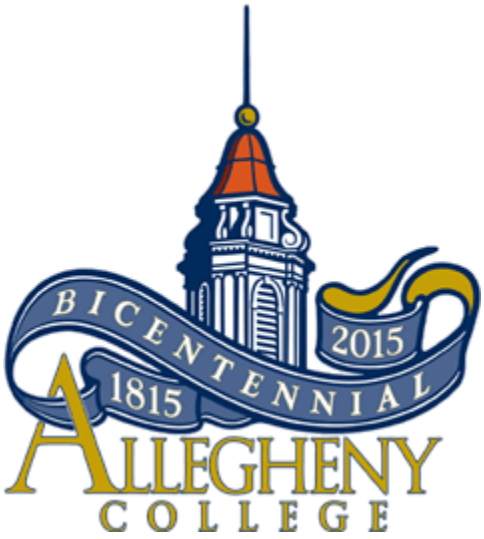
Quantifying the Relative Abundance of the Invasive Species *Neogobius melanostomus* throughout the French Creek Watershed via an Environmental DNA Analysis

By

Eric Michael Conners

May 3rd, 2016



Allegheny College

Department of Biology

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A Senior Comprehensive Project in Partial Fulfillment of the Requirements
for a Bachelor of Science Degree from Allegheny College

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.

_____

Pledge

Approval by the Senior Thesis Committee:

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Dr. Kristen Webb

_____

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Abstract

Recently, environmental DNA (eDNA) studies have emerged as an efficient and reliable method of detecting and quantifying the presence rare aquatic organisms, such as aquatic invasive species (AIS). One AIS, the round goby (*Neogobius melanostomus*), has proliferated throughout the Great Lakes region and has recently been sighted within the French Creek watershed (Waterford, PA). In this study, water samples from three locations within the watershed were analyzed for the presence of round goby eDNA. Additionally, negative controls were prepared to test for cross-contamination, while positive controls were analyzed to ensure the efficacy of the protocol. Quantitative PCR failed to amplify the target gene fragment in all environmental samples. Positive controls were successfully amplified and four negative controls exhibited evidence for the presence of round goby DNA. Thus, this study presents no molecular evidence for the organism's presence in the watershed. This is likely the result of false-negatives, as direct observation contradicts this conclusion. These false-negatives are likely due to insufficient concentrations of the target marker in the samples, degradation of the target marker in the environment, or poor project design. Future studies should investigate these variables in order to optimize the eDNA protocol.

Introduction

The effective management of rare species is essential to conservation efforts, as these typically include endangered native and introduced non-native organisms (Jerde et al. 2011). Early detection of non-native species is especially important, as these have the potential to integrate themselves within the existing ecosystem and negatively impact biodiversity through predation (Yonekura et al. 2007), competition (Blanchet et al. 2007), hybridization (D'Amato et al. 2007), habitat modification (McDowall 2006), and transmission of novel disease (Gozlan et al. 2006). For example, introduced predatory fish in the Little Colorado River preyed upon the native Humpback chub such that the population was at risk of severe depletion. From July 1991 to June 1995, monthly

river samplings indicated that Humpback chub comprised almost 14% of the non-native predators' diets (Douglas 1997). Management efforts aimed to control invasions such as this prove to be more time- and cost-efficient when the species is present at low densities and has yet to disperse (Gozlan et al. 2010). Despite the need for time- and cost-efficient detection procedures, traditional methods such as the use of nets and electrofishing are at a severe disadvantage, as they are most successful when the target organism is present at moderate to high density. This is especially true of aquatic environments, where organisms are not readily visible. Therefore, the use of traditional surveillance techniques proves to be challenging when the target organism is present at low frequency (Jerde et al. 2011). Typically, increased sampling effort is necessary to negate the low detection probability for rare species (McDonald 2004). This is generally infeasible, as these methods are time-consuming and cumbersome.

To combat the inefficiencies of traditional aquatic species surveillance methods and increase the chances of early detection, molecular tools have been applied as alternatives. In particular, environmental DNA (eDNA) has emerged as a promising counterpart to traditional observational methods. Environmental DNA refers to nuclear or mitochondrial DNA that is released from an organism via feces, skin, hair, carcasses, or scales (Ficetola et al. 2008). Consequently, eDNA can be extracted from aquatic and semi-aquatic environments for subsequent analyses, including species detection (Rees et al. 2014). To date, dozens of studies have utilized this approach of collecting and analyzing eDNA samples across a variety of organisms including reptiles (Piaggio et al. 2013), arthropods (Thomsen et al. 2012a), amphibians (Ficetola et al 2008; Goldberg et al. 2011), and fish (Dejean et al. 2011; Jerde et al. 2011). In general, short mitochondrial DNA markers are targeted for detection, as these allow for accurate and rapid species identification. More so, the mitochondrial markers that are targeted are typically short (90-150 base pairs), as larger fragments are less likely to remain intact upon being secreted into the water due to the rapid degradation of eDNA.

