

**TESTING ENVIRONMENTAL DNA TECHNIQUES TO ASSESS AMERICAN
EEL POPULATIONS IN THE BRONX RIVER**

A Final Report of the Tibor T. Polgar Fellowship Program

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ABSTRACT

The American eel (*Anguilla rostrata*) is a species of conservation concern, as it has declined drastically in abundance along the U.S. east coast, largely due to human habitat perturbations. Eels represent an integral part of the ecology of the Bronx River, a stream that flows through Westchester and Bronx counties before entering the Hudson Estuary. This case study developed the first genetic probes to detect eels using environmental DNA (eDNA). Water and sediment samples were compared for eDNA concentrations; it was found that the sediments samples provided higher DNA concentrations. Real time qPCR (RT-qPCR) was compared to standard PCR and was found to be a more sensitive technique for detecting low concentrations of eDNA. eDNA can be used in conjunction with RT- qPCR to identify eel habitats and conserve this population, as well as other rare, threatened, or endangered species. These techniques could potentially be used for earlier detection of invasive species, which would allow for better preventative and protective measures against them.

TABLE OF CONTENTS

Abstract	I-2
Table of Contents	I-3
Lists of Figures and Tables	I-4
Introduction.....	I-5
Methods.....	I-8
Results.....	I-15
Discussion.....	I-20
Acknowledgements.....	I-21
Literature Cited	I-22

LIST OF FIGURES AND TABLES

Figure 1 – Sampling locations	I-8
Figure 2 – How PCR works	I-11
Figure 3 – How qPCR its probe works	I-13
Figure 4 – Real-Time monitoring of qPCR	I-14
Figure 5 – Gel electrophoresis results for samples 1-14.....	I-17
Figure 6 – Gel electrophoresis results for samples 15-26 and negative control ..	I-18
Figure 7 – Preliminary real time qPCR test results.....	I-19
Table 1 – Nanodrop results for sediment samples	I-16
Table 2 – Nanodrop results for water samples.....	I-16
Table 3 – Nanodrop measurements taken before the preliminary real time qPCR test	I-18

INTRODUCTION

Environmental DNA (eDNA) is DNA from the nucleus or mitochondria that organisms shed into the environment (Shokralla et al. 2012; Rees et al. 2014). DNA enters the environment when organisms such as animals, bacteria, and viruses slough off skin, lose their hair and fur, and secrete waste, mucus or gametes. Samples of eDNA can be collected from water, soil, or feces, and analyzed using molecular techniques (e.g., PCR, qPCR, metabarcoding). Information that can be obtained from these eDNA samples can be useful for analyzing which organisms inhabit or migrate through an area, if there are any unknown organisms that have yet to be discovered or recorded, and which organisms are consumed by other organisms.

Applications of eDNA are still new and being tested. eDNA has primarily been applied to mesocosms (Kelly et al. 2014) and closed water systems such as ponds (Minamoto et al. 2012), and in detection of invasive species in the wild (Jerde et al. 2013). Among natural areas, open water systems, such as rivers, are complex due to variations in water flow that need to be considered and have thus far only been studied using calculations and artificial open water systems in labs. For example, samples collected downstream could contain eDNA from a discrete population upstream, which could lead one to conclude that the population is larger downstream than in actuality. The waterway studied, the Bronx River, is a more controlled environment for an initial inquiry into the applications of eDNA in an open water system because of the numerous man-made dams that impede the rate of the water flow. Very few studies so far have compared the effects of using sediment samples in comparison to water samples in detecting fish in the environment, but a recent study suggests sediment may contain more eDNA (Turner et al. 2015).

The organism of focus was the American eel (*Anguilla rostrata*) a species that is important ecologically and of conservation concern, having decreased in density throughout its range, largely over the past half-century (Haro et al. 2000; Limburg and Waldman 2009). American eels hatch in the salt water of the Sargasso Sea. They migrate to fresh waters such as the Bronx River, where they spend the majority of their lives, leaving only to spawn in the Sargasso Sea before dying. Eels disperse through a river watershed, with females moving inland. Because of the need to utilize freshwater habitats, dams on rivers are thought to be one of the primary causes for the decline of eel populations (Machut et al. 2007), as they block access to interior freshwaters by impeding upstream movements, which results in growth impedance, and decreased fecundity. Surveying eel populations above and below dams can give an indication of the effects of these structures on their passage success and consequent abundance. Based on the electrofishing survey of DeMarte et al. (2016) for the first six dams from tidewater, eels are abundant lower in the Bronx River (182nd Street dam), and density drops substantially with each upstream dam.

