# Developing a Pipeline for Environmental DNA Detection of Cnidarian Communities in New Jersey

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## Overview

Cnidarians are a critical part of New Jersey coastal ecosystems, serving as key predators and prey within the marine food web (e.g., Carman et al. 2017, Zarnoch et al. 2020). Several taxa, such as jellyfish, are increasing in number as they are tolerant to eutrophic conditions, respond favorably to increasing water temperatures associated with climate change, exploit anthropogenic habitat structures, and are easily moved into novel ecosystems as invasive species (Gaynor et al. 2016, Richardson et al. 2009). There is a growing need to characterize coastal habitats according to the cnidarians they support and the relationship between species composition and anthropogenic structures and stresses (e.g., presence of docks bulkheads; water temperature and nutrient content; DiBattista et al. 2020). There is also a need to evaluate the efficacy of management to suppress numbers of invasive and over-abundant cnidarians, and the response of native species to these restoration actions (Liu et al. 2020, Duarte et al. 2021).

Cnidarians present in the coastal waters of New Jersey typically have a bi-phasic life cycle, including a benthic and planktonic stage, making taxonomic identification of species difficult (Duarte et al. 2021). In addition, conventional surveys are relatively time consuming and expensive to mount given the range of species present, and their varied habitat associations (Darling 2015, Lopez-Escardo et al. 2018). These issues place real restrictions on the ability to sample for cnidarians across a large spatial scale and frequently through time, especially if there is a desire to capture the very cryptic and rare species that occupy any particular site (Darling et al. 2020a).

Environmental DNA (eDNA) overcomes these sampling limitations as it allows detection of species through the presence of their DNA within the water column rather than requiring an individual to be 'in hand' (Ponchon et al. 2013). All organisms release their DNA within their surrounding environment in the form of shed cells, mucus, saliva, and secretions (Ficetola et al. 2008). This eDNA is then present within the environment for days, weeks, or even years; depending on characteristics of the environment itself (Taberlet et al. 2012). In aquatic ecosystems, eDNA can typically be detected for about two weeks after initial deposition by an organism, with the primary factors influencing eDNA degradation being exposure to UV-B light, microbes, and high temperatures (Pochon et al. 2013). However, during that short time span, this shed DNA will diffuse through the water column or move with it via currents and tides making it broadly available for capture if that water is collected in a sample (e.g., 1L bottle; Barnes and Turner 2016). Using now-standard DNA sequencing or amplification techniques, followed by bioinformatics processing, this collected eDNA can be matched to the species from which it is derived (Gunther et al. 2018).

The value of eDNA surveys for detecting a range of aquatic organisms is now widely accepted, although its application for tracking composition of cnidarian communities is still somewhat rare (Gunther et al. 2018). The advantages to developing the capacity to deploy eDNA surveys for cnidarians in New Jersey lies in the ease with which water samples can be collected as compared to conventional sampling methodologies, and the streamlining of species identification

through the removal of the need for taxonomic experts and physical capture of the organisms themselves (Lopez-Escardo et al. 2018). Once an eDNA survey 'pipeline' is established, field surveys for cnidarians would simply involve collecting water samples and transporting these to an eDNA lab. Once at the lab, these water samples can be preserved or immediately processed. Typically, eDNA processing requires 1 to 4 weeks, with the output being a list of cnidarian species detected within each water sample (e.g., presence/absence). Some studies (Stoeckle et al. 2020, Andruszkiewicz et al. 2017) have also demonstrated that eDNA concentration is positively correlated with species abundance and/or biomass, which can serve as a means for quantification. This fast, cost-efficient and auditable eDNA survey process can substantially enhance the information base upon which management decisions are made (Darling et al. 2020a).

We sought to establish a cnidarian eDNA pipeline for the coastal ecosystems of New Jersey through pursuit of three objectives; (1) establishment of a DNA reference library of cnidarians found along New Jersey, (2) identification of a DNA region that can differentiate between the species in this library using only the eDNA fragments found in field water samples, and (3) establishment of a cost-effective method for extracting DNA from cnidarian tissues and from field water samples. This pipeline enables eDNA metabarcoding using high-throughput sequencing and associated bioinformatics.

Environmental DNA metabarcoding surveys perform optimally at species identification when the sequences obtained from field water samples are compared to a locally derived reference DNA library (Darling et al. 2020b). Although public databases continue to increase in the number of DNA sequences they house (e.g., GenBank), they typically do not have sufficient information to support solid species identification across all species in a particular geographical region (Stoeckle et al. 2020). This deficiency is particularly acute when considering more obscure groups such as cnidarians (Ponchon et al. 2013). Thus, we established the framework for, and begin to fill, a library of cnidarian DNA sequences from cnidarian tissue samples collected from New Jersey coastal waters.

The DNA region that we used to construct this library is of considerable importance as the region must be capable of differentiating between species, even when we can recover from water samples only a small number of DNA base pairs (Gunther et al. 2018). Other eDNA surveys use any one of a range of regions derived from either the mitochondria or ribosomes (e.g., Andruszkiewicz et al. 2017). We conducted a series of experiments that allowed us to choose the region of cnidarian DNA that best serves as the core of a pipeline and conducted initial tests of the efficacy of recovering small base pair segments of this region from water samples.

Finally, we established a cost-effective procedure for extracting high-quality DNA from cnidarian tissues, and from field water samples. Relatively large amounts of DNA must be recovered either from tissues or water samples to support high-throughput sequencing and associated bioinformatics (Ponchon et al. 2013). The amount of DNA extracted from either tissues or water samples can be strongly influenced by extraction or amplification inhibitors such as surface proteins (tissues) or sediment (water; Takahashi et al. 2020). We conducted a series of in-

lab experiments to deduce which of these factors are at play when extracting cnidarian DNA from environmental samples.

**Methods and Results**

*Pilot Sample Filtering*

To determine which filter materials and pore sizes would work well for eDNA sample collection, we conducted pilot testing of four filter types using representative water samples collected in May of 2021 by NJDEP staff. Three hundred milliliters of water from each of four water samples collected was thoroughly mixed and filtered using two replicates each of 0.45 µm PVDF, 0.6 µm PCTE, 1.0 µm PCTE, and 1.0 µm MCE filter membranes. DNA from these filters was then extracted using a DNeasy Blood and Tissue kit (Qiagen) per the manufacturer's instructions using minor modifications. We incubated all samples for 30 minutes at 95° C, during which time samples were vortexed every 10 minutes. The concentration of DNA in each extracted sample was quantified using a Qubit 2.0 fluorometer (Invitrogen, Thermo Fisher Scientific, Inc.). Accounting for the volume filtered using each filter type, the 0.45 µm PVDF filters had the lowest mean DNA yield (0.18 ng of DNA per milliliter filtered), and so we used a combination of 0.6 µm PCTE (mean: 0.65 ng/ml), 1.0 µm PCTE (mean: 0.41 ng/ml), and 1.0 µm MCE (mean: 0.33 ng/ml) filter membranes for the field eDNA samples collected in 2021.