Following extraction, the target fragment is amplified via polymerase chain reaction (PCR) utilizing species-specific primers; successful amplification of the target fragment provides evidence for the presence of the species of interest (Rees et al. 2014).

The application of molecular protocols based on eDNA is especially attractive for the management of invasive species, as these procedures allow for detection before the invading population becomes established or widely dispersed (Nathan et al. 2014). For example, bighead carp (*Hypophthalmichthys moltrix)* and silver carp (*Hypophthalmichthys molitrix*) (both will be referred to as Asian carps hereafter) have threatened the Great Lakes for over 25 years. Observational evidence suggested the low presence (bighead) or complete absence (silver) of Asian carps in the Chicago Area Water System (CAWS), a waterway branching off of Lake Michigan. However, a molecular analysis of water samples taken from the CAWS indicated the presence of Asian carps throughout the water system (Jerde et al. 2013). In a similar study, researchers investigated the distribution of the invasive bluegill sunfish (*Lepomis macrochirus*) in 70 ponds across seven locales on the Japanese mainland and the surrounding islands. Of the 70 ponds studied, 19 were confirmed to contain bluegill sunfish eDNA; previous observational studies had indicated that only eight of the ponds harbored the animal (Takahara et al. 2013). In summary, molecular analyses provided evidence for the presence of aquatic organisms where direct observation failed to do so.

Given that eDNA is a newly emerging approach to rare aquatic species detection, there are a handful of challenges and unknowns with respect to each step of the process that researchers must take into consideration. In particular, aquatic eDNA is unique in terms of its physical and biochemical interactions with the environment. For example, aquatic eDNA is prone to degradation due to the presence of DNases (enzymes that degrade DNA), radiation, acidity, and heat in the external environment. In addition, PCR inhibitors tend to be present in eDNA samples, thus impacting the efficacy of molecular analyses (Pilliod et al. 2013). Furthermore, lotic systems, i.e.

moving bodies of water, introduce numerous variables that can impact eDNA detection. These include but are not limited to the system's rate of flow as well as the distance the eDNA is carried from the source organism; lotic systems have the potential to carry viable genetic material up to and potentially beyond 240 m from the source organism (Jane et al. 2015). Additionally, the type of molecular analysis to be conducted must be taken into consideration. Generally, an eDNA study involves the collection of an environmental sample, extraction of eDNA, and the use of PCR to amplify the target gene. However, there exist three categories of PCR, each with various pros and cons: end point (traditional) PCR, quantitative PCR (qPCR), and digital droplet PCR (ddPCR). In the context of an eDNA study, traditional PCR can only be used to detect the presence of a particular species, while qPCR and ddPCR utilize different protocols and technologies to quantify the amount of target gene; these data can then be extrapolated to predict the abundance of the target species (Doi et al. 2015). Furthermore, both qPCR and ddPCR are more sensitive than traditional PCR, as they have been shown to detect as little as one copy number of target DNA (Doi et al. 2015; Takahara et al. 2013). This is especially important when the target organism exists in low quantities, as is the case when attempting to detect newly introduced invasive species (Turner et al. 2014).

This study will apply some of the aforementioned molecular techniques in order to detect the aquatic invasive species (AIS) *Neogobius melanostomus*, or the round goby, which represents a significant concern for conservation biologists. A bottom-dwelling fish, the round goby is native to the Ponto-Caspian region (Black Sea, Sea of Azov, and Caspian Sea) and was discovered in the Laurentian Great Lakes in June 1990 (Corkum 2004). Although the origins of the founding source population of the round goby in North America are unknown, it is recognized that it spread to all five Great Lakes in just five years. This presents numerous concerns for conservationists, as the round goby is known for its voracious feeding habits, ability to efficiently colonize thanks to high fecundity and a wide tolerance of abiotic factors, ability to transfer contaminants through the food web, and

sizable economic costs. Specifically, the spread of round gobies throughout the Great Lakes has resulted in the decline of mottled sculpin (*Cottus bairdii*) and logperch (*Percina* caprodes) and reduced the hatching success of native fishes by feeding on their eggs (Corkum 2004). One of the primary vectors of dispersal for invasive species such as the round goby is the commercial bait trade. Non-bait species can be distributed to retailers, sold to anglers, and disposed of by the anglers as excess bait either intentionally or unintentionally. Despite regulations prohibiting the capture and dispersal of non-bait species, 109 non-native fish introductions in the United States, including 18 in the Great Lake states, can be accounted for as a result of this behavior (Nathan et al. 2014). An eDNA based study of 525 retail bait shops throughout the Great Lake States confirmed the presence of at least one AIS for 27 samples, 8 of which showed evidence of round goby presence (Nathan et al. 2014). While this suggests that *N. melanostomus* are being spread via the commercial bait trade, there have been no eDNA studies of source water systems with respect to this animal. Thus, an eDNA-based study of round goby dispersal throughout the French Creek watershed is not only a novel application for this species, but a crucial step toward enacting meaningful conservation efforts in the area.

