Finding the optimum sample volume for eDNA detection of *Faxonius rusticus* (Rusty crayfish)

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Abstract

In this paper, we aim to find the optimum volume for detecting the presence of *Faxonius rusticus* in the streams and ponds in Ithaca, NY. To do this, we collected environmental DNA by filtering water, extracting, isolating and amplifying the DNA then running a quantitative PCR. There was no observable difference between the different volumes used. Though we detected the crayfish in all the samples, there was contamination in our field blanks, so we could not be confident in our results. However, we still recommend using a volume of 250-300ml when using eDNA to detect the presence of the *Faxonius rusticus*.

Introduction

Environmental DNA (eDNA) fragments are deposited into the environment (soil, water column, ground, etc) via cellular material shed by organisms such as mucus, feces and skin. eDNA can be used as a detection tool for species including invasive and difficult to detect organisms (Dougherty et al., 2016). Due to the nature of the cellular material, this method of detection is noninvasive and can, in some cases, be more effective than traditional detection methods (Jerde et al., 2011).

Because of the increasingly widespread use of this mini barcoding method, it is paramount that there is a level of standardization in how eDNA is being used in detection of species. This has led to the development of protocols such as the United States Fish and Wildlife Services Best Management practices (Bockrath et al., 2023). Guidelines such as these promote uniformity and confidence for researchers carrying out this novel species detection method. However, due to the precise nature of capturing eDNA and using it as a detection tool, the protocol must be altered on a case-by-case basis because of the fundamental differences that lie between each species and ecosystem. In addition to those differences, there are multiple factors to consider while carrying out this type of research including time of year, type of environment, weather, climate and access to resources.

In preparation for eDNA work on a cryptic and tropical marine invertebrate, our aim in this study was to first gain a sound understanding of eDNA and how it can be used to detect the presence of the Rusty crayfish in the streams and ponds of Ithaca, an abundant freshwater invertebrate. Working on a readily sighted and abundant species in the stream allows us to focus on learning the technique without worrying about false positives. Secondly, we investigated sampling volume; the use of active and passive sampling and testing the eDNA assay on a site where the crayfish presence is not confirmed. Exposure to these sampling aspects will allow us to design a suitable sampling strategy for *Tridacna crocea*. This research paper will be focusing solely on the optimal volume of sample collection from streams.

There have been multiple studies involving eDNA carried out on different organisms in both lentic (still waters) and lotic (moving water) ecosystems, each study has used different volumes of water with the average range from 250mLto 2L volumes (Mähler et al. 2015). Samples as large as 34L have been used in some eDNA studies, however, it was found that after a certain volume, there is not a significant increase in the efficiency of eDNA per unit of sample volume (Capo et al., 2019). There have also been studies which have used 15mLsuch as the study which aimed to track the southern river terrapin, an endangered organism. The researchers in this study were able to successfully detect the presence of Terrapins which were within 1km of the sample site (Wilson et al, 2017). Furthermore, a study investigating practical ways to carry out eDNA research found that 100ml syringe filtration coupled with ethanol sodium acetate precipitation to be the most efficient method in detecting fish species in both lentic and lotic water bodies (Muha et al, 2019). From this, we can see that smaller sample volumes are possible for detecting rare organisms, and the sample volume efficacy depends on the project being worked on.

For most of these studies, the main determining factor for the volume chosen is based on the logistics and the resources available (Mähler et al. 2015). For example, some field sampling sites may be a long distance away from the lab so transporting large volumes of water could be a potential issue, specifically with regards to cryptic organisms where a higher volume of water is potentially better as their exact location is not certain. Additionally, researchers must also consider that the eDNA degrades over time and in certain conditions, the rate of degradation can increase (Lamb et al., 2022). For example, tropical marine environments are at a higher risk of environmental degradation due to the UV radiation from the sunlight. Therefore, this information tells us that the volume chosen for eDNA studies has a big influence on the logistical feasibility and the quality of the research being carried out.