*Sample Collection and Filtering*

From May 18$^{th}$ through October 20$^{th}$, 2021, NJDEP staff collected 104 1-Liter water samples (including one cooler blank each day) from three sites along coastal New Jersey: Metedeconk River North Bank (40.056196, -74.066545), Metedeconk River South Bank (40.050150, -74.065525), and a salt pond in North Wildwood, NJ (39.007336, -74.790497). Following collection, water samples were transported on ice to the Rutgers eDNA lab for filtration and preservation. The 104 water samples were filtered with two to three different types of filter membranes each (0.6 µm PCTE, 1.0 µm PCTE, and 1.0 µm MCE), initially using a peristaltic pump (Pegasus Alexis, Proactive Environmental Products), and then a vacuum manifold (MultiVac 6 Place Aluminum Manifold, Rocker Scientific Co.) to allow for more efficient filtration of the collected samples. The volume filtered through each filter membrane was recorded and each filter was placed in a 1.5 ml tube filled with 1 ml of 100% (200 proof) ethanol. Movement of filters was accomplished using forceps that were sterilized between each sample using a 10-minute soak in 10% bleach, rinsed with DI water three times, and then flame sterilized three times. The samples were then stored frozen at -20° C. Including the field blanks and additional equipment negatives, this filtering effort generated 251 filter samples, consisting of 105 1.0 µm PCTE filters (the default filter type used across all field samples), 80 0.6 µm PCTE filters (to evaluate a membrane with a smaller pore size), and 66 1.0 µm MCE filters (to evaluate a different membrane material). The average volume filtered for each filter type was as follows: 0.6 µm PCTE: 250 ml (range: 15-780 ml), 1.0 µm PCTE: 357 ml (range: 100-970 ml), and 1.0 µm MCE: 541 ml (range:

230-800 ml). The 1.0µm MCE membranes were able to filter down samples most quickly but were the most difficult to handle in terms of placing in the storage tubes and during trial DNA extractions because they are thicker, more rigid, and more absorbent than the PCTE membranes tested.

*Cnidarian and Ctenophore Specimen Collection*

Specimens collected and identified by NJDEP staff for DNA extraction and sequencing (Table 1) were placed in 100% ethanol (200 proof, non-denatured) and stored at room temperature, with one species per container. After one week the ethanol was decanted, and each container refilled with fresh 100% ethanol to maintain a high ethanol concentration in each container. The larger bodied specimens became considerably brittle over the storage period (~one year), and so it is recommended that any species identification based on physical characteristics be performed prior to ethanol preservation.

**Table 1:** Cnidarian and ctenophore specimens provided for DNA extraction and sequencing.

| **Species** | **Common name** | **Quantity** | **Collection Location** | **Collection Date** |
|---|---|---|---|---|
| *Beroe ovata* | Brown Comb Jelly | 1 | North Wildwood | 6/22/2021 |
| *Chrysaora chesapeakei* | Atlantic Bay Nettle | 1 | Metedeconk River | 7/13/2021 |
| *Gonionemus vertens* | Clinging Jellyfish | ~12 | Metedeconk River<br>North Wildwood Salt Pond | 5/18/2021<br>6/22/2021 |
| *Mnemiopsis leidyi* | Warty Comb Jelly | 1 | Barnegat Bay | 6/16/2021 |
| *Nemopsis bachei* | --- | 3 | Barnegat Bay | 6/16/2021 |
| Unidentified species | --- | 2 | North Wildwood | 6/22/2021 |

*Genetic Reference Library*

NJDEP staff provided a list of eight common and six "obscure" cnidarian and ctenophore species (Table 2), and during the summer of 2022, using the "Nucleotide Search" feature within the Geneious Prime (Biomatters, Ltd.) software package, we used a variety of search term combinations (common name/scientific name + genetic marker name/abbreviations) to locate representative nucleotide sequences for each target species and various genetic markers (16S, 18S, 28S, COI, and Cytb) within the genetic sequence database GenBank (https://www.ncbi.nlm.nih.gov/genbank/). We downloaded these nucleotide sequences and used them to generate a reference library for each of the target species. The resulting sequences were then sorted into a "local" reference database, consisting of those derived from specimens collected along New Jersey and nearby states (260 sequences), and a "global" reference database, consisting

of a subset of sequences from specimens collected elsewhere (1,670 sequences). The sequences derived from the supplied specimens were also added to the local reference library, and a combined reference library was created with the lab, local, and "global" sequences. Table 3 contains sequence counts for each of the species-genetic marker combinations in the local database. These reference libraries can be used for future metabarcoding efforts along COI (Cytochrome c Oxidase subunit I) and to some extent, the other genetic marker regions.

**Table 2:** Common and obscure cnidarian and ctenophore species targeted for sequence aggregation.

| Occurrence | Species | Common Name |
|---|---|---|
| **Common** | *Aequorea* spp. | Crystal Jellies |
| | *Beroe ovata* | Brown Comb Jelly |
| | *Chrysaora chesapeakei* (formerly *quinquecirrha*) | Atlantic Bay Nettle |
| | *Cyanea capillata* | Lion's Mane |
| | *Mnemiopsis leidyi* | Warty Comb Jelly |
| | *Nemopsis bachei* | --- |
| | *Pleurobrachia pileus* | Sea Gooseberry |
| | *Rhopilema verrilli* | Mushroom Cap Jellyfish |
| **Obscure** | *Cladonema* spp. | --- |
| | *Clytia linearis* | --- |
| | *Eutima* spp. | Medusa Jellies |
| | *Obelia bidentata* | Double Toothed Hydroid |
| | *Rathkea octopunctata* | --- |
| | *Sarsia tubulosa* | Clapper Hydroid |

**Table 3:** Reference sequence counts for each of the target species along five genetic marker regions in the local sequence library. Counts annotated with asterisks denote supplemental sequences added from other geographic areas (totals for other geographic areas shown in parentheses).