Recently, the Pennsylvania Fish and Boat commission called upon anglers to address the spread of the round goby following a sighting in French Creek near Lake LeBoeuf (Waterford, Erie County, PA) ("Anglers urged," 2014). The Fish and Boat Commission posted notices informing anglers of existing laws that forbid the transport of round gobies and requested that anglers who catch them dispose of them in trash bins. Of particular concern was the potential for the fish to spread into French Creek. French Creek is known to contain threatened and endangered fish and freshwater mussel species as well as being one of the most ecologically diverse waterways in the Commonwealth ("Anglers urged," 2014). The Creek boasts more species of fish and freshwater mussels than any other similarly sized stream in the northwest United States. In total, there are 29

known freshwater mussel species, including two known to be endangered and 13 others considered threatened or endangered, as well as over 80 species of fish, including several threatened or endangered madtom, lamprey, and darter species (Smith et al. 2009). Numerous threats to these species exist, including invasive organisms such as the round goby.

Recent sightings suggest that round gobies have dispersed throughout Lake LeBoeuf to the mouth of the creek. In addition, round gobies were positively identified alongside Mystic Road, approximately six miles south of Lake LeBoeuf (personal correspondence, C. Bradshaw-Wilson). Based upon these observations, it is suspected that the round goby has migrated beyond Lake LeBoeuf into the main stem of French Creek (Figure 1) (personal correspondence, C. Bradshaw-Wilson). However, it is unclear what the distribution of fish may be throughout these areas. Furthermore, the extent to which the animal has migrated is unclear. Finally, while observational evidence has suggested that the round goby has moved into the main stem of French Creek, an eDNA study of this species has yet to be conducted on this body of water.



Figure 1. The portion of the French Creek watershed of interest. Round gobies have been sighted in Lake LeBoeuf (yellow) and along Mystic Road (purple).

This study attempted to answer questions regarding these unknowns using a molecular analysis of water samples taken from French Creek in order to corroborate evidence provided by direct observation. A molecular analysis of water samples collected from two locations within the watershed sought to elucidate the relative abundance of gobies throughout this portion of French Creek. It was expected that a molecular analysis of water samples collected from locations near Moore Road and French Creek Road, which are located south of Lake LeBoeuf, would provide evidence for the presence of round gobies in these areas. Furthermore, it was expected that the eDNA analysis would indicate that the abundance of gobies is decreasing further south of Lake LeBoeuf. Though inconclusive, these data highlight several important considerations when designing an eDNA study. A further refined molecular approach can be used in conjunction with evidence provided by observational studies utilizing traditional techniques in order to guide conservation efforts with the intention of controlling the spread of round gobies throughout French Creek. Furthermore, while the protocol detailed here is specific to *N. melanostomus*, the underlying principles can be applied to a wide variety of aquatic organisms. Thus, this study will serve as a benchmark for future eDNA-based studies at Allegheny College.

Methods

Study Area

The area of interest is a portion of the French Creek Watershed in Northwestern Pennsylvania. A negative field control was collected from French Creek near the Kenneth A Beers Jr. Bicentennial Park in Meadville, PA (GPS coordinates: 41.638, -80.162; not pictured), as it is suspected that round gobies have not migrated this far south in the watershed (personal correspondence, C. Bradshaw-Wilson). Thus, samples collected from this location should not contain round goby eDNA and therefore would not exhibit molecular evidence of goby presence. The presence of gobies has been confirmed as far south as Mystic Road (personal correspondence, C.

Bradshaw-Wilson) and as far north as LeBoeuf Lake ("Anglers urged," 2014) (Figure 2). Thus, experimental samples were collected along French Creek Road (GPS coordinates: 41.896, -79.989) and Moore Road (GPS coordinates: 41.909, -79.986), as these represent sites of suspected, but not confirmed, round goby presence (Figure 2). The French Creek Road sampling location is approximately 1,220 m downstream from the Moore Road site; the Moore Road site is approximately 3,200 m downstream of LeBoeuf Lake (Google® Maps 2016). Sampling sites were chosen so as to minimize the probability that positive detections were the result of genetic material being carried from one site to another via water flow while still being informative in terms of population location and density.