DeMarte et al. (2016) and subsequent sampling by J. Waldman on abundance and biomass of eels (unpublished data), provided sufficient data on upstream and downstream populations at six of the dams in the Bronx River, presenting the Bronx River as an ideal test case for evaluating the sensitivities of eDNA techniques in an open water system. Surveying eel populations above and below dams can give some indication of the effects of these structures on their abundance. The objectives of this study were to compare both water and sediment samples, as well as alternative amplification techniques, to detect American eel eDNA, using information from conventional sampling as reference data.

Another objective of this study was to develop eDNA techniques that could be applied to the diversity of taxa residing in the Hudson River waterway.

Given information on eel densities and biomass, it was hypothesized that higher levels of eDNA would be found downstream, because of a higher population density than upstream. The second hypothesis was that sediment samples would contain higher eDNA yields than water samples. This hypothesis is based on the benthic habits of American eels, which suggest the mucous found on their bodies could be left between the sediment layers after burrowing. In addition, sediment represents a more stable matrix to retain and preserve DNA compared with water. The last hypothesis was that real time quantitative polymerase chain reaction (RT-qPCR) would be more sensitive than the polymerase chain reaction (PCR) method in detecting eel DNA.

METHODS

Sampling Locations

Four dam locations were chosen along the Bronx River based on the research by DeMarte et al. (2016).

