BOP Teacher Training Materials
Environmental DNA Curriculum Overview

Purpose: The goal of these protocols is to take students through a series of steps in environmental DNA analysis, from study design, through sample collection, all the way to data analysis and presentation of results.
*Where noted below, steps can be omitted or outsourced to reduce time in the field or classroom.

Learning goals

- Learn the importance of environmental DNA as a method and its utility in studying biodiversity in aquatic ecosystems.
- Create a hypothesis and design an eDNA study using principles of study design (including replication).
- Collect samples and associated environmental data, extract DNA and amplify 12S rRNA genes.
- Analyze biodiversity data generated from eDNA to address an ecological question
- Learn common issues and pitfalls associated with environmental DNA

Table of Contents

I. Background on environmental DNA.

Efforts to quantify marine biodiversity and predator-prey distributions depend on adequate monitoring, but traditional surveys are often time-intensive, expensive, and in some cases can be destructive to biological communities (Baldwin et al. 2006; Bogich et al. 2008). However, a new method allows us to detect organisms from environmental samples via their DNA, without ever encountering the organisms directly. Individuals can shed DNA by sloughing off skin cells, hair, fur, and secretions; the collection of all of these samples of DNA from organisms such as animals, bacteria, and viruses in a site comprises what is called environmental DNA (eDNA) (Shokralla et al. 2012; Rees et al. 2014). Samples of eDNA may be obtained from water, soil, or feces and analyzed using molecular techniques (e.g., qPCR). From these samples one may ascertain what various organisms inhabit the one area, travel through the area and during what time of the year, if there are any unknown organisms that have yet to be discovered or recorded, and, from feces samples, one may be able to tell not only what organisms inhabit the area, but which organisms are consuming them. This method has strong potential benefits over capture-based surveys in that it typically requires fewer person-hours, can be more cost-effective, is less harmful to native wildlife (e.g. Rees et al. 2014), and can capture information about species composition and biodiversity from each water sample, allowing unprecedented data on community assemblages. The DNA collected from water samples can be analyzed using metabarcoding to assess the full suite of vertebrate species in those samples.

Two types of eDNA analysis are currently being used: metabarcoding (detection of multiple taxa) and quantitative PCR analysis (single-species detection). The current protocols below focus on metabarcoding for student projects and education. Metabarcoding data can be used to investigate ecological questions such as 1) temporal and spatial distribution of the presence and relative abundance of taxa of interest; 2) estimates of co-occurrence between prey and predator species, including key predators of management or conservation interest; 3) new occurrences of invasive species, which can directly aid management efforts and contribute to our understanding of the rate of spread of particular species; 4) community assemblage data for aquatic ecosystems, which can be compared with data from traditional surveys and used in conjunction with environmental data to construct predictive habitat models.

Applications of eDNA are still being tested. So far it has primarily been applied to mesocosms (Kelly et al. 2014) and closed water systems such as ponds (Minamoto et al. 2012), and detection of invasive species (Jerde et al. 2012), but a growing number of studies have applied it to ocean samples as well (e.g. Closek et al 2019). As eDNA becomes more widely used, it is important to consider how eDNA may be transported by different bodies of water such as oceans, lake and rivers. For example, oceans and rivers tend to be more complex systems than lakes due to hydrological flow, which needs to be factored in when interpreting the data. For example, samples collected downstream in a river could potentially contain eDNA from a population upstream.

Recommended reading (and see additional references at the end of the document)
High-throughput sequencing for Diversity Assays

1. Tsuji, S., Takahara, T., Doi, H., Shibata, N., & Yamanaka, H. 2019. The detection of aquatic macroorganisms using environmental DNA analysis—A review of methods for collection, extraction, and detection. Environmental DNA, 1(2), 99-108.

2. Caporaso JG, et al. 2010. QIIME allows analysis of high-throughput community

sequencing data. Nat. Methods 7:335-336.
3. Caporaso JG, et al. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc. Natl. Acad. Sci. U.S.A. 108 Suppl 1:4516-4522.
4. Soergel DAW, Dey N, Knight R, Brenner SE. 2012. Selection of primers for optimal taxonomic classification of environmental 16S rRNA gene sequences. ISME Journal 6: 1440-1444.