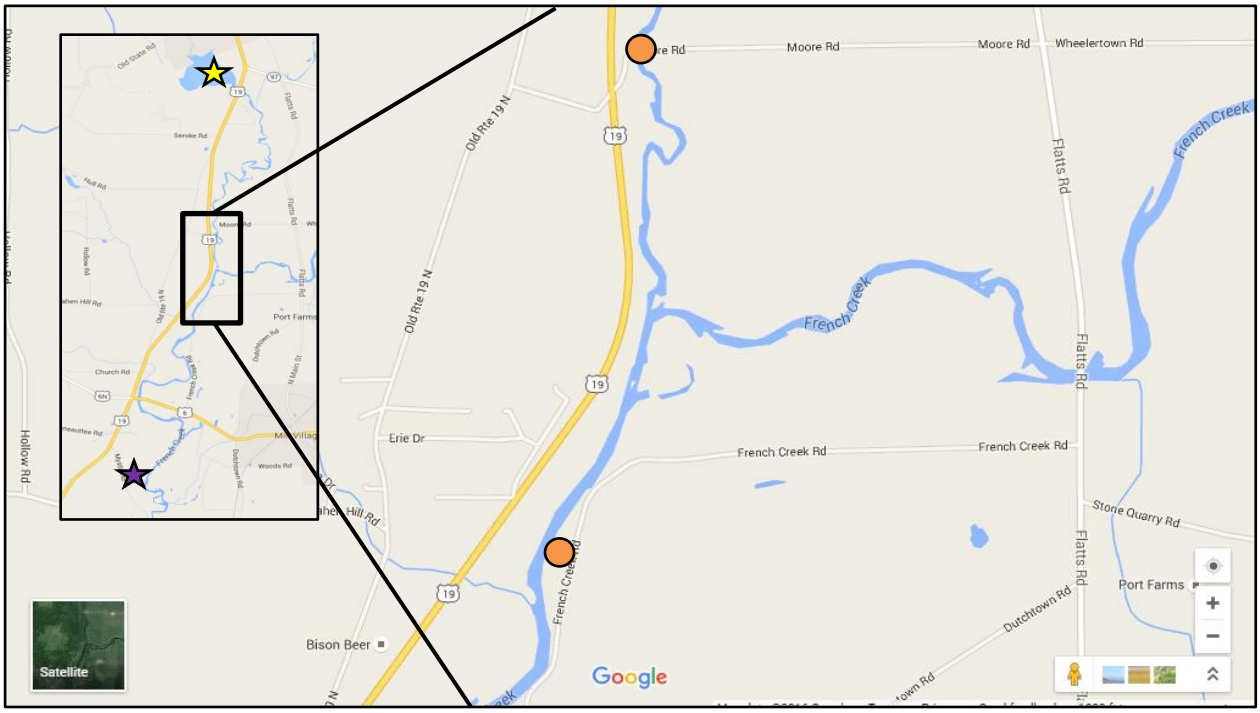


Figure 2. Round gobies have been sighted in Lake LeBoeuf (yellow star) and along Mystic Road (purple star). Sampling locations are represented as orange circles. An area along French Creek Road and the location at the intersection of the creek and Moore Road were sampled.

Collection and Filtration of Environmental Samples

Sample collection procedures mirror those of Jerde et al. (2011). From October to November, 2015, twelve 500 mL surface water samples were collected in autoclaved 500 mL Nalgene® bottles in addition to field controls. Nitrile gloves were worn during collections. Autoclaved Nalgene® bottles were opened and submerged below the surface in the middle of the creek to collect field samples. Field controls consisted of Nalgene® bottles filled with distilled water prior to leaving the lab that were opened at the sampling location, closed, and submerged below the surface of the water. This ensured that any subsequent positive detections were the result of valid sampling and not due to cross-contamination before or after sampling. Each location was sampled on different days and sampling equipment was sterilized in between sampling events via bleach soaking (waders) or autoclave (Nalgene® bottles) to prevent cross contamination. GPS coordinates for each collection site were approximated post-collection using Google® Maps. Five 500 mL water samples were associated with each collection site: four surface water samples (2 L total) and a fifth field control. Samples were transported from the collection site to Steffee Hall (Allegheny College, Meadville, PA) on ice in a cooler. Finally, multiple positive controls were prepared in the laboratory by placing one round goby in a 15 mL centrifuge tube filled with distilled water and allowed to sit overnight.

All samples were vacuum-filtered within 24 hours of collection using a peristaltic pump through 1.2 µm pore size GF/C glass microfiber filters (Whatman, GE LifeSciences, Pittsburgh, PA). Prior to filtration, all equipment that would come in contact with filter paper was sterilized via a 10 minute bleach soak. For each sample, an equipment control was processed prior to experimental samples and field controls; this consisted of a 2 L sample of distilled water that was run through the vacuum filtration apparatus and a piece of filter paper. This served as an additional negative control in order to ensure that subsequent positive detections were the result of goby eDNA being present

in the original sample, rather than as a result of cross-contamination with the apparatus. All filters containing sample filtrate were placed in individual 15 mL centrifuge tubes and stored at -20°C until further processing.

