from the larval fish because they are concentrated by the net and we are confirming the presence of those larval fish through traditional taxonomic methods.
- **Is metadata collection becoming standardized? If so, how?**
  - Both metadata and standardization are really important. Date, time and position are recorded for each sample. As the samples are processed in the lab, data like extraction concentration are recorded. Since there really aren't standardized methods, documenting what was done is crucial.

eDNA Questions and Answers

Questions:

- **How do you preserve the extracted eDNA until it's shipped to be sequenced? How do we deal with DNA degradation in eDNA samples?**
  - In this project we filter water samples within 24 hours, then freeze the filters until they're extracted. Extracts can be archived at -80 C for a long time (I'm not actually sure how long!). Ideally, samples would be field-filtered, flash-frozen with dry ice, then stored, but we found that wasn't realistic for our project. So there's always that tradeoff between optimal methods, and what's realistic for NERRS staff.

- **The anadromous fish work - a mix of freshwater and estuarine species. Was there a difference in above- and below-dam samples for eDNA? And did below-dam have all species, possibly from eDNA drift from freshwater upstream?**
  - Yes, the samples from below the dam contain both fresh and estuarine species, so there is a higher diversity there. Bearing in mind that the freshwater species are not actually living in the estuary, so in this case the DNA gives an inaccurate picture of diversity at the below dam sampling sites.



eDNA Questions and Answers

- **I'm surprised that the crabs don't shed DNA in their feces. Were they eating and defecating in the experiment?**
  - We're surprised too! Bear in mind that that was a really limited, short-term pilot, so it suggests that crabs don't shed a lot of DNA, buts it's absolutely not definitive. We plan to explore this more in the next year.
- **We are interested in monitoring water birds with eDNA. Would you have advice on bird primers?**
  - We haven't done any bird-specific monitoring although we do detect them sometimes with the primers we have. Ushio et al have a modified version of the MiFish 12S primer we use (called MiBird). We haven't tried it, but if it works let us know! https://www.nature.com/articles/s41598-018-22817-5
- **What primers did you use for the detection of hardshell crabs?**
  - We started with a CO1 primer designed for marine invertebrates (Lobo et al 2013). We also designed a dPCR probe that was validated on tissue extract (which is what we used in the graph I showed). Initially we thought it was an extraction or analysis problem, and we spent some time trying to optimize our sediment extraction and probe sensitivity. We use PowerSoil (for those who are interested), and we tried adding a metapolyzyme step to increase chiton breakdown, but it didn't help. Given the results of tank experiment, we think it's important to understand DNA shedding from crabs before we spend more time on lab methods. And if anyone else has ideas or suggestions we'd love to hear them!

Questions:

https://www.nature.com/articles/s41598-018-22817-5

Technical terms:

- **MetaPolyzyme**: A DNA isolation tool from Sigma-Aldrich consisting of a mixture of six enzymes.
- **dPCR**: Digital polymerase chain reaction; a more precise variant of PCR that uses thousands of fluorescence measurements instead of a single fluorescence measurement.

estuaries DNA

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eDNA Questions and Answers

Questions:

- **Do you think it's appropriate to compare traditional methods (seines, trawls, etc.) to eDNA methods? Seems as though they are completely different ways of measuring.**
  - Yes, they're different, and yes we still think it's appropriate. Traditional methods provide a baseline to understand how the methods differ. Most managers, and many regulations, rely on traditional methods for assessment, and it's important to understand how the methods compare. But you're right that it is really important to understand that the methods are fundamentally measuring different things, and we should not expect to see the same results.
- **Are you considering replication numbers and temporal frequency in your methods development?**
  - We usually collect three samples at each site (both water and sediment) but have collected up to six, and analyzed them all as separate extracts and pooled samples. We find, as others have, high variability in metabarcoding results from replicate samples. To be honest, some of this is probably part of our not being as rigid as we need to be in the lab (but we're getting better!), but part is real variability which is also seen in the much-more extensive freshwater literature. We hope to work on this more this year. For temporal sampling, South Slough collected samples at high and low tide, and we collected stream samples at the Great Bay dam sites over several weeks, but we haven't done estuarine time series. I'd love to, and I think it's really important, but we don't have the budget and capacity in this project.
- **Do you run PCR replicates independently? Do you have a threshold of presence to determine whether a species is present in a sample or not?**
  - At this point we're counting any detection as present, but are developing a 'confidence' scale, to differentiate detections that we're sure indicate a species is present, detections that are in the family but can't be resolved to species, detections that are likely due to contamination etc. With the metabarcoding we generally look for a sequence match above 98%, but in some cases even 99% is insufficient to distinguish very closely related species.

Thank you for joining us

Please complete the short survey at the end of the webinar