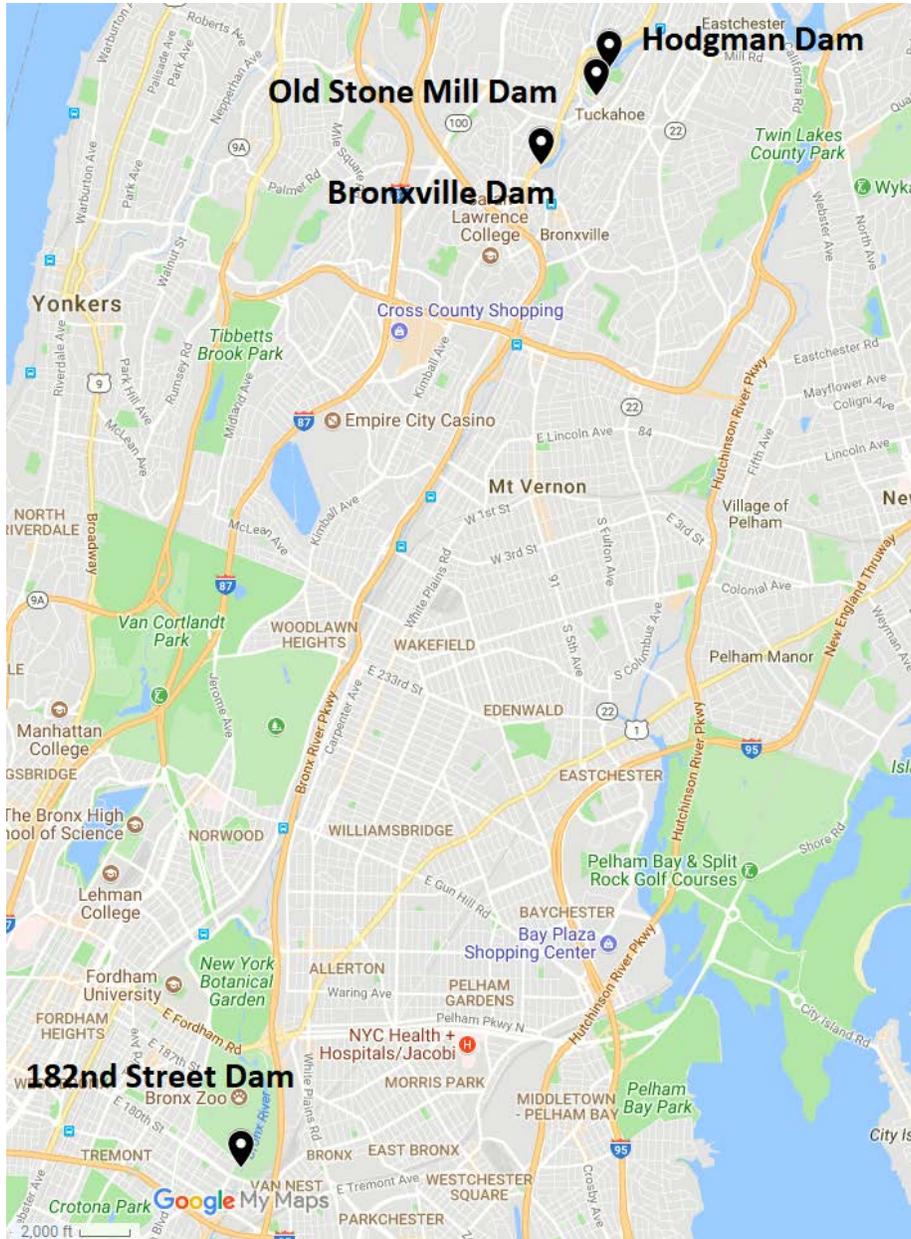


Figure 1: Sampling locations.

Materials and Sample Collection

Two 1-liter water samples were collected downstream of each dam into sterile Nalgene lab bottles. Five 10-ml subsurface sediment samples were collected along the banks downstream of each dam using sterilized scoops and placed into sterilized test tubes. Collected water samples were taken directly back to York College and were vacuum-filtered using two 0.22 µm membrane filters. The bottom filter was used to help prevent cross-contamination between each filtration and the top filter was used to collect the eDNA required for further testing. All equipment was sterilized with 10% bleach mixture between each sample. The membrane filters were then frozen at -80 degrees Celsius until the following laboratory meeting when DNA could be extracted. Sediment samples were refrigerated at 4 degrees Celsius until DNA extraction during the following laboratory meeting.

Primer Design

The original primers were designed according to specifications found in Jochen Trautner's 2006 study, "Rapid identification of European (*Anguilla anguilla*) and North American eel (*Anguilla rostrata*) by polymerase chain reaction." This pair of primers targets the cytochrome b gene, and amplifies a product length of 589bp.

Original Forward Primer:

1AngRosCytbF:

5'-CAAAGAAACATGAAACATTGGAGTC-3'

Original Reverse Primer:

6AngRosCytbR:

5'-TTGGTACTACTATTAGAACTAGAATA-3'

These primers worked well when using DNA from American eel fin clips; however, when used with eDNA samples, the quantity and quality of DNA obtained after PCR amplification was significantly lower. The eDNA obtained was too degraded for the primers to properly amplify the region specified, thus new primers had to be constructed. Mitochondrial cytochrome b genes from American eel samples in GenBank were compared, and new primers were designed using the program Primer3 to target a 150bp on cytochrome b. The new forward primer AngRosCytb506F and new reverse primer AngRosCytb656R were tested on fin clips from American eel and other fish species found in the Bronx River and environs, such as chub, carp, mummichog, striped bass, alewife, and blueback herring, to determine if the primers were specific enough to amplify only American eel DNA.

eDNA Techniques

Genomic DNA was extracted from the water filters using a Qiagen DNeasy extraction kit (including one blank filter as a negative control) and from the sediment samples using the MoBio Powersoil DNA extraction protocol (MoBio, Carlsbad, CA). DNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA). Both standard PCR and qPCR were carried out in the Alter laboratory at York College on an Eppendorf Mastercycler (Hamburg, Germany), and an Applied Biosystems Step One qPCR system (Foster City, CA), respectively, following the protocol of Thomsen et al. (2012). The PCR protocol used for this experiment was obtained from the Trautner (2006) paper.