Extracting DNA from Liver Tissues and Filter Paper

Approximately 100 round gobies were caught in French Creek by Dr. Casey BradshawWilson. Livers were dissected from three fish. Extraction of genomic DNA (gDNA) from goby liver tissues was accomplished using the DNeasy Blood and Tissue Kit following the Animal Tissues Spin-Column protocol (Qiagen, Valencia, CA). A 20 µL RNase A treatment was applied to each tissue extraction. Additionally, a negative control was prepared with all reaction components except for starting material (i.e. liver tissue). Extraction of eDNA from filter paper followed the procedure laid out by Goldberg (2015) using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Prior to beginning the extraction, the bench space was sterilized with 50% ethanol and two pairs of tweezers were soaked in a 50% bleach solution for 10 minutes. Filter paper was bisected using tweezers, with one half being used for extraction and the other half refrozen. The half used for extraction was torn into smaller pieces and placed into 180 µL Buffer ATL and 20 µL Proteinase K for an overnight incubation at 56° C. Following incubation, samples were transferred to Qiashredder spin columns (Qiagen, Valencia, Ca) and centrifuged for two minutes at 11,000 rpm. Then, 200 µL Buffer AL was added to each sample, at which point each sample was incubated at 70°C for 10 minutes. Following this incubation, 200 µL of 95% ethanol was added to each tube and the mixture was added to a DNeasy spin column (Qiagen, Valencia, CA) and centrifuged at 8,000 rpm for one minute. Spin columns were transferred to new collection tubes and 500 µL AW1 was added to each sample, followed by centrifugation at 8,000 rpm for one minute. The columns were transferred to new collection tubes, washed with 500 µL AW2, and centrifuged at 11,000 rpm for 3 minutes. Finally, eDNA was eluted

with 100 µL of Buffer AE (preheated to 70°C); after adding Buffer AE, the samples were incubated at room temperature for five minutes and centrifuged at 8,000 rpm for 1 minute.

Following extraction, Nanodrop spectrophotometry was conducted to estimate the concentration of gDNA or eDNA within each sample. Initial samples were run on a 1% agarose gel made up of 50 mL of 1x TBE buffer, 0.5 g agarose, and 5 µL SYBR Safe DNA gel stain for approximately 45 minutes at 74 volts to confirm the successful extraction of gDNA or eDNA. Subsequent samples were only subject to Nanodrop spectrophotometry to confirm sufficient concentration and 260/280 ratios.

Amplification of the COI Gene in Round Gobies via Quantitative PCR

Quantitative PCR was used to analyze both liver tissue extracts and filter paper extracts with the purpose of estimating the amount of goby eDNA present in environmental samples. All qPCR analyses were conducted using a StepOne™ Real-Time PCR Thermal Cycler and the corresponding StepOne™ Software (ThermoFisher Scientific, Chardon, OH). Previously published primers were used to target a 150 bp section of the mitochondrial cytochrome c oxidase subunit I (COI) gene (Table 1) (Nathan et al. 2014). Before the filtered environmental samples with unknown amounts of round goby eDNA were analyzed with qPCR, a standard curve was generated using a liver tissue extract. The tissue extract was added to the reaction wells such that each reaction well contained known amounts of DNA: 200 ng, 100 ng, 25 ng, 6.25 ng, or 0.06 ng. Each reaction consisted of 30 µL of Power SYBR Green PCR Master Mix Kit (ThermoFisher Scientific, Chardon, OH), 0.25 µM of each primer, and the volume of DNA corresponding to the known amount of DNA. Molecular grade water was added to each reaction to bring the total volume to 60 µL. All samples were set up in triplicate; for each sample, a 60 µL master mix with all reaction components was divided into three 20 µL reactions that comprised the triplicate. The standard curve generated from these reactions was

used to estimate the quantity of round goby eDNA in qPCR runs of the filtered environmental samples.

Table 1. Primers used to amplify a 150 bp section of the mitochondrial COI gene (Nathan et al. 2014).

Primer	**Primer Sequence (5' -> 3')**
Goby COI-F2	CTTCTRGCCTCCTCTGGWGTTG
Goby COI-R2	CCCWAGAATTGASGARATKCCGG

Following successful amplification of the tissue extract to generate a standard curve, filtered samples underwent qPCR with slight modifications to the reaction mix: each well consisted of 10 µL of Power SYBR Green PCR Master Mix Kit (ThermoFisher Scientific, Chardon, OH), 0.2 µM of each primer, 5µL of DNA template, and molecular grade water to bring the total reaction volume to 20 µL. Thermal cycler conditions were as follows: 50°C for two minutes, 95°C for two minutes, 55 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds, followed by a continuous melt curve of 60°C for one minute raised to 95°C at increments of 0.3%. Quantitative PCR products were run on a 1% agarose gel made up of 50 mL of 1x TBE buffer, 0.5 g agarose, and 5 µL SYBR Safe DNA gel stain for approximately 45 minutes at 74 volts to confirm the amplification of the COI gene based on banding at the expected base pair (bp) size (approximately 150 bp).

Determining the Amount of Round Goby eDNA in Environmental samples Using a Standard Curve Constructed from Liver Tissue Extract qPCR Data

Raw qPCR data for liver tissue amplifications was exported as an Excel spreadsheet for analysis. Outliers within each triplicate were excluded; a Ct mean within a triplicate that was larger or smaller than the other two Ct values in the triplicate by more than 2.0 was considered an outlier. For each sample, the log of the amount of gDNA from liver tissue extracts (in ng) was plotted against the mean of the threshold value (Ct mean). The equation for the linear standard curve was then used to determine the starting quantity of eDNA in each environmental sample by plugging in each

Ct mean generated by each experimental triplicate for "y" and solving for the log() of the amount of eDNA, the inverse log() of which represented the starting quantity of round goby eDNA (in ng) in each environmental sample.

