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Abstract

Environmental DNA, or eDNA, describes the presence of small fragments of an organism's genetic material left behind in their environment. For organisms in an aquatic environment, which includes both marine and freshwater ecosystems, these fragments of their eDNA can be released into the water column through shedded skin cells, waste, and mucus. These traces animals leave behind in their environment provide an effective way to monitor their presence in different environments, whether in the air, terrestrial or aquatic. This highly sensitive, non-invasive technique to monitor organism abundance is especially useful when looking for animals whose populations are of great concern, such as endangered, elusive, or invasive species. Originally from the Ohio River watershed, the rusty crayfish (*Faxonius rusticus*) is an invasive species in the Cayuga Lake watershed that has the potential to disrupt delicate balances within this freshwater system. This summer, as part of the Commons Biodiversity Project, I spent six weeks in the Cheong Lab at Cornell University, to train on eDNA techniques as a means to monitor the rusty crayfish and its whereabouts in the Cayuga Lake watershed. To do this, I worked with my research mentors Lee Yoke Lee and Soon Hon Cheong as well as Laidlaw scholars Jennifer Owiyo and Paige Yun to collect water samples from Fall Creek and two ponds in the Cornell Arboretum. We actively filtered each sample along with a control through filter paper using a hand pump apparatus, and then extracted the

DNA from it to have any collected genetic material in a higher concentration. Finally, with our products from DNA extraction, we performed a real-time quantitative PCR (qPCR) analysis to quantify any rusty crayfish eDNA we collected, and to find out which water bodies it was present in. Through our methods, we were able to make further progress in validating our eDNA techniques before applying them to next summer's work to conserve the endangered Crocus clam in Malaysia. Through our validation, we found that our technique was successful in confirming the presence of the rusty crayfish in Fall Creek, where we have sighted it before. With this, we are confident that the rusty crayfish is present in this system. However, because of amplification in our no-template controls and field blanks, we are not able to confidently conclude the eDNA assay's detection of this species in the Cornell Arboretum ponds.

Problem statement

The rusty crayfish (*Faxonius rusticus*), although native to the Ohio River watershed, is an invasive species that is present in the Cayuga Lake watershed. Introduced to the watershed as live bait for anglers, a rusty crayfish invasion can have detrimental effects on the ecosystem, including causing a decline in aquatic plants, crayfish that are native to the system, and macroinvertebrates. Management of the rusty crayfish in the Cayuga Lake watershed requires effective preventative measures and close monitoring of the species and its abundance. An effective methodology for detecting and monitoring benthic arthropods, especially those that are invasive, in an aquatic ecosystem is environmental DNA, also known as eDNA (Larson et al., 2017).

In addition to invasive species, environmental DNA can be used to track marine species that are rare, cryptic, small, or elusive (Bonfil et al., 2021; Bessell et al., 2023; Baker et al.,

2018). As the largest stationary molluscs found in coral reefs, giant clams (*Tridacna spp*) play a significant role in tropical coral reef ecosystems. Making up more than 60% of the biomass found in coral reefs (Li et al., 2024), giant clams can provide a home and breeding grounds for a wide variety of reef organisms, generate oxygen for the ecosystem through photosynthesis, promote the recovery of their system from nutrient pollution with their water filtering capabilities, and generate large amounts of calcium carbonate shell material that can later be incorporated into the reef framework. The many uses humans have found for giant clams, from aquarium trade to cuisine, have contributed to extensive exploitation of giant clam populations. Among these anthropogenic pressures on giant clams, overfishing in particular has led to giant clam abundance to drop to levels at which it is hard for populations to naturally replenish themselves, putting them at risk for extinction in the wild.

With its maximum size being up to 15 cm, the crocus clam (*Tridacna crocea*) is the smallest in the giant clam family (Neo et al., 2017; Li et al., 2024). Found in reefs in shallow waters, the crocus clam is known for its ability to burrow into coral reef substrates so that only its vibrant mantle is exposed. As it experiences anthropogenic pressures such as habitat destruction and over-harvesting, *T. crocea* has also experienced declines in its populations. In collaboration with a team of scientists at the Universiti Malaysia Terengganu, the Commons Biodiversity Project aims to apply eDNA techniques to help conserve the Crocus clams native to the area. With its mission of Crocus clam conservation, this project aims to foster and grow a passion for marine wildlife conservation in the local population of Terengganu, and highlights the importance of collaboration between scientists for opportunities rich in learning and sharing different techniques, asking questions together, and furthering a foundation for the project based on community involvement. This summer, as part of the Commons Biodiversity

Project, I participated in six weeks of research in the Cheong Lab, and under the mentorship of Lee Yoke Lee and Soon Hon Cheong, I worked with fellow Laidlaw scholars Paige Yun and Jennifer Owiyo to train on eDNA methodology. Our focus was on an abundant and easily found species - rusty crayfish (*Faxonius rusticus*), which has been visually confirmed in Fall Creek. My work this summer was to see if I could detect the crayfish in a waterbody where the species has not been sighted - Houston Pond in the Cornell Arboretum. Verifying the effectiveness of our method is an integral part of our preparation for the work we will be engaging in next summer to monitor the crocus clam in Malaysian waters.