| | | Genetic Marker | | | | | |
|---|---|---|---|---|---|---|---|
| **Common Name** | **Species** | **16S** | **18S** | **28S** | **COI** | **Cytb** | **Totals** |
| Crystal Jellyfish | *Aequorea spp.* | 3 | - | 1 | 72* | - | 76 |
| Brown Comb Jelly | *Beroe ovata* | - | 2 | - | 2 | - | 4 |
| Lion's Mane | *Cyanea capillata* | - | - | - | 1 | - | 1 |
| Bay Nettle | *Chrysaora chesapeakei* | 43 | - | 1 | 19 | - | 63 |
| N/A | *Clytia linearis* | 1 | 1 | - | 1 | - | 3 |
| Atlantic Sea Nettle | *Chrysaora quinquecirrha* | 9 | 3 | 5 | 7 | - | 24 |
| N/A | *Cladonema spp.* | - | - | - | 11* | - | 11 |
| N/A | *Eutima spp.* | - | - | - | 32* | - | 32 |
| Clinging Jellyfish | *Gonionemus vertens* | 1 | - | - | 123 | - | 124 |
| Sea Walnut | *Mnemiopsis leidyi* | - | 2 | - | 2 | 14 | 18 |
| N/A | *Nemopsis bachei* | - | - | - | 11 | - | 11 |
| Doubletoothed Hydroid | *Obelia bidentata* | 1 | 1 | - | 1 | - | 3 |
| Sea Gooseberry | *Pleurobrachia pileus* | - | 1 | - | 1 | - | 2 |
| N/A | *Rathkea octopunctata* | - | - | - | 7* | - | 7 |
| Mushroom Jellyfish | *Rhopilema verrilli* | - | - | - | 3 | - | 3 |
| Clapper Hydroid | *Sarsia tubulosa* | - | - | - | 21* | - | 21 |
| *From global database | Totals | 58 | 10 | 7 | 314 (143*) | 14 | 403 (143*) |

We then evaluated these reference libraries to determine the completeness and number of representative sequences for each species of interest. Given the number of available COI sequences within the assembled library and the limited number of specimens available for DNA extraction and sequencing, we chose to concentrate our sequencing and primer selection efforts within the COI region. The resulting sequence files for the (1) local, (2) global, and (3) combined reference libraries can be found in the supplemental material as .fasta files.

*DNA extraction methodologies*

We tested two DNA extraction methodologies to address the unique challenges of extracting DNA from cnidarian tissue (high water content and containing gelatinous proteins) and assessed each method's resulting DNA concentration and our ability to amplify the extracted DNA via Polymerase Chain Reaction (PCR).

We performed a pilot comparison between HotSHOT (Truett et al. 2000) and spin-column (DNeasy Blood and Tissue, Qiagen) extraction methodologies for their ease of use and low cost. This comparison was conducted using replicate tissue samples (mean: 14.6 mg, range: 12-18 mg) believed to be from *Aurelia aurita* that were 1) extracted fresh (wet), 2) dried using a vacuum centrifuge, or 3) immersed in 100% ethanol for 24 hours and then dried using a vacuum centrifuge. Three replicates of each tissue sample treatment category were extracted using HotSHOT and the Qiagen kit. The HotSHOT protocol (see Truett et al. (2000) for reagent preparation) consisted of

adding 100 µl of Alkaline Lysis Reagent to each piece of tissue, vortexing and centrifuging briefly, incubating on a dry bath (Digital Dry Bath, Thermo Fisher Scientific) for 30 minutes at 95° C, adding 100 µl of Neutralization Reagent, and then vortexing and centrifuging briefly. The Qiagen extractions were performed using the manufacturer's suggested protocol with minor modifications: we incubated all samples for 30 minutes, during which time samples were vortexed thoroughly every 10 minutes. Following DNA extraction, we quantified the DNA concentration of each sample using a Qubit 2.0 fluorometer.

Across treatments and adjusting for starting wet mass, the Qiagen-extracted samples were quantified to contain conservatively more than five times the amount of DNA than the HotSHOT extracted samples across all tissue preparation treatments (Figure 1). In follow up PCR tests, the HotSHOT samples failed to produce any amplification, while a subset of the Qiagen-extracted samples did produce amplified DNA. While the resulting PCR products from this extraction comparison test were non-specific (determined to be bacteria, fungi, or algae via Sanger sequencing), these results suggest the HotSHOT-extracted samples contained PCR inhibitors. Interestingly, the freshly collected and Qiagen-extracted samples contained more DNA than the dried and Qiagen-extracted samples, which contained more DNA than the samples placed in ethanol for 24 hours and then dried and extracted. These results suggest cnidarian samples are best extracted fresh using a Qiagen (or similar) extraction methodology. However, samples preserved in ethanol and extracted with the Qiagen kit still yielded more DNA than those that were extracted via HotSHOT across tissue preparation treatments.

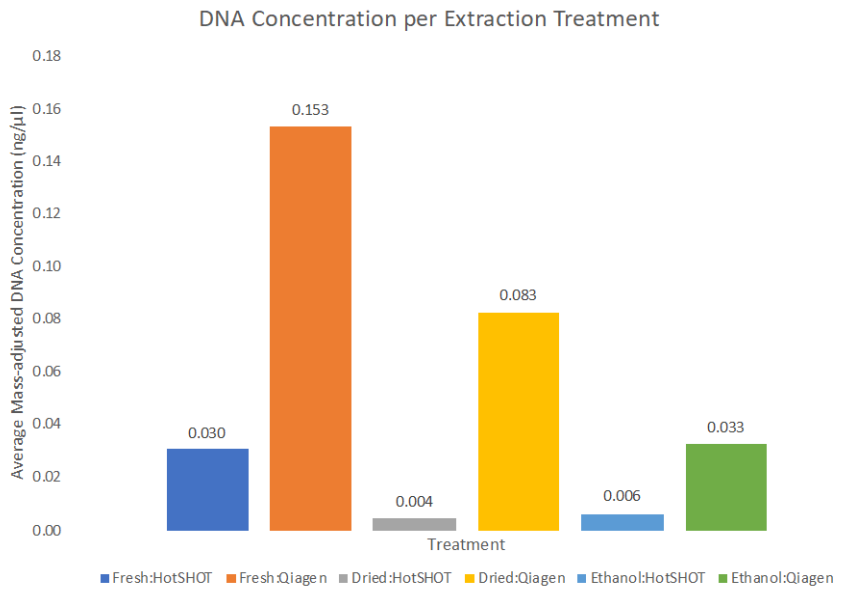



**Figure 1**: Average DNA concentration per extraction treatment, adjusted by starting wet tissue mass.

*Collected specimen tissue extractions*

Twenty-four tissue samples were collected from cnidarian and ctenophore specimens as above, but with a slightly larger starting mass than our pilot testing, when possible. Prior to DNA extraction, each specimen was removed from its storage container of 100% ethanol, rinsed briefly with DI water, and then a small piece of tissue (target: 0.3 cm or ~30 mg wet weight; actual mean weight: 29 mg, range: 4-59 mg) was removed using forceps and a scalpel that were cleaned using a 10 minute, 10% bleach soak, followed by a triple DI water rinse and triple flame sterilized. Between specimens, the forceps and scalpel were thoroughly cleaned with a bleach-soaked paper towel to remove any residual tissue before the sterilization process.