Sample Design and Replication

5. Lennon JT. 2011. Replication, lies and lesser-known truths regarding experimental design in environmental microbiology. Environmental Microbiology 13:1383-1386.
6. Prosser JI. 2010. Replicate or lie. Environmental Microbiology 12:1806-1810.

II. Overview of Protocols and Materials

The following represents a breakdown of steps in a semester-long (~12-week) project (assuming an average of 3-4 hours per week). A shortened version of this curriculum can be achieved by omitting certain elements of field or lab work as noted below.

1. Introduce students to environmental DNA (reading, PPT1, Worksheet #1)
2. Students work in groups or individually to develop a study question and hypothesis. Students present study proposal to class (Proposal Rubric #1)
3. Field work: Students collect water samples and associated environmental data (Protocol: Water collection; Field data sheet, PPT2) *Could be omitted; in this case, students would be provided with filters (move to step 4); or data (move to step 6)
4. Lab work: Students extract DNA from filters and (optionally) amplify 12S gene (Protocol: DNA extraction and amplification, PPT3) *Could be omitted; in this case, students would be provided with data (move to step 6)
5. Samples are sent to sequencing facility (e.g. Genewiz Inc, BGI, etc).
6. Data analysis: Students use one or more freely available bioinformatic tools (including CSHL's DNA Subway, NCBI database, MEGA, Excel, R) to analyze biodiversity data from samples (depending on the nature of projects). [Optional for classes or students desiring additional computational practice: Students process raw data using QIIME2] (Protocol: Data analysis using NCBI and example data; Analysis of Biodiversity Data using Excel and R)
7. Project presentations: Students present results in groups or individually (Final project rubric)
8. Students complete Post-study Worksheet

Materials needed:

All software and website listed here are freely available online.

Fieldwork will require: 1L polypropylene Nalgene bottles (wide-mouth are best); filtration apparatus and pump (vacuum-capable if filtering larger (1L) volumes; alternatively syringe filters can be used); ziploc bags or falcon tubes for storing filters; 45mm glass fiber filters (if not using syringe filters); falcon tubes (sediment only); scoopulas (sediment only); bleach for sterilization; cooler with ice (if in the field)

DNA extraction will require: Ethanol, Bleach, Qiagen PowerWater kit (includes Bead tube, Collection tubes, Spin filter, Solutions PW-PW6) as well as standard molecular lab equipment including Microcentrifuge, Hotplate or incubator, Vortexer, Micropipettors and tips.

III. Pre-study Environmental DNA Student worksheet and discussion.

a) Instructors: Provide students with the following papers to read (see reference list below): Tsuji et al. (2019) and Ruppert et al. (2019). Selected questions could also be used as questions for a writing exercise.

b) Place students in groups of 2-4 to answer the following discussion questions:

1. What are the pros and cons of using eDNA to measure biodiversity compared with traditional sampling methods? How would these pros and cons apply, in particular, to the ecosystem you are studying (e.g. oyster reef)?
2. What are the major factors that affect how long eDNA persists in the environment? How might these differ between different kinds of aquatic ecosystems (e.g. rivers, lakes, ocean, bays)?
3. What factors shape how eDNA gets transported? How might this influence your interpretation of biodiversity data from samples collected in a certain locale?
4. Given your answer above, if you were designing an eDNA study, how might you organize your sampling to try to ensure that your data most accurately reflect the biodiversity present?
5. Why is it necessary to filter or centrifuge water samples for eDNA processing?
6. Which genes are most commonly used to analyze eDNA? Why? Does the gene of choice change depending on the taxonomic group targeted (see, e.g. Figure 6 in Tsuji et al. 2019)?

c) Have each group report back their answers for one of the questions and discuss. Ask other groups to contribute additional thoughts.

IV. Protocols

A. Sample collection: Collecting and filtering eDNA samples.

General

**All sampler identification information and other field data should be recorded on the Field Collection Summary sheet.
**If relevant, water samples must be collected prior to sediment samples at each site.
**If sampled in flowing water, sampling should proceed in an upstream diagonal direction. The direction traveled for sampling should be recorded on the Field Collection Summary sheet.
** Equipment must be decontaminated between samples by submerging/scrubbing all non-disposable pieces with a solution of water and 10% bleach.
**Samples/filters should be kept on ice (e.g. in a cooler with ice) until they are able to be frozen at -20C.