Literature review

Environmental DNA, also referred to as eDNA, describes small fragments of mitochondrial or nucleic DNA that an organism has left behind in their habitat, whether it is on land, in the air, or underwater (Díaz-Ferguson & Moyer, 2014). These pieces of genetic material can be released into the environment in a variety of ways, including in the form of mucus, waste, and shedded skin cells. This released DNA can then be captured by researchers and used to monitor the organism, its population dynamics, and the biodiversity (and therefore health) of the ecosystem. In this process, these fragments of eDNA are used to detect an organism, also referred to as the target species, with the use of genetic markers that are specific to the target organism. Finally, the eDNA collected can be quantified through real-time quantitative PCR or qPCR (Díaz-Ferguson & Moyer, 2014).

eDNA techniques are effective for monitoring species that are rare, endangered, elusive, or invasive in an environment (Bessell et al., 2023). In aquatic environments, which includes both freshwater and marine ecosystems, eDNA methodologies for tracking organisms are especially useful, as more conventional methods of monitoring such as visual field surveys

and acoustic monitoring can be more costly and time-consuming (Díaz-Ferguson & Moyer, 2014). However, there are many environmental factors that can impact the effectiveness of eDNA techniques by affecting the persistence of the eDNA, which is how long eDNA fragments can remain in the ecosystem before degrading. Recognizing these environmental factors that may influence eDNA detections in an aquatic environment is important for designing effective eDNA technique procedures and interpreting eDNA results. With this, researchers can form a better understanding of how to draw conclusions from detections (Barnes et al., 2014). These environmental factors can be biotic or abiotic, and vary depending on the type of aquatic system being investigated.

The paper "Environmental Conditions Influence eDNA Persistence in aquatic systems" (Barnes et al., 2014) provides a foundation for understanding the ways in which environmental factors may impact not only the persistence of eDNA in the water column, but also the rate at which it degrades in different aquatic environments. The researchers divided environmental factors that impact eDNA degradation and persistence into three categories: the characteristics of the DNA molecule, abiotic factors in the environment, and biotic factors in the environment.

The way in which eDNA fragments can appear in a variety of lengths, shapes, and sequences, can affect how it degrades over time. More specifically, the length and shape of the fragment can influence the binding of the DNA to other particles in the environment, as well as the way in which it interacts with microbes in the water column. Given the way in which eDNA can be contained within whole cells or be extracellular fragments, the way in which eDNA appears can also influence its degradation. For instance, eDNA found inside whole, intact cells or organelles experience greater protection from external factors that can

contribute to its degradation.

Abiotic or non-living factors such as higher temperatures, salinity, and UV radiation exposure all increase eDNA degradation. Sediments present in the environment can also impact eDNA persistence in the water column. eDNA binds more strongly to clay particles as opposed to sand or silt, indicating the importance of sediment composition in eDNA persistence, regardless of whether it is suspended in the water column or settled to the bottom. The way in which eDNA interacts with sediments may be dictated by the pH and salinity of the system. Finally, high stream flow has the ability to dilute the eDNA present, increasing the chances for false negative detections of the target organism. The velocity of water flow in a freshwater system may also determine how far the eDNA can be carried within the stream (Shogren et al., 2018), and how much of the DNA settles onto the sediments at the bottom (Curtis et al., 2021).

Biotic factors that influence eDNA degradation include extracellular enzyme presence, tannins from plant matter (Díaz-Ferguson & Moyer, 2014), biofilm cover (Shogren et al., 2018), the size of the target organism, and the seasonal behaviors of the organism (Bessell et al., 2023). Among both freshwater and marine ecosystems, literature suggests that seasonal behaviors such as spawning can greatly affect the concentrations of eDNA in the water column.

These environmental conditions that impact eDNA persistence in aquatic ecosystems may vary depending on whether the system is freshwater or marine. For freshwater streams, stream flow and biofilm concentrations have the ability to influence the amount of eDNA found in the water column. However, in marine environments, environmental factors such as high salinity, pH, temperature, and currents may impact the rate at which eDNA can degrade.

These environmental factors were important to consider for our sampling sites and the persistence of eDNA in each system, as these measurements varied between each. With an average measured pH of 7.60, Houston Pond was found to be more acidic than Fall Creek, which had an average pH of 8.16 among the measurements collected at each site visit. Additionally, Houston Pond had a higher average temperature at 25.33 degrees Celsius, which was slightly warmer than the average temperature of Fall Creek, 22.08 degrees Celsius. While keeping these differences in pH and temperature occurring within our freshwater sampling sites, comparing these measurements to the conditions of the South China Sea off of the coast of Terengganu, Malaysia is important as well when planning for eDNA procedures next summer. In contrast to our freshwater systems sampled this summer, water temperatures in the South China Sea are about 27.94±1.01 degrees Celsius (Roseli et al., 2022). These higher temperatures in addition to higher salinity in the South China Sea have the potential to reduce the persistence of eDNA in the water column.

Knowing the ways in which environmental factors impacting eDNA detectability may vary between marine and freshwater systems is important when trying to optimize the effectiveness of our eDNA procedures in both types of aquatic systems. This drives questions about how these factors may vary within a single type of aquatic environment: freshwater systems. Given the significance of stream flow in eDNA concentrations in the water column, this summer I investigated the question: How do detections of the invasive rusty crayfish (*Faxonius rusticus*) compare between Fall Creek and two ponds in the Cornell Arboretum.

Method

To detect rusty crayfish presence in Fall Creek and the Cornell Arboretum ponds, our eDNA methodology consisted of three steps: collection of water samples at each site, extraction

of eDNA from each sample, and real-time quantitative PCR (qPCR) analysis of the extraction product to determine the presence of rusty crayfish eDNA at each site.