Results

Extraction of DNA from Liver Tissues

Genomic DNA extractions from round goby liver tissues were successful for three samples (Table 3). All DNA concentrations were above the minimum concentration requirement of 10 ng/µL and exhibited 260/280 ratios near 1.8 for pure DNA. Goby 7 yielded the highest concentration of gDNA and was utilized for subsequent qPCR reactions.

Extraction of Environmental DNA from Filter Paper

DNA concentrations were above 10 ng/µL and 260/280 ratios were near 1.8 for all but two samples, whereas equipment and field negative controls were below 10 ng/µL and 260/280 ratios

Table 3. Concentrations and purities of gDNA and eDNA extracts and corresponding controls

Sample Number	Sample ID	Concentration (ng/µL)	260/280 Ratio
n/a	Goby Liver Tissue 2	40.2	1.99
n/a	Goby Liver Tissue 7	57.8	1.95
n/a	Goby Liver Tissue 15	25.1	2.13
1	Lab Positive Control	201.4	2.14
2	Cabin Sample	37.4	1.49
3	Cabin Equipment Control	4.5	1.41
3	Cabin Field Control	3.0	1.55
5	Moore Road Sample	170.9	1.84
6	Moore Road Equipment Control	2.7	1.70
7	Moore Road Field Control	3.0	1.65
8	French Road Sample	90.8	1.63
9	French Road Equipment Control	3.3	1.59
10	French Road Field Control	3.1	1.34
Negative Control	n/a	2.2	0.83

lower than 1.8 (Table 3). The lab positive control and field samples indicated high concentrations of DNA with 260/280 ratios near or below 1.8. All negative controls (i.e. field and equipment controls)

returned very low concentrations of DNA and 260/280 ratios indicative of little to no DNA. Some 260/280 ratios are lower the expected threshold of 1.8 for pure DNA.

Constructing a Standard Curve from Liver Tissue Extract qPCR Data

Only three of the five triplicates were successfully amplified such that they fit the expected linear shape and were therefore used to construct the standard curve (Figure 3). Each of the three triplicates used to construct the standard curve produced amplicons of approximately 150bp as confirmed by agarose gel electrophoresis. The 200 ng and 100 ng triplicates resulted in Ct means well below the expected value and/or triplicates with high standard deviations within the Ct values such that a valid mean could not be calculated (Table 4). Therefore, only the 25 ng, 6.25 ng, and 0.06 ng triplicates were included in the standard curve.

Table 4. Ct means produced from various known quantities of DNA.

Sample	**Starting Quantity of DNA (ng)**	**log(starting quantity of DNA in ng)**	**Ct Mean**
Liver Tissue Extract 7	200	2.301	5.62[a]
Liver Tissue Extract 7	100	2.00	5.29[a]
Liver Tissue Extract 7	25	1.398	25.88
Liver Tissue Extract 7	6.25	0.796	27.06
Liver Tissue Extract 7	0.06	-1.222	32.33

[a] Ct means produced from the 200 ng and 100 ng samples did not fit the expected linear curve and were excluded from the standard curve

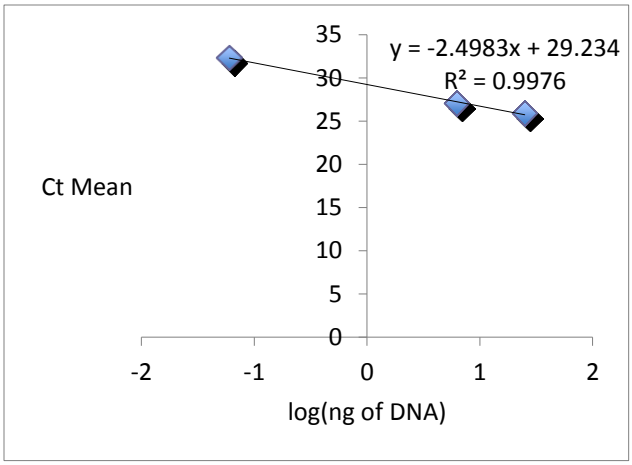


Figure 3. The standard curve generated from qPCR amplification of the COI gene in various quantities of round goby DNA.

Determining the Quantity of Round Goby eDNA in Filtered Samples

All environmental samples failed to produce detectable Ct thresholds, while the positive lab control produced a Ct mean of 51.2527, corresponding to a starting concentration of $1.538e^{-9}$ ng of DNA (Table 5). Visual inspection of the amplification plots generated by the StepOne™ Software confirmed that all experimental samples failed to amplify successfully. This indicated that the qPCR reaction was successful in generating detectable Ct values in a filtered sample known to contain round goby DNA (i.e. the positive control despite the failure to amplify the COI gene fragment in environmental samples). Additionally, two equipment control replicates and two field control replicates produced detectable Ct values; however, these were excluded as outliers, as the other two reactions in the triplicate did not (Table 5). Agarose gel electrophoresis confirmed the amplification of the COI gene in the lab positive control, as