Materials needed: 1L polypropylene Nalgene bottles (wide-mouth are best); filtration apparatus and pump; ziploc bags or falcon tubes for storing filters; 45mm glass fiber filters; falcon tubes (sediment only); scoopulas (sediment only); bleach for sterilization; cooler with ice (if in the field)

Water

1. Sample labels should be affixed to the outside of sterile 1L bottles prior to going into the field. Bottles will be labeled with an appropriate ID that does not indicate location (to allow blind processing). We generally use a numbering scheme as follows: YYYYMMDD-SSS, where YYYY is the year (such as 2015), MM is the month (such as 11 for November), DD is the day (such as 13), and SSS is the sample ID number (such as 001, 002, 003, etc). An example for a sampling event scheduled for 11 June 2009 would be: 20090611-001, 20090611-002, etc. The SSS numbers will be consecutive starting from 001 and increasing to the maximum number of samples taken that day (999).
2. When arriving at a sample location, the lead sampler and sampling assistant 1 should put on sterile exam gloves (powderless latex or nitrile). REMINDER – Gloves must be changed before each new site to prevent cross contamination. The same gloves may be worn when collecting duplicate or blank samples in tandem with a regular sample in a transect.
3. Going in consecutive numerical order based on the bottle labels, the lead sampler will remove a labeled 1L sample bottle from the sample cooler. Just prior to collecting the sample, the lead sampler will unscrew and remove the lid from the sample bottle. The lead sampler will then reach over the upstream side with the 1L sample bottle and fill the bottle by skimming the surface of the field water.
4. Once the sample bottle is filled (approximately 1 in. of space should be left within the sample bottle), the lead sampler will screw the lid back on to the bottle until it is tight. The closed bottle will then be returned to the sample cooler.
5. While the lead sampler is collecting the water sample, sampling assistant will take habitat measurements: water temperature, approximate depth, GPS coordinates in Decimal Degrees, time of sample, location (e.g., east bank, center of transect), notes on habitat or other (eg over fine gravel) and record the information on the datasheet next to the appropriate sample ID.

6. Negative control samples should be taken at each site as follows: the sampler will unscrew the lid and remove to expose the bottle contents to the atmosphere for 5 sec, reseal the bottle, fully submerge the bottle in the field water, and return the bottle to the sample cooler from which it was removed. The lead sampler should relay to sampling assistant that the sample was a blank, so that it can be recorded on the data sheet next to the appropriate ID.
7. Once sampling is complete, ice should be added to the sample coolers as soon as possible. Enough ice should be added to each cooler to completely surround each sample bottle and maintain an inside temperature of 4.4°C. If at any time during transport the inside temperature of the cooler(s) rises above 4.4°C, additional ice should be added.

Filtration:
** Filtration equipment should be decontaminated between samples by submerging/scrubbing the filter frit, clamp, and open beaker (the three topmost pieces of the setup) with a solution of water and 10% bleach, then rinsing with deionized water. It is not necessary to dry equipment between samples.
**Before beginning, label clean and sterile falcon tubes, Ziploc bags or Powerwater bead tubes with the appropriate sample names.
**For water samples that contain a lot of algae or sediment, it may be necessary to filter 1L water in 2-3 batches in order to move all the sample volume through.
Set-up: Ensure equipment is sterilized via bleach cleaning or UV exposure. Attach the filter frit to the Ehrlenmeyer flask and attach the vacuum pump hose. Using sterile forceps, place a clean 45mm filter (glass fiber) on the filter frit. Check that it is centered properly before placing the beaker on top and then clamp the apparatus together using the metal clamp. Slowly pour the water sample into the open beaker and turn on the vacuum pump. Continue to allow a steady but slow stream of water into the beaker until the sample is done. Wait until the sample has flowed completely through the filter but do not allow the pump to continue running once the water has flowed through.
Use sterile forceps to remove the filter, fold it with the exposed surface on the inside, and place it in a clean and labeled Ziploc bag, falcon tube or a PowerWater bead tube. Clean the equipment and move on to the next sample.