Water sampling

Water sample collection from field sites (see **Figure 1**) Fall Creek (42.4540029, -76.4569845), Houston Pond (42.4508221, -76.4555532), and Houston Pond Adjacent (42.452086, -76.4545786) were collected during the weeks of June 10 and July 8, 2024. During the first week of sampling, 18 field samples were collected in total, each with its own negative control of 300 mL autoclaved distilled water. During the second week of sampling, four field samples were collected in addition to a control sample for each. Each of the three sampling sites were sampled in either triplicate or duplicate by choosing sites at least 50 feet apart. With this, Fall Creek had three selected replicate locations referred to as A, B, and C, and both Houston Pond and Houston Pond Adjacent had 2 replicate locations: A and B. Each time field sites were visited, environmental factors were recorded, including weather, the type of water body, pH, water temperature, water flow, human use of the area, turbidity, and tree cover.

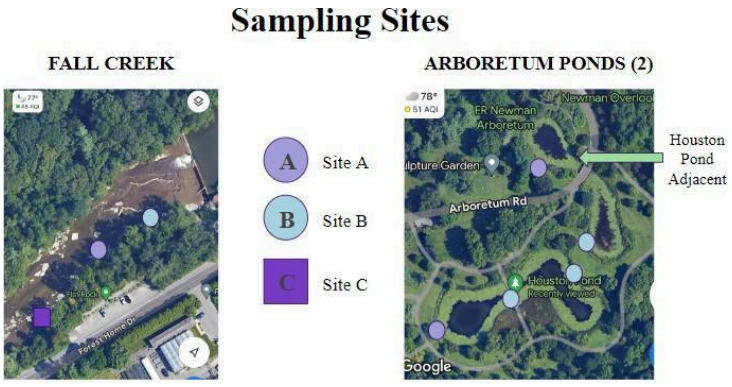

Figure 1. Stream vs. Pond sampling sites Fall Creek (left), Houston Pond (bottom right), and Houston Pond Adjacent (top right).

Water samples collected were filtered using active filtering. For this, an apparatus

(**Figure 2**) was constructed on a flat surface using an Erlenmeyer flask, a Büchner funnel, a metal ring stand, a rubber stopper, a plastic manual hand pump, and plastic tubing from the Eisco economical filter kit.

Figure 2. Active filtering apparatus assembled at Fall Creek.

Collection, filtering, and handling of field samples was conducted using clean nitrile gloves. Field sample collection techniques varied between each week of sampling. For the first week, weather permitted both sampling *and* filtering in the field. With this, water samples were filtered immediately after collection to reduce the amount of time the eDNA within the sample had to degrade, therefore allowing us to maximize the amount of eDNA captured in the filter paper for each sample. During the week of July 8, 2024, however, water filtration was done in the lab to avoid being in the field for too long during heat advisories. Thus, during this final week of sampling, only autoclaved 1 L bottles were brought to the field to collect one liter samples, instead of a field bag filled with all necessary materials for both sampling and filtering at the sample site. To reduce the rate of eDNA degradation in these samples brought back to the lab for filtration, samples were filtered within two to three hours following collection in the field. Bottles of sampling site water that were not going to be immediately

filtered in the lab were placed in the fridge prior to their filtration.

To filter field samples, a filter paper was carefully placed inside the Büchner funnel with a pair of clean forceps previously dipped in 8.25% household bleach solution and rinsed into a waste container with nanopure water. Samples and negative field controls were filtered through filter papers of various pore sizes, including 0.45 um, 0.2 um (Immobilon PVDF), and Whatman filter paper number 1 (11 um). For both field samples and negative field controls, water was added slowly for the hand pumping of the sample until the required volume was collected in the Erlenmeyer flask below. Before filtering samples, negative field controls were filtered first to verify if the filtration apparatus was properly decontaminated for any eDNA remaining from previous samples. This was done by manually hand pumping 300 mL of autoclaved distilled water. For the field samples, 1 L of the water collected at the sampling site was filtered. Once these volumes were collected in the Erlenmeyer flask at the bottom of the apparatus, the filter paper was carefully folded using two pairs of clean forceps in order to fit into a 15 mL conical tube containing 4 mL of Longmire Buffer. The Longmire Buffer was added to the tube with a plastic dropper drawing from an aliquot of the stock solution. The filter paper was inserted into the test tube over the Büchner funnel and until it was submerged in the Longmire Buffer. These test tubes containing filter paper used for both controls and samples were immediately stored in the lab refrigerator at 4 degrees Celsius.

Filtering apparatuses differed between the filtering of pond water and stream water. Given the way the water samples collected in the pond field sites consisted of much more sediments and biological matter such as vegetation and daphnia, a pre-filter (**Figure 3**) of unknown pore size was placed on top of the filter paper inside the Büchner funnel at the top of the apparatus to collect these sediments and biological matter from the pond samples to

avoid PCR inhibition later on.

Figure 3. Active filtering apparatus with pre-filter assembled at the Cheong Lab.

DNA Extraction

Following sample collection, DNA was extracted from each conical tube for both controls and samples using the QiaAmp DNA Mini Kit. Before DNA extraction, lab bench surfaces that were going to be used were sprayed with 70% ethanol solution before clean paper towels were placed on top and sprayed with 70% ethanol. Micropipettes and racks for holding shipping tubes and centrifuge tubes were also sprayed with 70% and wiped dry prior to their use. After decontamination of surfaces, the samples and controls were retrieved from the fridge and warmed in the wells of a heating block at 65 degrees Celsius for 10 minutes.