PCR is depicted in summary in Figure 2. The DNA extracted from the water filters and soil samples serve as template DNA, the original DNA to be replicated. DNA

primers are made to bind to a specific complementary region of the template DNA and added along with the template DNA and many nucleotides into a PCR tube. Step 1 is denaturation, during which the reaction is heated to a high temperature, which disrupts the hydrogen bonds between the nucleotides that make up the template DNA. Step 2 is annealing, during which hybridization occurs between the primers and the template DNA. Step 3 is elongation, during which, DNA polymerase (not present in Figure 2.) adds nucleotides complementary to the template in a 5' to 3' direction. These steps are repeated for several cycles until the desired product is obtained. After successful PCR, amplifications were sequenced at Genewiz, Inc. (Plainview NJ) to ensure that the correct target species was obtained.

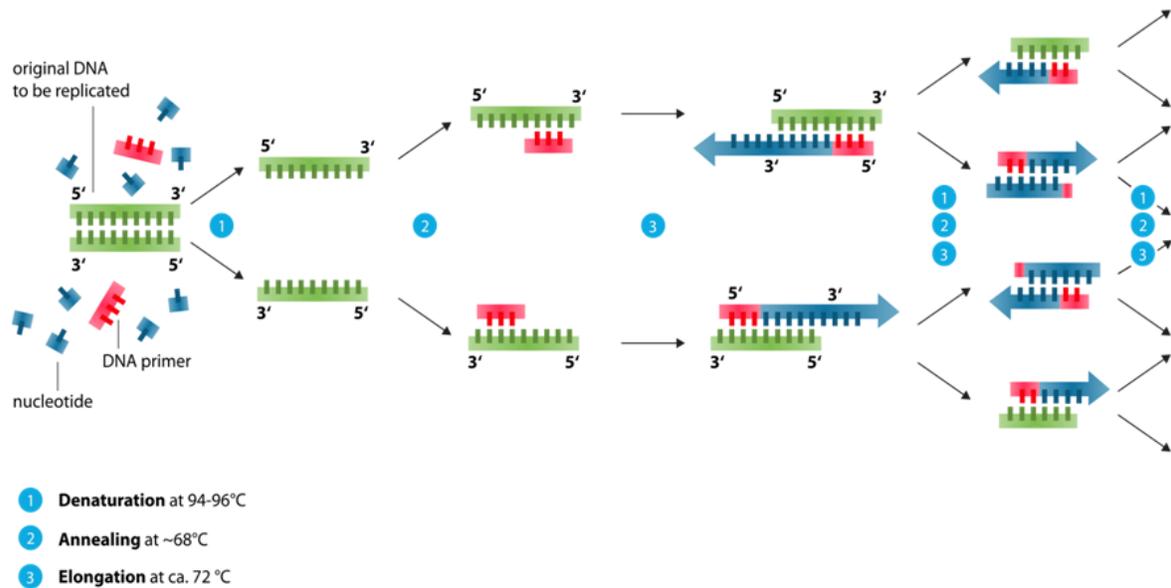


Figure 2: How PCR works (Image credit: https://upload.wikimedia.org/wikipedia/commons/thumb/9/96/Polymerase_chain_reaction.svg/2000pxPolymerase_chain_reaction.svg.png).

As seen in Figure 3, the DNA extracted from the water and sediment samples are the template DNA. Data from American eel samples found in GenBank were used to design the primers for PCR and the probe for qPCR. A probe is designed to hybridize with a sequence on one of the template strands. The sequence where the probe hybridizes must be downstream of the sequence where the primer hybridizes on that same template strand, and within the length that will be amplified. A probe has two main features: the fluorophore, which emits fluorescence, and the quencher, which absorbs fluorescence emitted from the fluorophore as long as the two are close together. During the reaction, similar to PCR, there is the denaturation phase, followed by the annealing phase during which the forward and reverse primers and the probe hybridize to the template DNA, followed by the elongation phase. During the elongation phase, seen in the third step, DNA polymerase (not pictured in Figure 3.) adds nucleotides to the template strand, and works as an exonuclease, which removes the nucleotides in its way, and continues with elongation. As seen in step 4, the probe has been cleaved, and the fluorophore and quencher are no longer in close proximity for the quencher to act on the fluorophore, thus allowing for fluorescence to be emitted, signaling that one round of PCR has been completed.