Sediment

1. Sediment collections will be taken using either use Wildco sediment corers fitted with sterile, disposable plastic liners or sterile scoops/15-50mL Falcon tubes (depending on availability/depth of soft sediment). Sampler will use sterile gloves. Taking as much care as possible not to disturb surrounding sediment, the sampler will use a sterile 15-mL or 50-mL collection tube to scoop sediment from the top 2 cm of the sediment surface. Tubes should be labeled as above with an additional SED on the end of the sample name.
2. Tubes will be stored in their own sterile Ziploc bags before being placed into the cooler for transport back to the lab.
3. Negative control samples will be taken at each site, following the same protocol listed above for water samples: the sampler will unscrew the lid and remove to expose the bottle contents to the atmosphere for 5 sec, reseal the bottle, fully submerge the bottle in the field water, and return the bottle to the sample cooler from which it was removed. The lead sampler should relay to sampling assistant that the sample was a blank, so that it can be recorded on the data sheet next to the appropriate ID.

B. DNA extraction and amplification

The following DNA Extraction protocol requires the PowerWater (Qiagen) Kit and is modified for use with Glass Fiber Filters (Millipore). Students who have not been trained in using pipettors should first be taught to practice moving water from 1.5 mL tubes, and watch the following video demontrating good pipetting practice: https://www.youtube.com/watch?v=QGX490kuKjg.

NB: This is a modified protocol for use with larger glass fiber filters that otherwise soak up too much buffer and leave minimal supernatant.

Materials needed (per sample):

In PowerWater kit: 1 Bead tube, 5 Collection tubes, 1 Spin filter, Solutions PW-PW6
Microcentrifuge
Hotplate or incubator
Vortexer
Micropipettors and tips

Procedure:

1) Roll filter and place in bead tube using sterile forceps.
Warm PW1 and PW3 at 56 degrees C in hotplate or incubator until precipitates dissolve and the reagent appears totally clear.
2) Add 1.5mL PW1 to bead tube
3) Secure bead tube to vortexer and agitate at maximum speed for 10 minutes (
4) Centrifuge bead tubes at 4000 x g for 2 minutes
5) Transfer all supernatant to a collection tube
6) Centrifuge at 13,000 x g for 1 minute
7) Transfer supernatant to new collection tube
8) Add 200uL of PW2
   - Vortex to mix
9) Incubate at 4°C (fridge) for 5 minutes
10) Centrifuge at 13,000 x g for 1 minute
11) Transfer supernatant to new collection tube
12) Add 650uL of PW3
   - Vortex to mix
13) Load 650uL of mixture onto a spin filter
14) Centrifuge at 13,000 x g for 1 minute
15) Discard flow through
16) Repeat 13-15 until all of the mixture has been filtered
17) Place the spin filter into a new collection tube
18) Shake PW4 to mix it
19) Add 650 uL of PW4
20) Centrifuge at 13,000 x g for 1 minute
21) Discard flow through
22) Add 650uL of PW5

23) Centrifuge at 13,000 x g for 1 minute
24) Discard flow through
25) Centrifuge at 13,000 x g for 2 minutes
26) Place spin filter into a new collection tube
27) Add 100uL of PW6 to the filter membrane
28) Centrifuge at 13,000 x g for 1 minute
29) Discard filter and retain flow-through = purified extracted DNA

Use extracted DNA in amplification directly, or store between -20 to -80C for later use.

C. Data analysis using NCBI and example data.
Using Environmental DNA (eDNA) to assess biodiversity in NYC waterways

In this exercise, students will investigate the fish (and other vertebrate) biodiversity of an aquatic ecosystem or sampling site in New York City using environmental DNA (eDNA) data.
Each of the datasets represents a subset of sequences obtained from water samples from an aquatic site in NYC (including outer boroughs).