To process each sample, a 2 mL dolphin microcentrifuge tube was labeled with permanent marker as ‘Reaction tube’ and a second dolphin tube was labeled ‘Elution tube’ and a spin column was prepared. The label for the Elution tube was more detailed (date, sample site, initial of collector) whereas the label Reaction tube and spin column has sufficient information to avoid being confused with other samples. 20 uL of Proteinase K was placed in each Reaction tube.

The samples were vortexed with a Vortex-Genie Mixer at speed 8 for 5 minutes. 400 uL

of the sample was transferred to the Reaction tube using a 1 mL serological pipette and pipette aid, ensuring that no bubbles were captured. This was followed by the addition of 200 uL Buffer AL, and 400 uL 100% Ethanol. The Reaction tube was vortexed for at least 10 seconds each time a new reagent was added to ensure it was properly mixed.

550 uL of the sample in the reaction tube was added to the spin column, which was then centrifuged at 6000 x g for 1 minute. After centrifuging, filtrate or liquid captured in the collection tube of the spin column was discarded in a liquid waste bottle. Next, the remainder of the reaction tube mixture was added to the spin column to be centrifuged at 6000 x g for 1 minute. The filtrate from the spin column was again removed from the collection tube. In a similar manner, 500 uL Buffer AW1 was added to the spin column and centrifuged. Then, 500 uL Buffer AW2 was added to the spin column, to be centrifuged at 17,000 x g for 3 minutes, after which the collection tube was replaced with a new one. With the new collection tube, the spin column was centrifuged again at the same speed for 1 minute repeatedly until there was no more liquid appearing in the collection tube. Finally, after placing the spin column into the sample's Elution tube, 100 uL RNase-free water was added to incubate for 5 minutes at room temperature. Incubation was followed by a final centrifuge at 6000 x g for 1 minute, after which the eluted DNA was stored in the freezer at -20℃.

qPCR Analysis

To quantify the amount of DNA found in the samples, qPCR analysis was done with each round of sample collection for the weeks of June 10 and July 8, 2024. Before running the qPCR analysis of samples after DNA extraction, a 96-well plate map was drawn out, to plan which samples were going to be included in the assay, and calculate the volumes of reagents needed to create master mix that would be put in each well.

When designing a plate map, the number of total wells needed for the assay was determined by counting the number of field samples and controls to be included, and then adding wells for their qPCR replicates (denoted by subscripts: i and ii), one spiked sample well for each field sample/control and its qPCR replicate, 16 wells for serially-diluted Standard, and three wells for no-template controls (NTC). For each plate map, a code was used to denote samples or controls, sample site, and the date sampled, as indicated in the key below.

qPCR Plate Map Key	
qPCR plate symbol	*Meaning*
1, 2, 3	At the beginning of sample name, representing day of the week sample was collected
s__	At the beginning of SPIKED sample name
F	Fall Creek
H	Houston Pond
S	Houston Pond Adjacent
A	Field replicate A
B	Field replicate B
C	Field replicate C
x	Negative field controls
ii	Second qPCR replicate of sample/control

Figure 4. qPCR plate map key describing code used to represent samples in both qPCR assays.

Figure 5. 96-well qPCR plate map including a representation of the samples collected the week of June 10, 2024.

A representative subset of samples from the week of June 10, 2024 was selected to be

included in the 96-well plate for the first qPCR run. This assay was done alongside my two fellow scholars' samples. In subsequent assays, we ran our own samples in our respective plates. The first Stream vs. Pond qPCR assay (for plate map see **Figure 5**) included six samples and controls from each sample site. To determine the source of DNA contamination, a simpler assay (**Figure 6**) performed on July 3, 2024 only included one sample and control from Fall Creek replicate A, and one sample and control from Houston Pond replicate B. Finally, for the week of July 8, 2024, all eight field samples and controls including replicates A and B were included in the plate map for the final Houston Pond assay (**Figure 7**).

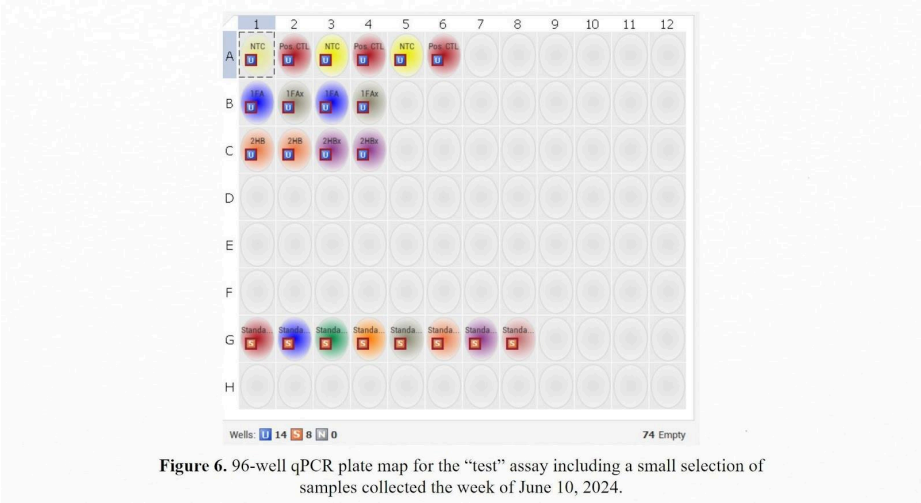

Figure 6. 96-well qPCR plate map for the "test" assay including a small selection of samples collected the week of June 10, 2024.