Methods

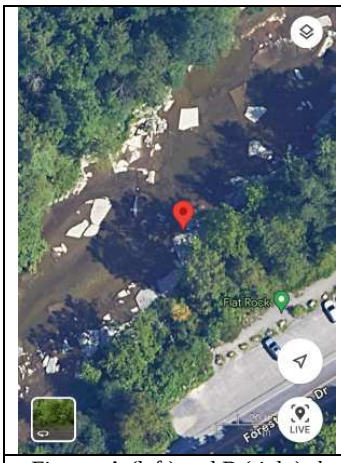
2.1 Study Species

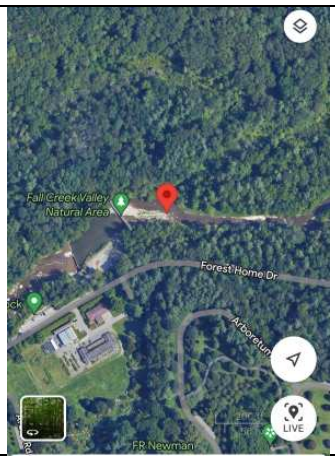
The *Faxonius rusticus* (Rusty crayfish, FaRu) is an invasive species that is native to the Ohio River Basin. They were first documented outside the Ohio River Basin as early as 1897 but widespread occurrence was not recorded until 1990s through to the 2000s (Kvistad et al., 2021). The introduction of these Rusty crayfish species into other water bodies is thought to be mainly due to anglers using the crayfish as bait (Olden et al., 2011). The Rusty crayfish can be found in the ponds and streams in Ithaca, NY (US Geological Survey, 2017 and 2020).

2.2 Sample collection and filtration

The sampling method for this project was adapted from an existing protocol from FishTracker, a local citizen science program. The sampling visits were done between June 24th and June 26th with each volume taken at the same location on Fall Creek in Ithaca, NY (coordinates:

42.4551391, -76.454919). Four volumes were collected (0.5L, 1L, 1.5L and 2L), each with three replicates collected on different days of the week. Between July 8th and July 9th, the sampling protocol was repeated on Fall Creek but a few hundred meters upstream (coordinates: 42.4538346, -76.4567406), with some modification to the numbers of samples and the volumes of samples being collected. There was no significant difference between the different volumes tested in the June samples, therefore we were interested in seeing whether smaller volumes still detect the crayfish. The volumes collected for the July samples were 300mL, 1L and 1.5L.





Figures A (left) and B (right) show the sampling locations in Ithaca NY. The coordinates are (42.4551391, -76.454919) and (42.4538346, -76.4567406) respectively.

Extensive steps were taken to ensure that the sampling equipment is free of any carryover FaRu DNA, also known as DNA contamination. The bottles used to collect the water samples were autoclaved the day prior and kept in an oven until the time of use. Also, prior to filtration, the Buchner funnel used was soaked in concentrated 8.25% household bleach for at least 30 minutes and rinsed in distilled water, the rest of the equipment was sprayed with 70% ethanol and wiped down. Immobilon PVDF filter paper (Millipore Sigma) was used with a pore size of 0.2um.

Figure C, set up of the sampling station on the field site. Figure D shows sampling station in lab. The filter paper goes in the Büchner funnel (the white funnel), water sample is poured down the funnel and the blue hand pump is used to create vacuum in the flask that will draw the water through the filter.

Upon arrival at the field site, the autoclaved bottles were used to collect the sample water from the streams' edge. The field blanks were filtered first; this process involved filtering distilled, autoclaved water using the set up shown in figures C and D. The autoclaved collection bottles were always handled with sterile nitrile gloves. After collection, the samples were taken back to the laboratory for filtration. There was a 30-minute travel time between the site of collection and the site of filtration at the laboratory. Additionally, we used Zip loc bags to collect water samples as they were presumably free of FaRu DNA since they were new, cheaper, more readily available (as they did not have to undergo the autoclaving process). However, we could only use the Zip-loc bags when we sampled on the field site, as they leak water when being transported

over distances, therefore we used the autoclaved bottles when sampling in the lab. Moreover, because the field blank samples were autoclaved, they had to be carried in the glass bottles.

Upon arrival into the laboratory, the samples were immediately filtered and those waiting were put in a refrigerator at 4° Celsius. The maximum waiting time for these samples was 1.5 hours. The filtration setup, shown in Figure C, consisted of a Buchner funnel secured into a conical flask with a rubber stopper. A tube off the side of the conical flask was then connected to a hand pump, which created a vacuum when pumped, drawing the water through the filter paper. The apparatus was secured on a stand. The forceps and hemostat used to handle the filter paper were soaked in 8.25% bleach when not in use and rinsed with distilled water before use. After filtration, the filter paper was transferred into a sterile 15mL conical tube containing 4mL of Longmire Buffer solution and stored in a fridge with temperatures of 4°C until DNA extraction.