Table 5. Quantitative PCR Results of the Lab Positive Control, Environmental Samples, and Negative Controls

Sample Name	Ct value	Ct Mean
Lab Pos Ctrl	50.85154	51.2527
Lab Pos Ctrl	51.65387	
Lab Pos Ctrl	Undetermined[a]	
Cabin Sample	Undetermined	N/A
Cabin Sample	Undetermined	
Cabin Sample	Undetermined	
Cabin Eq Ctrl	Undetermined	N/A
Cabin Eq Ctrl	Undetermined	
Cabin Eq Ctrl	23.80709[b]	
Cabin Field Ctrl	Undetermined	N/A
Cabin Field Ctrl	Undetermined	
Cabin Field Ctrl	Undetermined	
Moore Rd Sample	Undetermined	N/A
Moore Rd Sample	Undetermined	
Moore Rd Sample	Undetermined	
Moore Rd Eq Ctrl	Undetermined	N/A
Moore Rd Eq Ctrl	Undetermined	
Moore Rd Eq Ctrl	Undetermined	
Moore Rd Field Ctrl	Undetermined	N/A
Moore Rd Field Ctrl	Undetermined	
Moore Rd Field Ctrl	51.92508[c]	
French Rd Sample	Undetermined	N/A
French Rd Sample	Undetermined	
French Rd Sample	Undetermined	
French Rd Eq Ctrl	Undetermined	N/A
French Rd Eq Ctrl	Undetermined	
French Rd Eq Ctrl	21.76376[b]	
French Rd Field Ctrl	28.73798	N/A
French Rd Field Ctrl	Undetermined	
French Rd Field Ctrl	Undetermined	

[a] Replicate failed to amplify and was considered an outlier

[b] Equipment control replicate for the Cabin and French Road Samples produced a detectable Ct value and was considered an outlier

[c] Field control replicate for the Moore Road and French Road samples produced a detectable Ct value and was considered an outlier

qPCR products generated the expected banding corresponding to a qPCR product of length 150 bp (Figure 4). Varying band brightness is indicative of varying concentrations of target DNA in the qPCR product. In general, all bands were faint, indicating low concentrations of the target fragment. In particular, the third reaction in the lab positive control triplicate produce extremely faint banding.

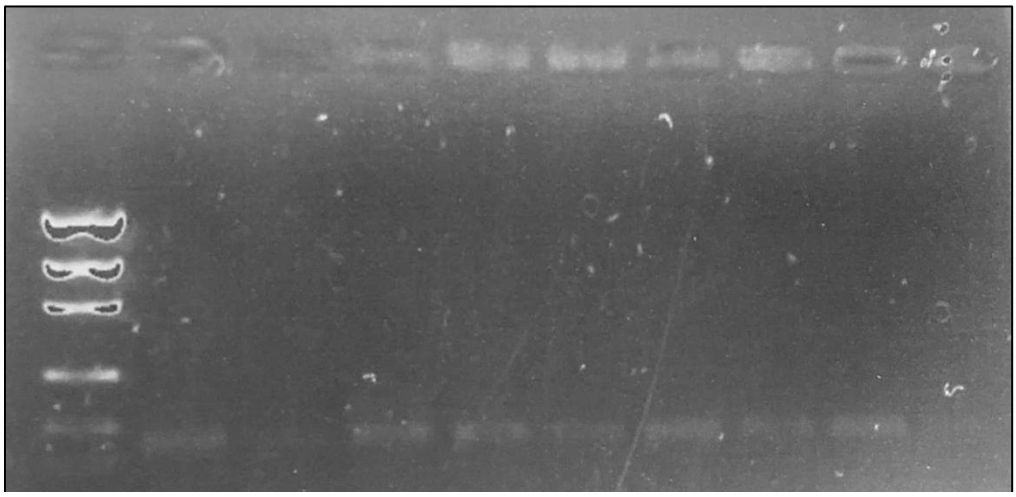


Figure 4. Agarose gel results. The lab positive control was run alongside the PCR products for each triplicate of the liver tissue extract samples for 25 ng and 6.25 ng, which served as positive controls for the gel electrophoresis analysis. Well setup is as follows: A: Ladder; B-D: Liver tissue extract, 25 ng; E-G: Liver tissue extract, 6.25 ng; H-J; Lab positive control.

Discussion

The eDNA evidence reported here cannot confirm the presence of round gobies in the French Creek watershed. Although direct observation suggests that the animal has migrated into the main stem of the creek (C. Bradshaw-Wilson, personal correspondence), this molecular analysis does not support the claim. It is important to note that these results do not provide evidence of absence; the discrepancy between the molecular and observational data highlights the importance of optimizing the eDNA protocol such that subsequent eDNA studies at Allegheny College can be conducted efficiently and effectively.