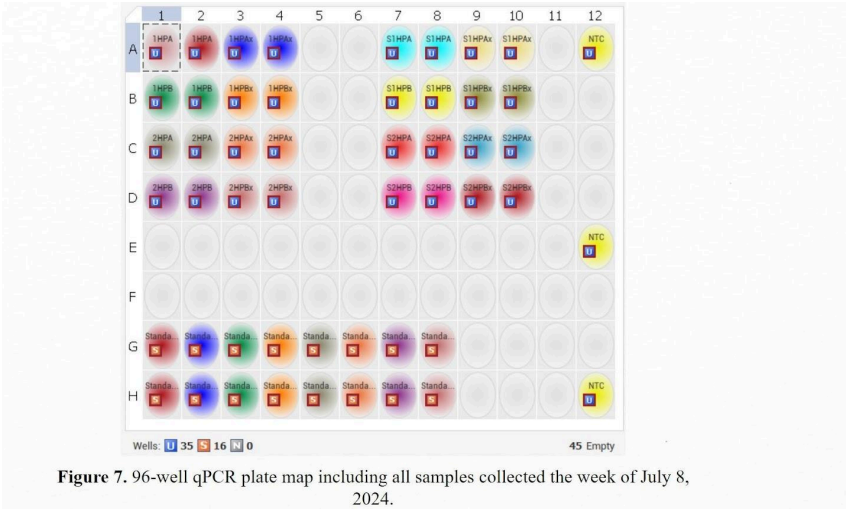

Figure 7. 96-well qPCR plate map including all samples collected the week of July 8, 2024.

The same decontamination and preparatory qPCR procedure was used for both Stream

vs. Pond assays. For the first qPCR assay, plate preparation was performed on a lab bench, however, with contamination observed in the analysis afterwards, the second qPCR plate preparation was done in a Biological Safety Cabinet. With surfaces decontaminated, micropipettes were also sprayed with 70% ethanol and wiped dry prior to their use.

After decontamination of surfaces and pipettes, field samples from DNA extraction, probes, and primers were removed from the freezer, and master mix was removed from the fridge. For each qPCR analysis, we used a custom Taqman FaRu-probe, Environmental Taqman Mastermix, and FaRu primers. The FaRu probe had the sequence 5' - ACTGAGCCAAGAATAGAAGAAACCC - 3', the *forward* FaRu primer had the sequence 5' - GGGCGTCAGTAGATTTAGGTATT - 3', and the *reverse* FaRu primer had the sequence 5' - GTCATTCCCGTAGCTCGTATATT - 3'. These qPCR primers were used to target the cytochrome oxidase I (COI) gene region (Coster et al., 2021) in the mitochondrial DNA of the rusty crayfish. Mitochondrial eDNA was chosen as the focus for our qPCR assays because it is more abundant in cells in comparison to nucleic eDNA. Targeting mitochondrial eDNA allowed us to optimize our chances of finding the genetic material of the rusty crayfish in our samples, thus reducing the risk of having false negative results. Samples, probes, and primers were warmed, rubbing them in between hands and occasionally finger-vortexed to ensure that it was fully thawed. After they were thawed, primers and probes were centrifuged for at least 10 seconds so that all liquid inside the capsules was collected at the bottom of the container.

Prior to master mix preparation, calculations were performed to determine the necessary volumes of each reagent. For these calculations, the total number of filled wells in the 96-well plate map in addition to three extra reactions was multiplied by the required volume of each reagent for master mix required for each well (see **Figure 8**).

Component	(Required volume per rxn) * (total # rxns)	*TOTAL* Volume Needed
Taqman master mix	7.5 μL * (94 rxns)	705 μL
RNase-free water	6.4 μL * (94)	601.6 μL
Forward primer	0.03 μL * (94)	2.82 μL
Reverse primer	0.03 μL * (94)	2.82 μL
Probe	0.03 μL * (94)	2.82 μL

Figure 8. Calculations of volumes for master mix reagents based on required volumes per reaction and total number of reactions (plus extra to account for pipetting error) in the 96-well plate for the first Stream vs. Pond assay.

The calculated volumes for the master mix reagents were added to a 1.5 mL dolphin tube, beginning with the largest volumes, and then vortexed to mix. With 14 uL master mix pipetted into all necessary wells, 1 uL of each field sample and control to be represented in the assay as represented on the plate map was pipetted into their corresponding well. In addition to samples, the plate map also included No-template controls (NTCs). For NTCs, 1 uL of RNAse-free water was added to the 14 uL master mix present in the well. Including No-template controls in each assay allows us to verify if any of our qPCR reagents were contaminated with rusty crayfish DNA.

After plating the NTCs and samples, these portions of the plate were covered with tin foil to prevent DNA contamination, and the serial dilution of the standards was performed. For the first qPCR assay, an eight-step serial dilution was performed for the plate standards. The standards used in each assay are synthetic amplicons of the rusty crayfish's mitochondrial DNA and would help us to quantify the eDNA copy numbers. After adding 9uL of RNAse-free water into each dolphin tube, 1 uL of the standard was added to the first tube and mixed by pipetting up and down. Then, 1 uL of this solution from the first tube was pipetted into the next, which was then mixed. This process continued for all eight tubes. However, after

the first qPCR assay, we observed that the first tube had too high of a concentration of standard. Thus, the final Houston Pond qPCR assay standards were prepared in a 12-step serial dilution, and then plated beginning with the fourth serial dilution from the previous assay. 1 uL of each serial dilution was added to the 14 uL of master mix in the well. Different pipette tips were used for each well.