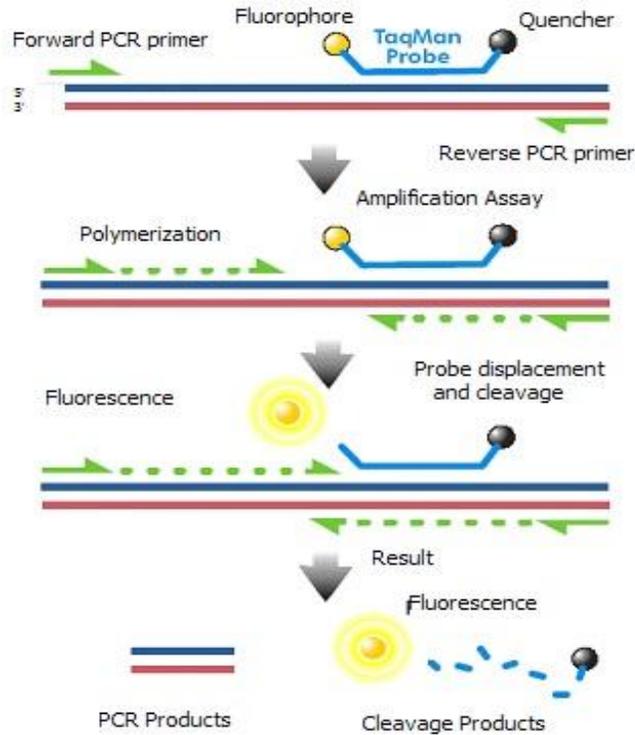


Figure 3. How qPCR and its probe work.

In Real Time qPCR, the fluorescence is tracked with the Applied Biosystems Step One qPCR system and plotted on a xy graph. As shown in Figure 4, the x-axis is time, which represents the rounds of PCR (cycle number), and the y-axis is amount or intensity of fluorescence detected by the system. Each time a new amplicon is produced, another fluorescent marker is separated from its quencher; therefore, with each round of PCR, the number of fluorophores released and amount fluorescence emitted doubles, increasing the amount fluorescence detected by the program. If the starting concentration of DNA in the sample is low, it takes several more rounds of PCR before the amount of amplicons reach the threshold and is detected. If the starting concentration is high, it takes fewer rounds of PCR before the threshold is reached and the amount of amplicons is detected.

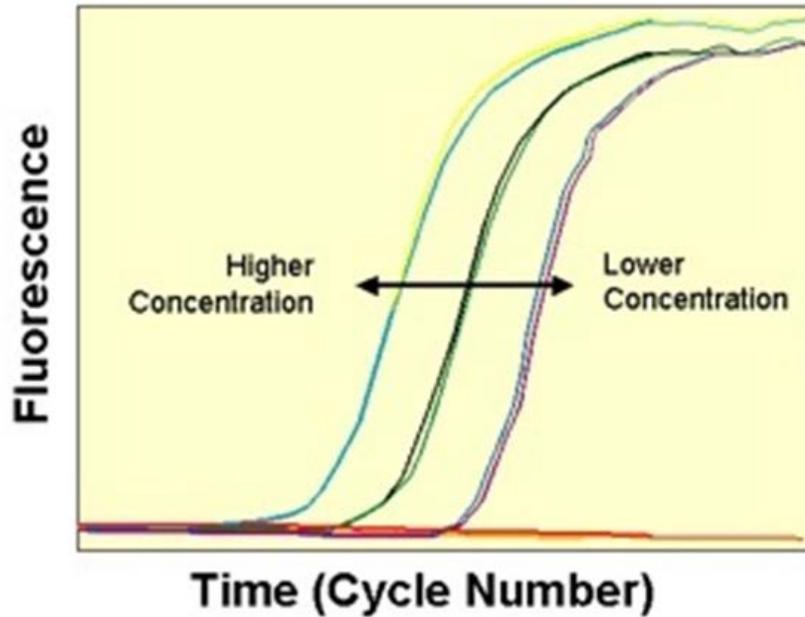


Figure 4: Real-Time monitoring of qPCR (Photo credit: https://dna.utah.edu/Image/Quantification/real-time_curve_web.png).