Each tissue sample was then placed in a 1.5 ml tube and dried thoroughly using a vacuum centrifuge (Vacufuge Plus, Eppendorf) set to 30° C for 30 minutes, to remove any residual water or ethanol (which inhibits the initial steps of the DNA extraction process). Samples were then extracted using a DNeasy Blood and Tissue kit (Qiagen) for PCR testing and sequencing purposes. We quantified the concentration from these extracted samples using a Qubit 2.0 fluorometer, and on average the 200 µl extraction volume contained 8.16 ng/µl of genomic DNA (range: 4.360 - 11.20 ng/µl). There was not a significant relationship between the starting amount of wet tissue and the extracted DNA concentration, likely due to differences in the tissue type used between samples, as the range of body sizes necessitated use of different tissue types between species. The samples provided did yield far more DNA per sample than those from the *Aurelia aurita* specimens in our extraction methodology comparison, even at comparable tissue masses, despite being preserved in ethanol and then dried prior to extraction. Given the relatively low concentration of DNA extracted from the *Aurelia aurita* specimens compared to the target species across extraction treatments, we suggest that the fresh vs. dried vs. ethanol tissue preparation comparison be repeated using other species of cnidarians that have been freshly collected. Based on the above results, we recommend the use of a spin column DNA extraction kit (such as the Qiagen DNeasy Blood and Tissue kit) for cnidarian tissue DNA extractions, whether the specimen is fresh or preserved in ethanol.

*Polymerase chain reaction (PCR) primer selection*

We conducted a brief literature search to identify appropriate primer sets for amplifying cnidarian and ctenophore DNA along the COI, 16S, and 18S marker regions based on genomic and eDNA studies. We tested the following primer sets (see Table 4) on the provided cnidarian and ctenophore tissue samples: LCOI1490F + HCO2198R (Folmer et al. 1994), 18S-4F + 18S-5RC (Machida and Knowlton 2012)), 16S U16F + 16S U16R (Bridge et al. 1995), nsCOIFO + mlCOIintK (Leray et al. 2013; Gunther et al. 2018), jgLCO1490 + jgHCO2198 (Geller et al. 2013), Jellyfish_CO1_F + Jellyfish_CO1_R2 (Minamoto et al. 2017), for the "Leray" Fragment of COI: mICOIintF-XT + jgHCO2198-XT (Wangensteen et al. 2018), and specifically for ctenophores:

F259Bfor + R1060Mod4 (*Beroe Ovata*) and MnLeLCO1490 + MnLeHCO2198 (*Mnemiopsis leidyi*) (Christianson et al. 2022).

**Table 4:** PCR primers used and their respective genetic marker regions.

| Primer Name | Marker | Forward Sequence | Reverse Sequence | Reference |
|---|---|---|---|---|
| u16F / u16R | 16S | TCGACTGTTTACCAAAAACATAGC | ACGGAATGAACTCAAATCATGTAAG | Bridge et al. 1995 |
| 18S-4F / 18S-5RC | 18S | AGGTCWGTRATGCCCTYMG | TGYACAAAGGBCAGGGACC | Machida and Knowlton 2012 |
| nsCOIFO/ mlCOIintK/ mlCOIintR | COI | THATRATNGGNGGNTTYGGNAAHTG | K: GGRGGRTAWACWGTTCAWCCWGTWCC<br>R: GGRGGRTASACSGTTCASCCSGTSCC* | Gunther et al. 2018<br>*Leray et al. 2013 |
| jgLCO1490 / jgHCO2198 | COI | TANACYTCNGGRTGNCCRAARAAYCA | TNTCNACNAAYCAYAARGAYATTGG | Geller et al. 2013 |
| LCOI1490F / HCO2198R | COI | GGTCAACAAATCATAAAGATATTGG | TAAACTTCAGGGTGACCAAAAAATCA | Folmer et al. 1994 |
| Jellyfish_CO1_F / Jellyfish_CO1_R2 | COI | KKTCAACAAAYCATAAAGATATWGG | GGAACTGCTATWATCATWGTWGC | Minamoto et al. 2017 |
| mICOIintF-XT / jgHCO2198-XT | COI | GGWACWRGWTGRACWTTTAYCCYCC | TATACYTCTGGRTGTCCRAARAAYCA | Wangensteen et al. 2018 |
| **Ctenphore-specific Primers** | | | | |
| MnLeLCO1490 / MnLeHCO2198 | COI | TTTCTACTAATCACAAAGATATAGC | TAAACTTCTGGATGGCCAAAAAATCA | Christianson et al. 2022 for *Mnemiopsis leidyi* |
| F259Bfor / R1060Mod4 | COI | GCTGATATGTGYCTTCCTAG | TWCCAGAYARRCCWCCAAAAGT | Christianson et al. 2022 for *Beroe ovata* |
| F259 / R1060 | COI | GCWGATATGTGTTTACCYMG | ACCRGAYARGCCACCAAAAGT | Christianson et al. 2022 for *Beroe ovata* |

All PCR tests were carried out using the AmpliTaq Gold DNA Polymerase Kit (Thermo Fisher Scientific), which allows the user to customize the concentration of each reagent when preparing a PCR master mix. This PCR kit was used with a variety of cycling parameters and reagent concentrations depending on the target marker, primer set, and specific application. All of the resulting PCR products were handled in a dedicated post-PCR lab, and amplified DNA was visualized using 1-1.5% agarose gel electrophoresis.

*Sanger sequencing*

Amplified PCR products were enzymatically cleaned using ExoSAP-IT (Thermo Fisher Scientific) per the manufacturer's protocol and prepared for bi-directional Sanger sequencing using separate forward and reverse reactions with the PCR primers and nuclease-free water. Samples were sent to an external lab for Sanger sequencing. The resulting sequences were manually trimmed, inspected for incorrect or ambiguous nucleotide base calls, and then the forward and reverse sequences were aligned using the Geneious software platform.

The results of each of our PCR trials and sequencing efforts can be found in the supplementary materials ("Cnidarian and Ctenophore PCR Log"). Overall (and with limited testing), we were unable to use the 16S and 18S primer sets to amplify cnidarian or ctenophore DNA, nor produce usable sequences. Given the availability of reference sequences and existing metazoan primer sets along the COI marker that have successfully amplified cnidarian DNA from "bulk" eDNA samples (Leray and Knowlton 2015; Wangensteen et al. 2018, etc.), we chose to focus our efforts on COI amplification and sequencing. We had early success with general "Folmer" COI primers using certain reagent concentrations and cycling parameters, but the most successful primer sets were derivatives of the Folmer primers that have been tailored to target cnidarians. The "Jellyfish" primers (Minamoto et al. 2017), produced the longest and highest quality sequences overall, and we also had success with the "jg" primers (Geller et al. 2013). Although they specifically target just the ~313 bp "Leray" fragment of COI, the "Leray-XT" primers (Wangensteen et al. 2018) also reliably produced high quality sequences from cnidarian samples.