Each sample and control included in the assay had a corresponding spiked sample as a positive control to check for PCR inhibition. This inhibition can be caused by naturally occurring chemicals and biological matter in the stream and pond. For the spiked samples, 1.5 mL dolphin tubes were labeled to match the samples and controls on the plate map. To each of these dolphin tubes, 4 uL of RNAse-free water and 0.5 uL of the standard was added, making sure to use new pipette tips each time the standard was added. After the RNAse-free water and standard were added, 0.5 uL of the sample or control was added to its respective dolphin tube, using a different pipette tip between samples. Each dolphin tube was vortexed to mix the components before adding 1 uL of the spiked sample solution to its corresponding well on the plate.

After samples, spiked samples, standards, and NTCs were all plated, all primers, probes, samples, and standards were returned to the freezer. The plate was carefully sealed with Axygen Microplate Sealing Film, which was carefully applied to fully cover all wells before being smoothed down applying even pressure across the plate to ensure a secure seal over each well and remove any air bubbles along the edges of the plate. The sealed plate was then centrifuged for at least 10 seconds to ensure that all liquid inside remained at the bottom of each well.

Before qPCR analysis, the plate map for the assay was set up in the QuantStudio 3

Design & Analysis Software v1.5.3 and saved onto a USB drive to be inserted into the QuantStudio 3 qPCR machine. In the program, a previously made pre-run template file was opened, and the "Properties" and "Method" pages were updated prior to entering the plate map. The "Properties" page was updated to include a file name reflecting the purpose of the assay and date of qPCR analysis, and display a 96-Well 0.2-mL Block type, Standard Curve for experiment type, Taqman® Reagents for chemistry, and a Standard run mode. Additionally, under the "Method" page, the volume was set to 15 uL. With the "Properties" and "Method" tabs displaying the correct information, the plate map designed prior to plating and analysis was replicated in the "Plate" tab of the file. For each filled well on the plate map, "Taqman" was selected as the Target. To add standards to the plate map, "Define and Set Up Standards" was selected from the "Action" dropdown menu. This page was updated to ensure that the number of wells designated for standards was eight with two replicates, all serially-diluted with a serial factor of 1:10, and with Taqman (with FAM as the reporter) as the target. Standards were then manually selected and labeled with their respective dilution step numbers. Similarly, samples and NTCs were also labeled to match the previously designed plate map. With the plate map finished, the file was saved to a qPCR Pre-runs folder on the USB drive. The QuantStudio 3 program was closed prior to ejecting the drive.

With the drive inserted, the qPCR machine was turned on, and the plate was inserted with a layer carefully placed on top to prevent any reflection and inhibition during the run. The Pre-run file was selected and the run was started. Once the machine was finished, the run was saved to the USB drive in a qPCR Runs folder, to later be opened in QuantStudio 3 to view the amplification graphs. After the run was completed, the plate was covered with tin foil, labeled according to the assay, and placed in the fridge at 4 degrees Celsius.

Findings/Results

For each week of sampling, a qPCR run was completed. For the week of June 10, 2024 sample assay, the qPCR produced the following amplification plot.

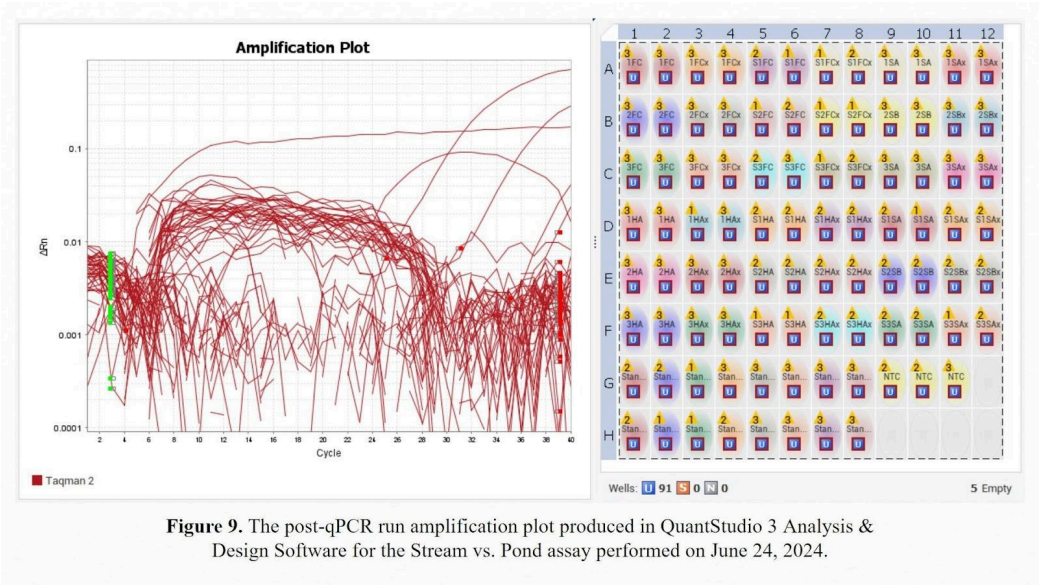

Figure 9. The post-qPCR run amplification plot produced in QuantStudio 3 Analysis & Design Software for the Stream vs. Pond assay performed on June 24, 2024.