1. Select a dataset from Site #1-Site #5 (if you finish your selected site early, feel free to explore another).

2. Go to the following url: https://blast.ncbi.nlm.nih.gov/Blast.cgi
Select Nucleotide Blast.

3. Cut and paste one of your sequences in the box labeled "Enter accession number(s), gi(s), or FASTA sequence(s)".

3. Under "Choose Search Set" select "Others" for Database and leave the rest of the settings in the Default value.
This is the option for searching for nucleotide sequences with a nucleotide sequence, but other options (such as searching for translated sequences, searching within the human genome, or searching for really close matches quickly) are available.

5. Under "Program selection" select "Highly similar sequences (megablast)".

6. Click BLAST button and wait for results (usually several seconds to one minute)
Once the search is done, you can check out which sequences were found that generated significant alignments with your query sequence by scrolling down the page. You can also see the alignments with these sequences that the BLAST algorithm generated as well. There is a graphical representation (near the top of the results page) that shows where the various hits could be aligned with the query sequence and how good that alignment is.

The BLAST algorithm calculates similarity scores for local alignments (i.e., the most similar regions between 2 sequences) between the query sequence and subject sequences using specific

scoring matrices, and returns a table of the best matches ("hits") from the database. The hit table includes several useful pieces of information, including the similarity score, query coverage (percent of the query sequence that overlaps the subject sequence), E-value (see below), and max identity (percent similarity between the query and subject sequences over the length of the coverage area). The Graphic Summary displays all the results with how well that matched to the query sequence (your input), the length as well as the color indicate where and how the sequences align.

What is an E-value?
From the NCBI website: "The Expect value (E) is a parameter that describes the number of hits one can "expect" to see by chance when searching a database of a particular size. It decreases exponentially as the Score (S) of the match increases. Essentially, the E value describes the random background noise. For example, an E value of 1 assigned to a hit can be interpreted as meaning that in a database of the current size one might expect to see 1 match with a similar score simply by chance.
The lower the E-value, or the closer it is to zero, the more "significant" the match is. However, keep in mind that virtually identical short alignments have relatively high E values. This is because the calculation of the E value takes into account the length of the query sequence. These high E values make sense because shorter sequences have a higher probability of occurring in the database purely by chance. For more details please see the calculations in the BLAST Course.
The Expect value can also be used as a convenient way to create a significance threshold for reporting results. You can change the Expect value threshold on most BLAST search pages. When the Expect value is increased from the default value of 10, a larger list with more low-scoring hits can be reported."

How many hits did you get?
For each sequence results, scroll to the table of GenBank matches. Read through the top 10-20 matches.

a. What is the top identity of the species that is best matched with the unknown sequence? (Record the accession number – the last column listed in the table).
Look in particular at the E-value and the Ident columns.
b. Are there multiple species with the same scores for the E-value and Ident columns? What does this tell you?
c. For each record, click on the Accession Number to pull up the record for that sequence in Genbank. This record will tell you the organism that it was sequenced from, the publication or project it is associated with, and sometimes other relevant info like collection locale. Click on "Taxonomy Reports" if you want to learn more about the organism that matched the query sequence. Now, repeat the BLAST exercise for the eDNA sequences from your site in NYC!
d. Google the top hits for species to find out more about their habitats and environmental requirements.
e. Repeat for all the sequences from your site. Do you notice a difference in the number of exact matches in shorter versus longer sequences?
f. Once you have a list of potential species matches, make an educated guess about the location or habitat type that this water sample was taken from.

V. Post-study worksheet and discussion.

Instructors: Once students have wrapped up data analysis and/or final presentations, they can work individually or in groups to answer the following post-study questions:

1. How many taxa did you identify to the species level in your study? Genus? Family? [If time allows: compare your list of taxa against available Bioblitz data, iNaturalist data, or regional species checklists such as Waldman et al. 2016. Where are the commonalities, and where do the lists differ?]

2. If you had used different methods of bioinformatic identification, or a different reference database, do you think you might get different results? Why or why not?

3. In 1-2 sentences, summarize the central finding from your results. What do your results indicate about your ecosystem?

4. Did you detect sources of contamination in your results? What were they and from where do you think they might have come?

5. What other sources of error might be present in your data? How might those sources of error affect your interpretations of the data?

6. Briefly, describe one or more follow-up studies that you would design to further explore the results that you found.

VI. References.

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