*Ctenophore troubles*

We experienced difficulty amplifying and sequencing DNA from the two provided ctenophore specimens (*Mnemiopsis leidyi* and *Beroe ovata*). PCR trials using the aforementioned primer sets and a variety of cycling parameters (see supplemental "PCR Log") failed to amplify ctenophore DNA. During our experiments, we tried several PCR cycling parameters and reagent concentrations successfully employed by others for ctenophores (Song et al. 2011; Gunther et al. 2018; Christianson et al. 2022), and while these efforts produced some usable cnidarian sequences, they tended to use lower annealing temperatures or higher reagent concentrations, leading to non-specific amplification of bacteria, fungi, or algae from the ctenophore samples as determined by Sanger sequencing. We surmise these non-specific sequences are from organisms that were on, or in, the gut of the dissected ctenophores. We also added more template DNA to each PCR reaction or added bovine serum albumin (BSA) at two different levels (0.04 µl and 0.16 µl of 50 mg/ml BSA) to our PCR reactions in an attempt to enhance annealing of the primers to the target sequences but were unsuccessful in amplifying DNA from the two ctenophore species. Finally, we ordered primer sets that were specifically designed to amplify the COI region of *Mnemiopsis leidyi*

and *Beroe ovata* respectively (Christianson et al. 2022), but these primer sets also proved to be unsuccessful.

Although we extracted a high amount of DNA from the two ctenophore specimens (mean: 6.27 ng/µl), we chose to re-extract another batch of both cnidarian and ctenophore specimens for further testing. While the newly extracted cnidarian samples amplified well using the "jg" COI primer set, as before, the newly extracted ctenophore samples failed to amplify DNA. Future efforts should include redesigning primers for these species along a different portion of COI, trialing other species-specific primer sets (Pett et al. 2011), or increasing the concentration of $MgCl_2$ in each reaction, as our group has done this successfully in the past to enhance the amplification of difficult target sequences.

*Successful PCR Parameters*

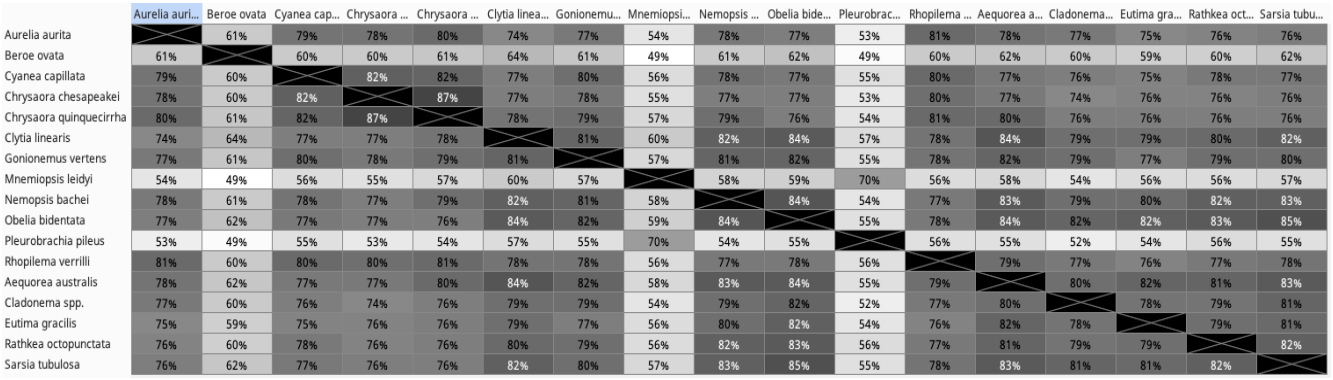
The following reagent concentrations and cycling parameters worked reliably well for the "Jellyfish" and "jg" primer sets for the cnidarians: 20 µl reaction volumes per sample containing 12.7 µl of nuclease-free water, 2 µl of PCR buffer, 0.3 µl of dNTPs, 0.4 µl of each primer (10 µM), 0.2 µl of Taq Polymerase, 2 µl of $MgCl_2$, and 2 µl of DNA template solution. The following PCR cycling conditions were used for these reactions: 96° C for 10 minutes, then 45 cycles of: {96° C for 30 seconds, 45° C for 40 seconds, and 72° C for 90 seconds}, followed by a final extension step of 72°C for 5 minutes, and then a 4°C hold. Using these methods, we were able were to successfully generate representative COI sequences for *Chrysaora chesapeakei, Gonionemus vertens, Nemopsis bachei,* and the "unidentified" specimens. The sequences generated from the three identified species were highly similar (97% or greater identity) to those in the local COI reference database, suggesting they were correctly identified. We were ultimately not able to generate sequences for the ctenophores provided (*Beroe ovata* and *Mnemiopsis leidyi*). Sanger sequencing of the "unidentified" specimens generated COI sequences that were 93% similar to *Aequorea australis*.

*Metabarcoding Primer selection and initial testing*

Segments of the 648 bp portion of COI, often referred to as the "Barcode of Life" (Herbert et al. 2003; Ratnasingham and Herbert 2007) are often used for metabarcoding efforts and may yield species-level taxonomic resolution with an appropriate sequence reference library. Our efforts yielded lab-generated sequences for 4/16 target species of cnidarians captured in New Jersey coastal waters, and local reference sequences for 11/16 species. By using sequences from other geographic locations for the remaining species (5/16), we have representative COI sequences for all cnidarian and ctenophore species of interest.

We investigated interspecific genetic variation to determine if COI could be used for species-level identification for a metabarcoding sequencing effort. Comparing the representative sequences for each species, the COI region provides good taxonomic resolution for COI overall

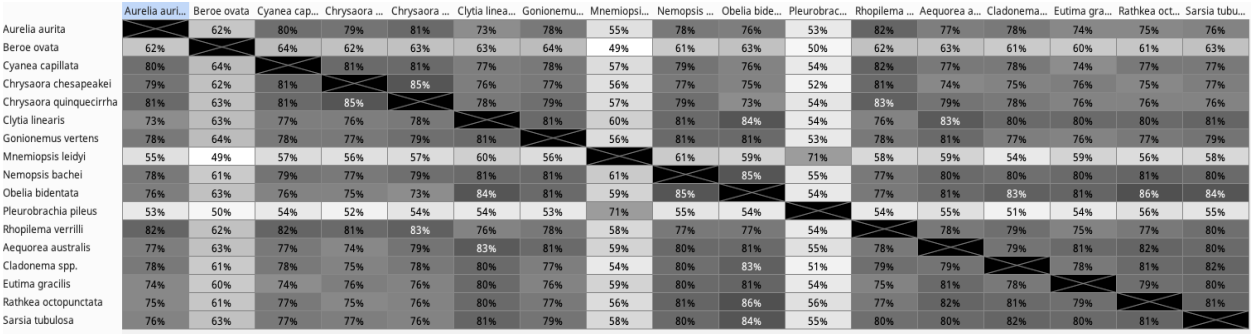
(maximum similarity: 85%) except for representative sequences from *Chrysaora chesapeakei* and *Chrysaora quinquecirrha*, which were recently identified as two separate species (Bayha et al. 2017) (Figure 2).