2.3 DNA extraction and qPCR

The QIAAmp DNA mini kit (Qiagen, Hilden, Germany) containing the AI buffer, proteinase K, AW1, AW2 buffers, and spin columns was used to carry out the extraction. The 15mL conical tubes containing the filter paper and Longmire's preservation buffer were warmed at 65°C for 10 minutes then vortexed for 5 minutes to shake out the DNA fragments from the filter paper. 400uL of the solution from each 15mL conical tube was collected for extraction of each sample. 20uL of Qiagen proteinase K was added to each tube and the samples were vortexed for 10 seconds; 200uL of AI buffer was added to each tube then vortexed for 10 seconds; 400uL of 100% ethanol was added to each test tube and vortexed for 10 seconds. The solution was then added to a spin column and centrifuged for 1 minute at 6000g. 500uL of buffer AW1 was added to the solution then centrifuged at 6000g for 1 minute; 400uL of AW2 was added then centrifuged at 17000g for 3 minutes. The solution was further centrifuged at 17000g for 1 minute

until there was no more residue on the collection tube. 100uL of RNase free water was left to incubate for 5 minutes and then centrifuged at 6000g for 1 minute. The columns were then labeled and stored in a fridge at a temperature of -20℃.

Quantitative PCR (qPCR) was carried out on a 96- well plate. We prepared a master mix solution which contained 7.5uL of Applied Biosystems™ TaqMan™ Environmental Master Mix 2.0 (Thermofisher, Waltham, MA, USA), 0.03uL of TaqMan® probe (Thermofisher, Waltham, MA, USA) with the purpose of emitting a fluorescent signal during the DNA amplification process in the qPCR, 6.4uL of RNase-free water, forwards and reverse primers (0.03uL each). The primer pair design targeted the FaRu mitochondrial DNA and was based on the work of Larson et. al 2017. Mitochondrial DNA was chosen as opposed to genomic DNA in this study because an organism's cell naturally has more mitochondria and only one nucleus. Mitochondrial DNA has higher copy numbers therefore increasing the probability of detection in qPCR. The volumes of the components of the master mix previously mentioned were measured per reaction. Therefore, each well contained 1uL of the extracted DNA, and 14uL of the Master Mix solution resulting in a total volume of 15uL. Standards are synthetic sequences of short fragments of the FaRu DNA that will allow for the absolute quantification of eDNA copy numbers. To create our standards for this experiment, we tested sequences using a computer model (PrimerQuest™ Tool) and the sequence was developed from Coster et al 2017. The standard was diluted 1:10 eight times to create a standard curve. Additionally, for each one of the samples being tested we included a spiked sample which consisted of 1uL of the sample, 0.5uL of the FaRu Standard and 4uL of RNase free water. Spiked samples contain a concentrated amount of the DNA/RNA sequence section investigated in a qPCR analysis, they are included in the assay as a positive control, to check whether there were any inhibitors in our field samples. Some of the inhibitors which may

occur in streams include algae, glycogen and Fulmic acids (Schrader et al. 2012). qPCR controls were included on the well plate as No Template Controls (NTCs) made up of RNase-free water, this helps us to detect contamination in both our qPCR plating process and the material used to plate the DNA. Quant Studio 3 Design & Analysis Software was used to set up the parameters of the qPCR run and to map the well plate. For the fluorescence to be detected in the qPCR machine, the DNA goes through thermal cycles which is a process of repeatedly changing the temperature to achieve certain results. Thermal cycling conditions included 2 minutes at 50°C and 10 minutes at 95°C. This was then followed by 40 cycles of 15 seconds denaturing at 95°C and 1-minute extension at 60°C.

Figure E shows the stages of the thermal cycle.

Figure F shows the plate map designed on Quant Studio 3 Design & Analysis Software for the second week of experiments.

Results

In both weeks of sampling, we detected the presence of the Rusty Crayfish in all our volume samples. However, in both weeks we also detected contamination in our field blank samples. In

the qPCR amplification plot, each one of the field sample blanks showed amplification curves above background levels. Two out of three of our no template controls also showed amplification in the first week and all three no template controls in the second week.