For qPCR, there were extra steps that needed to be carried out on the Applied Biosystems Step One qPCR system before a qPCR could be done for the samples collected. First, a standard curve analysis was built by using a standardized dilution series of eel DNA obtained from an American eel fin clip. Step One software was then used to determine the threshold, cycle threshold, and ΔR_n values for the samples and the negative control, double distilled water (ddH₂O). The threshold is a horizontal line at which all the samples are straight and parallel to each other, which allows for analysis of the data. The cycle threshold (C_T) is the value at which fluorescence can be detected above the threshold and considered a real signal. ΔR_n is the amount of fluorescence at a given time point.

RESULTS

The results show that the eDNA method successfully detected American eel DNA from both water and sediment in the Bronx River. The Nanodrop spectrophotometer results for both the sediment and water samples in Tables 1 and 2 show their concentrations and their sample purity values, which are consistent with the second hypothesis that sediment retains more DNA than water. The purity levels for most of the samples indicate that the samples are within the accepted purity range of 1.8-2.0. The concentrations for the sediment samples were, overall, significantly higher than the concentrations for the water samples, confirming that sediment samples contain higher eel eDNA yields than water samples.

Sample Number	Sample Identification	Concentration (ng/μL)	A260/280
1	Bronxville Sediment 1	75.3	1.86
2	Bronxville Sediment 2	3.8	1.75
3	Bronxville Sediment 3	135.5	1.89
4	Bronxville Sediment 4	49.1	1.80
5	Bronxville Sediment 5	62.9	1.82
6	Old Stone Mill Sediment 1	45.7	1.82
7	Old Stone Mill Sediment 2A	12.7	1.82
8	Old Stone Mill Sediment 2B	6.4	1.63
9	Old Stone Mill Sediment 3	40.1	1.82
10	Old Stone Mill Sediment 4	37.0	1.86
11	Old Stone Mill Sediment 5	18.6	1.86
12	Hodgman Sediment 1	45.9	1.83
13	Hodgman Sediment 1	63.1	1.84
14	Hodgman Sediment 1	56.0	1.84
15	Hodgman Sediment 1	6.0	1.63
16	Hodgman Sediment 1	57.7	1.83
23	182 nd Street Sediment 1	19.8	1.85
24	182 nd Street Sediment 2	47.7	1.80
25	182 nd Street Sediment 3	41.7	1.91
26	182 nd Street Sediment 4	72.4	1.82

Table 1: Nanodrop results for sediment samples.

Sample Number	Sample Identification	Concentration (ng/μL)	A260/280
17	Hodgman Water 1	23.1	1.88
18	Hodgman Water 2	19.3	1.93
19	Old Stone Mill Water 1	34.6	1.83
20	Old Stone Mill Water 2	25.7	1.92
21	Bronxville Water 1	17.9	1.83
22	Bronxville Water 2	17.9	1.82

Table 2: Nanodrop results for water samples.

The gel electrophoresis results from Figures 5 and 6 confirm that there is eel DNA in all the samples except for the negative control (distilled water). This confirmation allowed for the continuation onto the next experiment, the RT-qPCR experiments. Because the sediment samples had higher concentrations, one sediment sample was used for a serial dilution; it was diluted tenfold for four serial dilutions. The data from these serial dilutions can be seen in Table 3. The concentrations for D2 to D4 are negative numbers because the nanodrop is not sensitive enough to detect any DNA, and the concentrations of the samples could not be calculated accurately. After recording the nanodrop data, the original sample along with its four dilutions, and three other samples were placed into the Applied Biosystems Step One qPCR system to test and compare the sensitivity of qPCR to PCR.