| | Aurelia auri... | Beroe ovata | Cyanea cap... | Chrysaora ... | Chrysaora ... | Clytia linea... | Gonionemu... | Mnemiopsi... | Nemopsis ... | Obelia bide... | Pleurobrac... | Rhopilema ... | Aequorea a... | Cladonema... | Eutima gra... | Rathkea oct... | Sarsia tubu... |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Aurelia aurita | | 61% | 79% | 78% | 80% | 74% | 77% | 54% | 78% | 77% | 53% | 81% | 78% | 77% | 75% | 76% | 76% |
| Beroe ovata | 61% | | 60% | 60% | 61% | 64% | 61% | 49% | 61% | 62% | 49% | 60% | 62% | 60% | 59% | 60% | 62% |
| Cyanea capillata | 79% | 60% | | 82% | 82% | 77% | 80% | 56% | 78% | 77% | 55% | 80% | 77% | 76% | 75% | 78% | 77% |
| Chrysaora chesapeakei | 78% | 60% | 82% | | 87% | 77% | 78% | 55% | 77% | 77% | 53% | 80% | 77% | 74% | 76% | 76% | 76% |
| Chrysaora quinquecirrha | 80% | 61% | 82% | 87% | | 78% | 79% | 57% | 79% | 76% | 54% | 81% | 80% | 76% | 76% | 76% | 76% |
| Clytia linearis | 74% | 64% | 77% | 77% | 78% | | 81% | 60% | 82% | 84% | 57% | 78% | 84% | 79% | 79% | 80% | 82% |
| Gonionemus vertens | 77% | 61% | 80% | 78% | 79% | 81% | | 57% | 81% | 82% | 55% | 78% | 82% | 79% | 77% | 79% | 80% |
| Mnemiopsis leidyi | 54% | 49% | 56% | 55% | 57% | 60% | 57% | | 58% | 59% | 70% | 56% | 58% | 54% | 56% | 56% | 57% |
| Nemopsis bachei | 78% | 61% | 78% | 77% | 79% | 82% | 81% | 58% | | 84% | 54% | 77% | 83% | 79% | 80% | 82% | 83% |
| Obelia bidentata | 77% | 62% | 77% | 77% | 76% | 84% | 82% | 59% | 84% | | 55% | 78% | 84% | 82% | 82% | 83% | 85% |
| Pleurobrachia pileus | 53% | 49% | 55% | 53% | 54% | 57% | 55% | 70% | 54% | 55% | | 56% | 55% | 52% | 54% | 56% | 55% |
| Rhopilema verrilli | 81% | 60% | 80% | 80% | 81% | 78% | 78% | 56% | 77% | 78% | 56% | | 79% | 77% | 76% | 77% | 78% |
| Aequorea australis | 78% | 62% | 77% | 77% | 80% | 84% | 82% | 58% | 83% | 84% | 55% | 79% | | 80% | 82% | 81% | 83% |
| Cladonema spp. | 77% | 60% | 76% | 74% | 76% | 79% | 79% | 54% | 79% | 82% | 52% | 77% | 80% | | 78% | 79% | 81% |
| Eutima gracilis | 75% | 59% | 75% | 76% | 76% | 79% | 77% | 56% | 80% | 82% | 54% | 76% | 82% | 78% | | 79% | 81% |
| Rathkea octopunctata | 76% | 60% | 78% | 76% | 76% | 80% | 79% | 56% | 82% | 83% | 56% | 77% | 81% | 79% | 79% | | 82% |
| Sarsia tubulosa | 76% | 62% | 77% | 76% | 76% | 82% | 80% | 57% | 83% | 85% | 55% | 78% | 83% | 81% | 81% | 82% | |

**Figure 2:** Percent similarity of representative COI sequences between target cnidarian and ctenophore species.

Given the availability of COI sequences for the target species and a reliable process for extracting and sequencing tissue samples from cnidarian specimens, we suggest the use of a COI primer set for future metabarcoding efforts. The "Leray" fragment is a ~313 bp segment of COI that has been demonstrated to perform well across metazoan diversity for metabarcoding efforts (Leray et al. 2013), and more recently, Wangensteen et al. (2018) have improved the universality of the Leray primer set (designing "Leray-XT" primers). Along the Leray fragment for each of the target species, the closest similarity is 86% (Figure 3), meaning that setting the identity threshold at 90% or higher for sequences resulting from a metabarcoding sample run would yield species-level resolution for the target species. To date, the Leray primer set and its derivatives have been successfully used to detect cnidarian species in "bulk" eDNA samples (Leray and Knowlton 2015; Wangensteen et al. 2018) and from a variety of water eDNA samples in tropical settings (Nichols et al. 2022). However, our results suggest that using the Leray-XT primer set as currently designed would not amplify the two target ctenophore species, as a PCR using the Leray-XT primer set and DNA extracted from ctenophore tissue samples resulted in no amplification of the target. Along the Leray fragment sequences for *Beroe ovata*, we identified a 6 bp insertion along the forward primer binding location, which is likely contributing to our difficulty amplifying *Beroe ovata* DNA using the Leray-XT primer set. However, the available COI reference sequences for *Mnemiopsis leidyi* do not have this insertion, but also failed to amplify using the Leray-XT primer set.