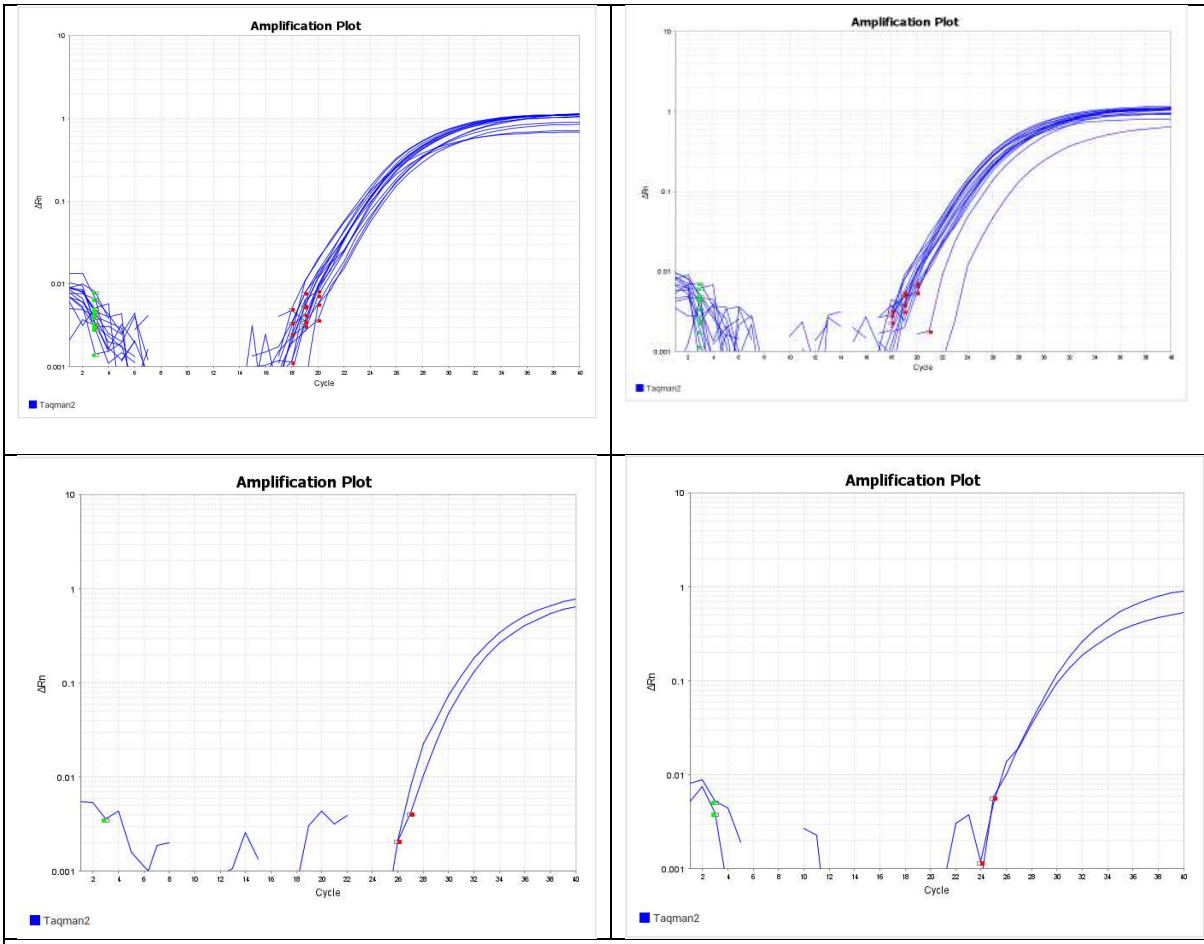


Figure G (top left): Sample amplification from week 1 results. Copy numbers start at 20 which indicates that there is contamination by the FaRu standards as copy numbers tend to be later (cycle 28-30). Figure H (top right) shows the field blanks which show amplification, further indicating that there is FaRu DNA contamination in the samples. Figure I (bottom left) show the amplification of the second week sample for 3oomL. Figure J shows the amplification of second week sample for 1.5L.

In both weeks' samples the first few standards showed no amplification beyond background levels, while the last four dilutions had normal amplification curves, with each dilution succeeding in cycle number where true amplification was detected. Upon further investigation of

this result, we looked at the multicomponent plot of the standard curves which shows the fluorescence at each cycle. It showed us that the initial concentration of FaRu standards was too high therefore there was only a slight increase in amplification throughout the qPCR run. This suggests that the initial concentration of the FaRu standards was too high and needed to be diluted more for proper amplification to be detected. Note that there was variation in the standards' amplification curves (i.e. the successive standards did not produce the successive curves that indicate a regular 1:10 dilution) in the second week. This is due to the inaccuracy of the pipetting.