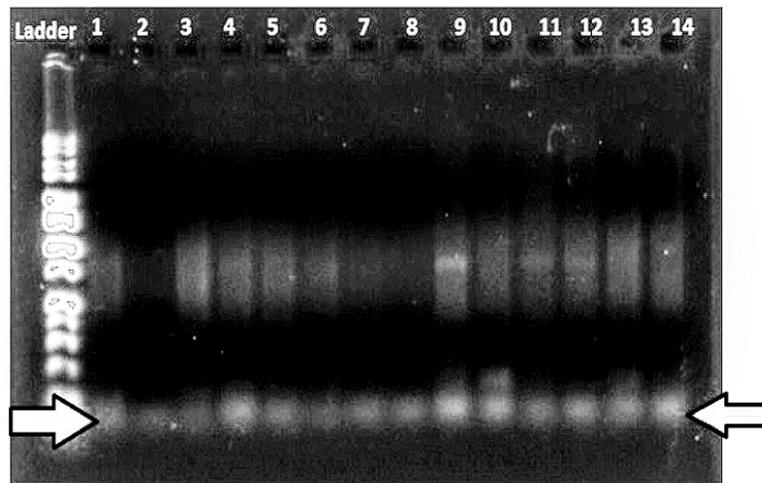


Figure 5: Gel electrophoresis results for samples 1-14.

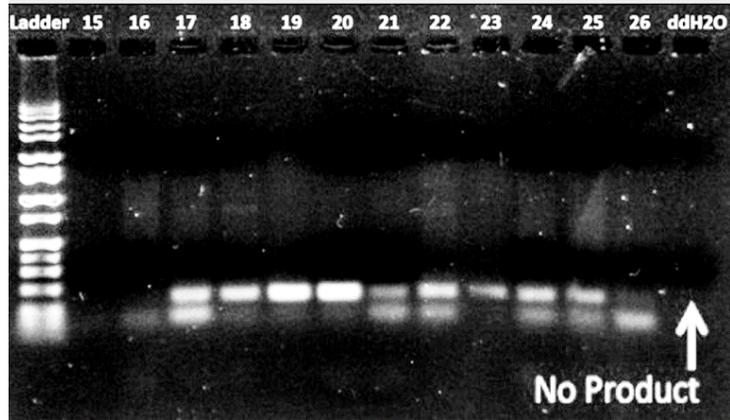


Figure 6: Gel electrophoresis results for samples 15-26 and negative control.

Sampling Identification	Concentration	A260/280
Hodgman Sediment	48.7	1.94
Hodgman Sediment Dilution 1 (D1)	3.4	1.43
Hodgman Sediment Dilution 2 (D2)	-0.9	3.76
Hodgman Sediment Dilution 3 (D3)	-0.6	-5.76
Hodgman Sediment Dilution 4 (D4)	-1.2	2.43

Table 3: Nanodrop measurements taken before the preliminary real time qPCR test.

As seen in Figure 7, the four full-concentration original samples for 182nd ST sediment, Hodgman water, 182nd ST water, and Hodgman sediment were amplified as expected, and surpassed the threshold. The negative control, ddH₂O, did not amplify as was expected, and confirmed that there was no contamination. The results for the dilutions show that there was genetic material in at least the first and second dilutions, the third dilution failed, and if the test had been allowed to continue past where the other samples plateau, the fourth sample may have reached the threshold. The results from this RT-qPCR test are consistent with the last hypothesis, in that the concentrations of the dilutions would not have been detected within the limits of PCR because of the number of cycles required to obtain a sufficient amount of PCR product to be detected in the

nanodrop tests and for use in gel electrophoresis; however, the results were not entirely consistent with the hypothesis that downstream locations will harbor more eel DNA. Figure 7 shows inconsistent results between water and sediment samples. Interestingly, the sediment samples show the expected relationship (greater concentration of eel DNA in the downstream compared with upstream site), whereas the water samples indicate the opposite. This could result from a longer residency time of eel DNA in sediment; however, more samples and sampling sites will be needed to determine whether these results are representative of true concentrations in the Bronx River.