| | Aurelia auri... | Beroe ovata | Cyanea cap... | Chrysaora ... | Chrysaora ... | Clytia linea... | Gonionemu... | Mnemiopsi... | Nemopsis ... | Obelia bide... | Pleurobrac... | Rhopilema ... | Aequorea a... | Cladonema... | Eutima gra... | Rathkea oct... | Sarsia tubu... |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Aurelia aurita | | 62% | 80% | 79% | 81% | 73% | 78% | 55% | 78% | 76% | 53% | 82% | 77% | 78% | 74% | 75% | 76% |
| Beroe ovata | 62% | | 64% | 62% | 63% | 63% | 64% | 49% | 61% | 63% | 50% | 62% | 63% | 61% | 60% | 61% | 63% |
| Cyanea capillata | 80% | 64% | | 81% | 81% | 77% | 78% | 57% | 79% | 76% | 54% | 82% | 77% | 78% | 74% | 77% | 77% |
| Chrysaora chesapeakei | 79% | 62% | 81% | | 85% | 76% | 77% | 56% | 77% | 75% | 52% | 81% | 74% | 75% | 76% | 75% | 77% |
| Chrysaora quinquecirrha | 81% | 63% | 81% | 85% | | 78% | 79% | 57% | 79% | 73% | 54% | 83% | 79% | 78% | 76% | 76% | 76% |
| Clytia linearis | 73% | 63% | 77% | 76% | 78% | | 81% | 60% | 81% | 84% | 54% | 76% | 83% | 80% | 80% | 80% | 81% |
| Gonionemus vertens | 78% | 64% | 78% | 77% | 79% | 81% | | 56% | 81% | 81% | 53% | 78% | 81% | 77% | 76% | 77% | 79% |
| Mnemiopsis leidyi | 55% | 49% | 57% | 56% | 57% | 60% | 56% | | 61% | 59% | 71% | 58% | 59% | 54% | 59% | 56% | 58% |
| Nemopsis bachei | 78% | 61% | 79% | 77% | 79% | 81% | 81% | 61% | | 85% | 55% | 77% | 80% | 80% | 80% | 81% | 80% |
| Obelia bidentata | 76% | 63% | 76% | 75% | 73% | 84% | 81% | 59% | 85% | | 54% | 77% | 81% | 83% | 81% | 86% | 84% |
| Pleurobrachia pileus | 53% | 50% | 54% | 52% | 54% | 54% | 53% | 71% | 55% | 54% | | 54% | 55% | 51% | 54% | 56% | 55% |
| Rhopilema verrilli | 82% | 62% | 82% | 81% | 83% | 76% | 78% | 58% | 77% | 77% | 54% | | 78% | 79% | 75% | 77% | 80% |
| Aequorea australis | 77% | 63% | 77% | 74% | 79% | 83% | 81% | 59% | 80% | 81% | 55% | 78% | | 79% | 81% | 82% | 80% |
| Cladonema spp. | 78% | 61% | 78% | 75% | 78% | 80% | 77% | 54% | 80% | 83% | 51% | 79% | 79% | | 78% | 81% | 82% |
| Eutima gracilis | 74% | 60% | 74% | 76% | 76% | 80% | 76% | 59% | 80% | 81% | 54% | 75% | 81% | 78% | | 79% | 80% |
| Rathkea octopunctata | 75% | 61% | 77% | 75% | 76% | 80% | 77% | 56% | 81% | 86% | 56% | 77% | 82% | 81% | 79% | | 81% |
| Sarsia tubulosa | 76% | 63% | 77% | 77% | 76% | 81% | 79% | 58% | 80% | 84% | 55% | 80% | 80% | 82% | 80% | 81% | |

**Figure 3:** Percent similarity of representative Leray-XT fragment sequences between target cnidarian and ctenophore species.

To further investigate whether use of the Leray fragment would yield species-level assignments for sequences produced from a metabarcoding effort, we conducted a Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) search of the ~313 bp "Leray fragment" portion of the COI region for each of the target species, as one would expect a metabarcoding sequencing effort could yield sequences similar to those of the target species from other organisms present in ocean water. The following target species' sequences also yielded BLAST results with >95% similarity to the target species, meaning that during a bioinformatics analysis, assignment to the species level may be inconclusive:

- *Cyanea capillata*: *Cyanea lamarckii* (95.31%)
- *Sarsia tubulosa*: *Sarsia princeps* (95.31%): *Sarsia lovenii* (95%)
- *Chrysaora chesapeakei*: *Chrysaora quinquecirrha* (99.69%)

For these species, identification could be inferred based on location (when appropriate) or by a detailed look at the resulting sequences from a metabarcoding effort. It should be noted that utilizing another portion of the COI marker region would not improve taxonomic resolution, as these three species pairings have a ~95% or greater similarity along the entire COI region. Other marker regions could be tested for a metabarcoding effort to detect cnidarians and ctenophores and have been used by others (e.g., 18S: Laroche et al. [2020]), but representative specimens would need to be sequenced to complete the reference library for these markers.

One large question that remains is whether the eDNA "signal" from cnidarians and ctenophores would be proportionally small compared to that of other organisms in coastal water samples. Given that rare species are sometimes not well represented in sequence lists generated by metabarcoding efforts, a COI fragment primer set that more specifically targets cnidarians and ctenophores may be appropriate. Should the relative quantity of cnidarian DNA in environmental samples prove to be low, other methods, such as autonomous reef monitoring structures (ARMS) or surface sampling could be used to create "bulk" eDNA samples from areas of interest to help

"concentrate" target organisms for identification via eDNA metabarcoding (Leray and Knowlton 2015).

*eDNA Filter Testing*

We selected 26 eDNA filters generated from the water samples collected in 2021 and extracted them using the Qiagen DNeasy Blood and Tissue kit following the manufacturer's suggested protocol with minor modifications: all filter samples were incubated for 30 minutes, during which time samples were vortexed every 10 minutes. The extracted DNA samples were then measured for DNA concentration using a Qubit 2.0 fluorometer. While the purpose of these extractions was to test the suitability of the Leray-XT primer set and not directly compare DNA yield from the three filter types used, we note that the 0.6 µm PCTE filters yielded the highest mean DNA concentration (5.73 ng/µl), with the 1.0 µm MCE (mean: 4.90 ng/µl) and 1.0 µm PCTE (mean: 4.84 ng/µl) filters yielding slightly less DNA. Accounting for the volume filtered, the mean DNA concentration per milliliter of water filtered was as follows: 0.6 µm PCTE (2.80 ng/ml), 1.0 µm PCTE (2.03 ng/ml), and 1.0 µm MCE (1.15 ng/ml) (Figure 4). Given the DNA yield from this small subset of filters, it appears that the PCTE filters of both pore sizes more effectively captured DNA from these water samples, although formal testing with a larger sample size and from matching field samples is suggested.