In this first qPCR assay, there was no amplification in any Fall Creek Site C samples or controls for that qPCR run, and the same was observed for the Houston Pond Site A and Houston Pond Adjacent samples and controls. However, for the standards included in the assay, only Standard 3 had amplification, while the other standards did not.

To account for any plating error and potential contamination occurring during the plating process impacting assay results, an additional qPCR run was performed using a small selection of Fall Creek and Houston Pond samples and controls collected the week of June 10, 2024. In the plate map for this assay, positive control wells consisted of 14 uL of master mix and 1 uL of standard. In the following amplification plot from this test assay, amplification was observed in NTCs, all controls and samples included, and standards 3-8.

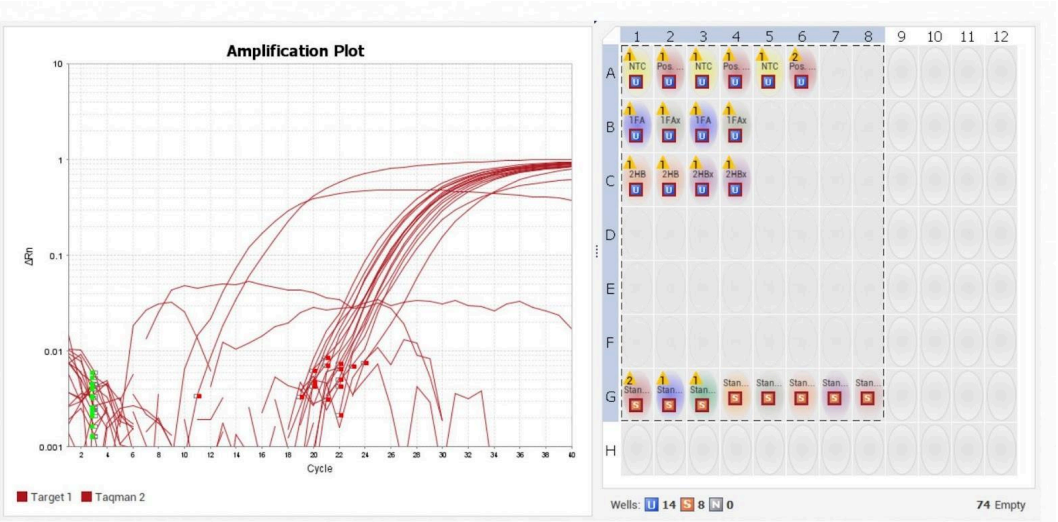

Figure 10. The post-qPCR run amplification plot produced in QuantStudio 3 Analysis & Design Software for the Stream vs. Pond test assay performed on July 3, 2024.

The figure below shows the overall amplification plot for Houston Pond qPCR assay performed on July 12, 2024. In this assay, we observed amplification in samples, field blanks, and NTCs. However, the spiked samples did not appear at all on the amplification plot.

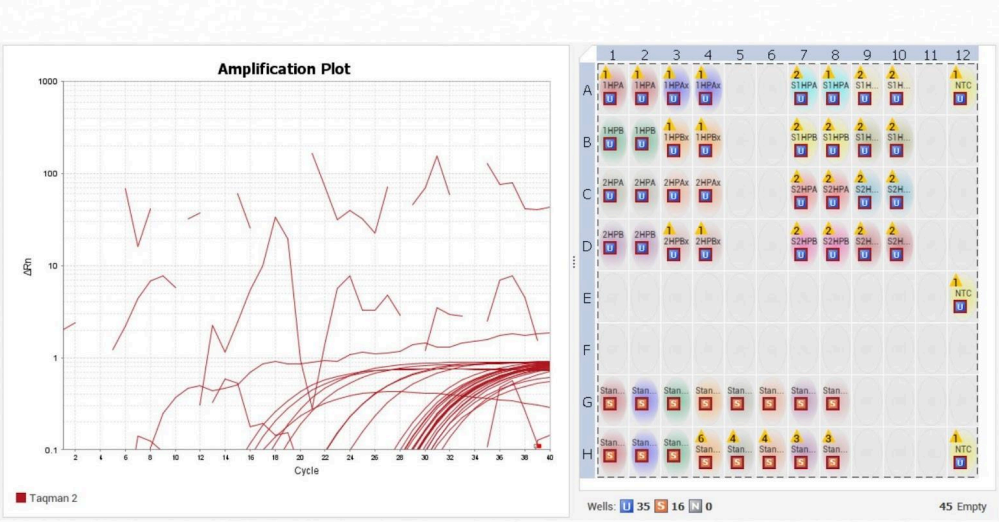

Figure 11. The amplification plot for the Houston Pond assay performed on July 12, 2024.

Discussion

Given that rusty crayfish were observed through opportunistic sightings while sampling in Fall Creek, it is likely that our positive detections of rusty crayfish eDNA in Fall Creek in the qPCR assays can be attributed to rusty crayfish presence in that aquatic ecosystem. However, with amplification observed in field blanks and lack of visual sightings in Houston Pond and Houston Pond Adjacent, it is hard to be just as confident in positive detections in the samples from these two sites indicating the presence of the species in the Cornell Arboretum Ponds. With this, we suspect the amplification in these samples to be from contamination of the field blanks during the plating process, as sampling equipment was thoroughly decontaminated prior to use. The contamination of field blanks and NTCs in the qPCR assays likely occurred during the plating process, while standards containing high concentrations of rusty crayfish DNA were being handled as well. DNA can become aerosolized when opening dolphin microcentrifuge tubes, allowing it to settle into the sample, control, and NTC wells of the plate. Additionally, using the same set of micropipettes for samples, qPCR reagents, and standards earlier on may have contributed to contamination of micropipettes with rusty crayfish DNA. Additional qPCR assay on just the primers, probe, and Taqman master mix confirmed this suspicion. The hydrolysis probe used is highly sensitive. It certainly did not help that the concentration of the standards used was too high for the assay. Thus, contamination during the qPCR procedure is difficult to get rid of, as each qPCR assay amplifies the copy numbers of the rusty crayfish DNA despite thorough measures taken to decontamination of surfaces and equipment leading up to plating with these materials.