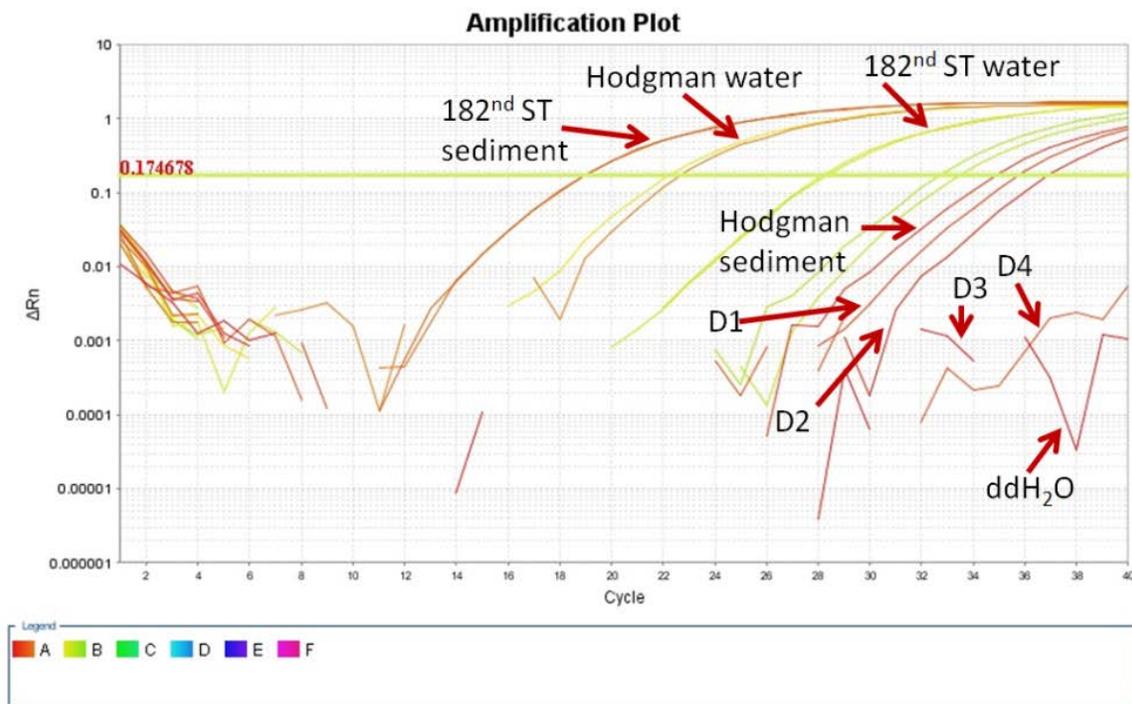


Figure 7: Preliminary real time qPCR test results.

DISCUSSION

Regarding applications of this study to other eDNA studies for known species that have been recorded into GenBank, primers and probes can be designed as easily as they were for the American eel. However, for species that reside in an unexplored environment and have yet to be discovered, have not been documented as inhabiting that area, or were thought to be extinct, a different approach must be taken. With the metabarcoding results, the unknown species mentioned above can then be studied as in this case study.

Further studies will be required to assess the relationship between river flow rate and eDNA concentrations. It seems likely that high flow rates dilute eDNA concentrations in the water column, but less so or not at all in the sediments. If this experiment were replicated, how different would the water sample results be if on-site filtration were used? Yamanaka et al. (2016) compares on-site filtration, filtration after 6 hours in an ice bath, and filtration after 6 hours at room temperature. The results show that the on-site filtration is the most assured method to obtain the greatest concentration of eDNA. A follow-up study on American eels would involve tank tests and eDNA degradation. Eels of the same size will be placed into temperature controlled tanks and removed after a certain amount of time to look at the degradation rates of American eel eDNA over different periods of time. Degradation rate by eel size will also be compared to determine how the size of eels modulate eDNA concentrations, i.e., if eDNA concentrations are more sensitive to eel numbers or biomass.

Using the data from DeMarte et al. (2016), this study, and an eDNA degradation study, mathematical calculations can be used to predict river eDNA concentrations at

distances from the known eel territories and compared to the actual data to further develop the use of eDNA in open water systems. While eDNA holds great promise as a tool for biodiversity analysis and conservation, there is much more to be learned about both its potential and its limits as a tool. Studies such as this one that are beginning to compare eDNA results with reliable information on the characteristics of the organism of study in natural systems are one way that progress can be made.

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