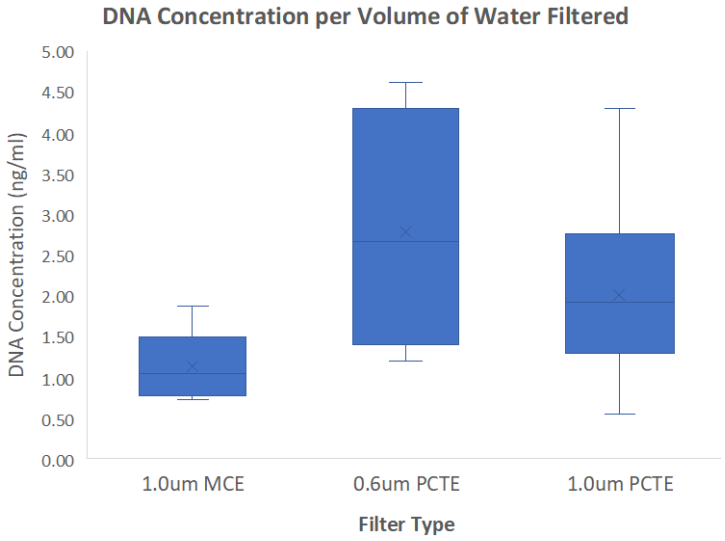



**Figure 4:** Plot showing DNA concentration from extracted filter samples per milliliter of water filtered.

We then conducted a series of test PCR runs based on the parameters used in Wangensteen et al. (2018): 20 µl reaction volumes with 12.5 µl of nuclease-free water, 2 µl of PCR buffer, 0.3 µl of dNTPs, 0.5 µl of each primer (10 µM), 0.2 µl of Taq Polymerase, 2 µl of $MgCl_2$, and 2 µl of

DNA template solution. The following PCR cycling conditions were used for these reactions: 96°C for 10 minutes, then 45 cycles of: {96° C for 1 minute, 45° C for 1 minute, and 72° C for 1 minute}, followed by a final extension step of 72° C for 5 minutes, and then a 4° C hold. We ran our PCR reactions for the extracted filter samples along with a positive control of extracted *Chrysaora chesapeakei* DNA. We conducted an initial PCR run, consisting of 12 field samples, the positive control, and an extracted filter sample that was created by filtering seawater used to transport *Gonionemus vertens* specimens to the Rutgers eDNA lab. Only the *Chrysaora chesapeakei* positive control and one out of twelve field samples in this initial test generated a faint, visible band. The DNA amplified in the field sample was sequenced via bi-directional Sanger sequencing and generated sequences with an 85% similarity to a species of dinoflagellate (*Heterocapsa circularisquama*). Given that Sanger sequencing of an eDNA sample using a general primer set may amplify sequences from any number of species present in an eDNA sample, we did not expect to amplify cnidarian DNA per se, but this does demonstrate that eDNA can be amplified from these field samples using the Leray-XT primer set. However, given that the extracted filter samples each contained a measurable amount of DNA (mean: 4.99 ng/µl, range: 2.82 – 7.68 ng/µl) and only 1 of 12 samples demonstrated amplification using the Leray-XT primer set, these results suggest the presence of PCR inhibitors present in the field samples.

*PCR Inhibitor Testing*

Given the lack of amplification in these 12 field test samples and the *Gonionemus vertens* transport water, we conducted another PCR with the same cycling parameters and reagent concentrations, but with multiple PCR replicates per sample (up to six) and "spiked" four field samples (which did not exhibit amplification during the previous PCR experiment) with DNA extracted from *Chrysaora chesapeakei* to test for the presence of PCR inhibitors. We then pooled the PCR replicates for each sample by combining all three reaction volumes into a single tube. These cnidarian-spiked field samples demonstrated amplification for two of the four samples, however the two samples that did amplify showed much fainter bands than the positive control samples with the same amount of *Chrysaora chesapeakei* DNA. These results further suggest that there are fairly high levels of PCR inhibitors present in the extracted field samples.

We conducted a follow-up PCR with multiple PCR replicates of the remaining, extracted field samples using the same cycling parameters and reagent concentrations, but with the addition of BSA (which is thought to enhance the PCR reaction in the presence of inhibitors [e.g., Plante et al. 2010]). However, this PCR did not yield defined bands of the correct size, but rather smears of approximately 600-1,100 base pairs, suggesting the future trials should reduce the amount of BSA used, or that the PCR annealing temperature should be increased.

## Summary and Future Steps

We identified a cost-effective extraction methodology that results in high quality genomic cnidarian DNA that can be readily sequenced. DNA quantity was assessed using a Qubit 2.0 fluorometer, and DNA quality was gauged by our ability to successfully amplify regions of interest using PCR and gel electrophoresis. Our results suggest use of the Leray-XT primer set as the basis for an eDNA cnidarian metabarcoding pipeline, with the caveat that ctenophore species are unlikely to be detected using this primer set. While some genetic regions may be readily amplified from genomic DNA extracted from tissue samples, regions that persist in the environment can be more difficult to isolate and amplify. We found that water samples taken from New Jersey coastal waters likely had a high level of PCR inhibitors. We aggregated available sequence data from cnidarians likely to be found in New Jersey waters and sequenced specimens collected and identified by NJDEP personnel via Sanger sequencing. This database includes 16 cnidarian species likely found in New Jersey coastal waters and exhibits a strong ability to differentiate between species. This reference DNA library should be augmented through the continued collection and sequencing of local cnidarian species, especially those that are rare, but can now serve as the basis of a cnidarian eDNA pipeline.

Future work could include spike testing of field samples with BSA (at varying concentrations) included in the PCR master mix. Additionally, our results suggest either the use of a DNA extraction kit with additional inhibitor removal steps (such as the Qiagen Powersoil DNA extraction kits) for field samples or field samples could be treated post-extraction using a bead clean up or commercially available inhibitor removal kit (e.g., Zymo OneStep PCR Inhibitor Removal Kit) to better allow for the amplification of target DNA in field samples. Our group has had success with such post-extraction clean up kits for samples that had previously demonstrated no amplification during spike testing. Post inhibitor removal, we suggest testing sets of field samples spiked with several different concentrations of extracted cnidarian DNA to ensure concentrations of DNA expected from the target species in field samples can be successfully amplified. Other commercially available PCR master mixes that are designed to amplify DNA in the presence of environmental PCR inhibitors may also prove successful.

Future efforts towards realizing the goal of developing a pipeline for eDNA detection of cnidarian communities may include:

- Testing various inhibitor treatments for field samples.
- Quantifying the relative quantities of cnidarian DNA in estuarine and ocean samples.
- Collection and sequencing of more tissue samples from cnidarians and ctenophores found in New Jersey coastal waters to ensure correct taxonomic assignment of sequences.
- Exploring the use of surface water sampling using polyethersulfone (PES) filters, which have been demonstrated to successfully filter larger volumes of water than the filter materials tested here.

- Developing other ctenophore-specific primers based on the available genetic sequences, or collaborating with laboratories that have experience amplifying and sequencing ctenophore DNA from the target species.
- Developing custom cnidarian/ctenophore primer sets that target shorter fragments while still maintaining species resolution, as these shorter fragments may persist for longer periods of time within the environment.
- Testing metabarcoding primer sets for PCR primer bias using synthetic DNA sequences derived from the reference library.
- Developing qPCR assays or qPCR assay panels for specific cnidarian species of interest, should cnidarian and ctenophore DNA prove to be difficult to detect using metabarcoding methods due to low concentrations in water samples. ARMS or surface "bulk" eDNA sampling could also be used to characterize a subset of the cnidarian community.

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