In addition to working to avoid contamination during the plating process, we also

worked to find out why some spiked samples and standards were not amplifying or did not appear on the amplification plot altogether. This lack of amplification was attributed to there being too high of a concentration of rusty crayfish eDNA being used in the serial dilution of the standards. In the first two qPCR runs, the 1:10 serial dilution was performed using eight microcentrifuge tubes, from all of which 1 uL was used to plate into their corresponding wells.

However, in the final qPCR run for Houston Pond, twelve microcentrifuge tubes were used for the serial dilution, and the plating of these standards began with the fourth microcentrifuge tube. When the plated standards began with the fourth step in the serial dilution, all showed amplification. To determine the reason behind this change, we examined the multicomponent plot of the spiked samples and standards that were originally not showing any amplification in the first two assays. With this, we found that their concentrations of rusty crayfish DNA began at such a high concentration, very little amplification was able to occur, leading to a lack of amplification in these wells in the amplification plot. This is demonstrated by **Figures 12** and **13** below.

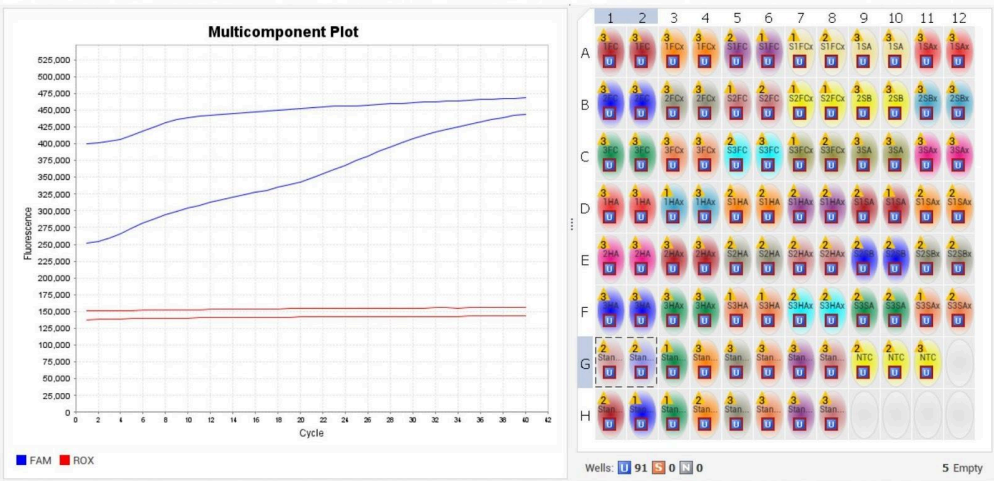

Figure 12. The multicomponent plot for the first two standards in the *first* Stream vs. Pond assay performed on June 24, 2024.

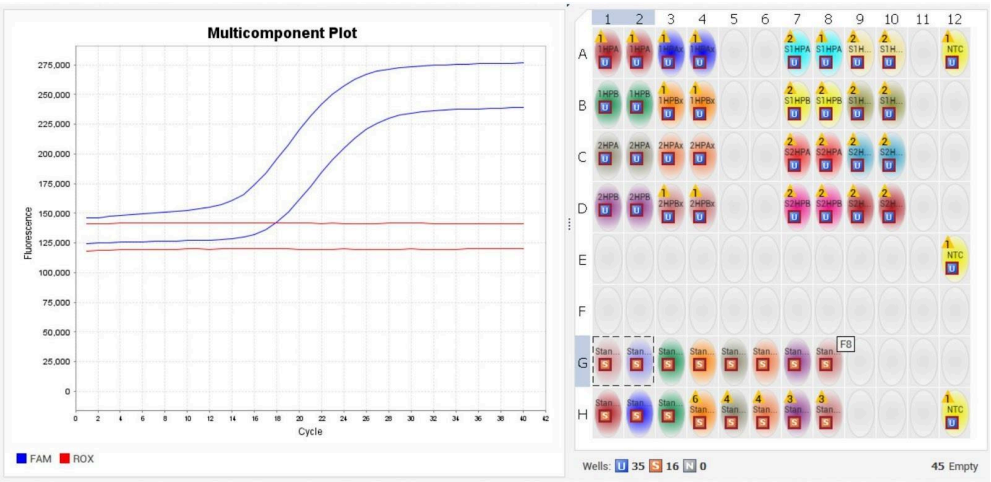

Figure 13. The multicomponent plot for the first two standards in the Houston Pond assay performed on July 12, 2024.

As we prepare for next summer's work in Malaysia to conserve the elusive Crocus clam, we will apply these takeaways regarding proper decontamination, plating techniques, and sources of contamination. In this, we will continue following the appropriate DNA decontamination procedures in each step of our methodology, but also make sure to apply these results regarding the sources of contamination in our assays to achieve qPCR analysis results we can be confident in when tracking the presence of our target species in the marine environment.

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