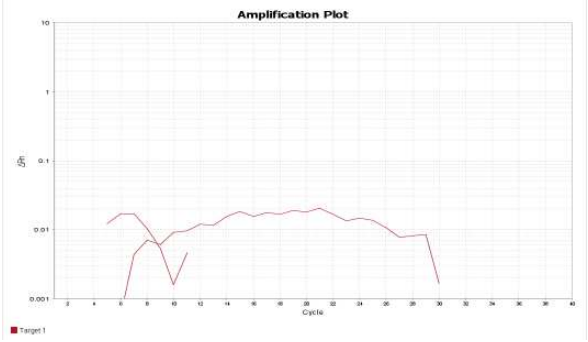
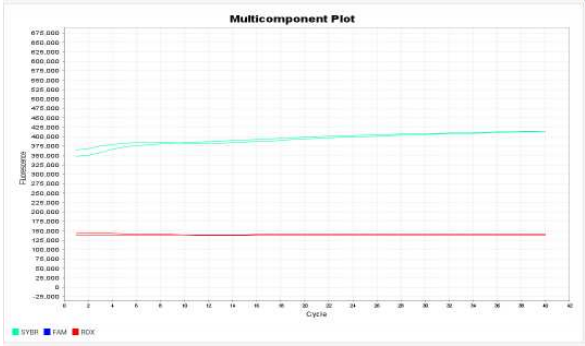
	

Figure G shows the plot for the first standards, which shows no proper amplification beyond background levels.	Fig H shows the multicomponent plot which indicated that the initial fluorescence levels were too high as there is no significant change as the cycle number increases.

In our method, we used the undiluted version of the FaRu standards in our preparation of the spiked samples and consequently, there was no amplification detected in the spiked samples in both weeks of sampling.

Discussion

Visual sighting of *Faxonius rusticus* confirms its presence in Fall Creek Ithaca, NY. The cycle threshold value (Ct) for sample volume of 300ml was around 30 cycles, similar to sample volume of 1.5L.

We also detected amplification in our field blanks and in our qPCR plating blanks (No Template Controls). This tells us that there was contamination in our method, and because of this, we cannot be certain that our positive sample plots are also not the result of contamination as well. We identified some potential flaws in our decontamination protocol after the first segment of the research project. Some of the main contamination points are because we did not carry out our plating in a Biosafety cabinet, a tube opener was not used to open the dolphin tubes which may have caused aerosolization of the FaRu DNA standards, and we did not cover sections of the plate while using FaRu DNA standards. Because of our mishandling of the standards, we suspect that there may have been some carry-over contamination whereby the initial contamination by aerosolization gets on multiple surfaces, gloves and pipetting tools causing contamination in all reagents/materials which are handed afterwords (Chanplot et al., 2010).

Upon further validation with regards to the contamination of our results, we run a qPCR analysis on the primers, master mix and probe to test whether there was any amplification. It was found that the primers were contaminated with FaRu standards, the probe and master mix were also contaminated but did not have as many copy numbers as the primers. This tells us that they all contained some degree of contamination which would explain our results. Though we went through a thorough decontamination process, specifically in the second week of our experiment, qPCR is a highly sensitive process, and any contamination will be amplified and carried onto the next assay.

If we were to carry out this research further, strict measures regarding the use of the standards would be put in place. This involves storing and handling the standards in a separate room, with separate pipettes, gloves and lab coats. After this, cover the plate with tin foil while plating everything else. Additionally, we recommend using an aliquot of a much less diluted version of the standard. We plan on carrying out our research further on the Giant Clam (*Tridacna crocea*) found mainly in the Indian Ocean, Western Pasific and South China Sea (Li et al., 2024). Because it will be a marine environment, we must consider factors such as eDNA degradation due to heat, humidity and UV radiation given the distance and method of travel between the sample sites in the ocean and the lab where the sampling will be carried out. We would strongly consider filtering water samples as soon as logistically possible. Once filter papers are in Longmire buffer, they can be placed in cooler box during transit. This giant clam is often found in shallow depths (0-20m) and buried in corals (Ikeda et al. 2017). Moreover, the spawning season, where there is likely to be a higher amount of eDNA in the water due to the release of eggs, has been found to be between April and July (Shaw-Huai et al. 1998). With this in mind, we can use methods such as sounding weights and satellite data to sample from shallow waters containing coral beds as well as sample in the months between April and July. With close adherence to the life cycle and habits of the giant clam I believe that we can use volumes as small as 250ml to detect the presence of *Tridacna crocea*.

Despite the lack of certainty about our positive results, we believe that volumes as small as 250ml and 300ml are sufficient for detecting the presence of the abundant *Faxonius rusticus* based on the literature present and the observed sample results (Muha et al, 2019).

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