In 2014, the Pennsylvania Fish and Boat commission indicated concern that the round goby had made its way into French Creek near Lake LeBoeuf (Waterford, Erie County, PA) ("Anglers urged," 2014). While the Fish and Boat Commission had attempted to prevent the organism's spread into other parts of the watershed, sightings in more southern branches of the creek indicated that it had in fact migrated into the main stem. While the fish was confirmed to be present in Lake LeBoeuf and approximately six miles south alongside Mystic Road (C. Bradshaw-Wilson, personal correspondence), qPCR analysis presents no evidence that the round goby is in the watershed, specifically at the sampling sites near Moore Road and French Creek road. Due to the discrepancy in these data, one possible conclusion is that the evidence presented here is the result of false negatives, which can occur if too few copies of the COI target marker were present in the environmental samples, the COI target marker copies was degraded beyond detectability, or the laboratory analyses were improperly executed (Schultz and Lance 2015). Each of these considerations is discussed below.

Considerations for Sample Collection

Collection of field samples for subsequent molecular applications was based upon the methods laid out by Jerde et al. (2011). As with any eDNA study, careful attention must be granted to the process of sample collection. Of particular concern is the risk of contamination. When collecting aquatic samples, it is imperative that cross-contamination be avoided, as this can generate false-positive detections. For example, target DNA may be unintentionally carried from one sampling location to another if multiple locations are sampled consecutively (Thomsen and Willerslev 2015). This can be alleviated by proper sterilization of equipment via bleach soaking as well as standing downstream of the actual point of collection (Goldberg 2015). If possible, collection can be done on separate days, with all equipment being sterilized overnight via autoclave (Nalgene bottles) or soaked in bleach (waders, forceps). In addition, negative field controls are essential in

order to detect potential contamination, as successful amplification of target DNA in a negative control is indicative of cross-contamination. In this study, two equipment negative control and two negative field control triplicates contained a single reaction that produced a Ct threshold value indicative of starting amounts of genetic material within the range of 6.25 ng to 25 ng. The presence of genetic material in negative control replicates suggests contamination. Specifically, it indicates that round goby DNA is present in the control. In any case, the possibility of contamination is a crucial consideration in any eDNA study. Additionally, the Lab Positive control was known to have a gDNA concentration of 200 ng/µL, with each qPCR reaction well receiving 5 µL of sample, or 1000 ng of gDNA. However, the Ct mean generated suggested a drastically lower concentration of gDNA. This inconsistency suggests that the qPCR reaction failed to accurately quantify the amount of starting material in the positive control. In general, sample collection, extraction, and amplification steps should be optimized so as to prevent cross-contamination as well as the accurate quantification of starting genetic material.

In addition, sample site location must be taken into account during any aquatic eDNA study involving lotic systems, as this inevitably affects the interpretation of results. Here, two locations served as experimental sites (Moore Road and French Creek Road), while one site (Kenneth A Beers Jr. Park) served as a negative field control. Experimental sites were approximately 1,220m from one another, while Moore Road is approximately 3,200 m from LeBoeuf Lake. Given the distance between Lake LeBoeuf and the sampling sites, as well as the distance between the sites themselves, it is important to factor in the upper limits of eDNA detection in aquatic systems. Environmental DNA is detectable at least 240 m from the site at which the genetic material was released from the source organism. Importantly, the upper limit to detection has not been confirmed and is likely beyond 240 m (Jane et al. 2015). In this study, sites were chosen so as to minimize the likelihood that any positive detections were the result of viable goby DNA being carried downstream from

either Lake LeBoeuf or an upstream sampling site. In other words, any goby DNA detected at the French Creek Road sampling site, which is downstream of Moore Road, should not have come from Moore Road. However, because the upper limits of detection are not yet known, this cannot be confirmed. Having said that, it is well understood that aquatic eDNA is prone to degradation due to the presence of DNases (enzymes that degrade DNA), radiation, acidity, and heat in the external environment (Pilliod et al. 2014). More so, small (100 bp) aquatic eDNA fragments degrade beyond detectability within a few (0.9 – 6.7) days in marine environments (Thomsen et al. 2012b). Although these limits to detectability were determined in a marine environment as opposed to a freshwater system, this suggests that any aquatic DNA can and will degrade beyond detectability. Therefore, it is reasonable to assume that there exists a maximum distance at which eDNA remains detectable before succumbing to these factors. When trying to corroborate the direct observation of gobies with the evidence provided in this paper, it is entirely possible that any goby DNA that may have been collected in each sample was degraded to the point at which it was no longer detectable. It is worth noting that the mitochondrial genome is several thousand base pairs in length as well as being circular; these factors play a role in minimizing the likelihood of rapid degradation. Thus, mitochondrial gene markers should remain the target of eDNA studies.

Related to sampling location is sampling effort. In this study, two sites were sampled in addition to a negative control and various lab controls. At each site, 2 L of water was collected. Future eDNA projects might consider an increased sampling effort, as the probability of detection increases with the number and volume of water samples, especially when the target marker is at a low concentration (Schultz and Lance 2015). Thus, it is recommended that future eDNA projects increase the number of sampling locations; specifically, 3 L should be collected at up to 10 different locations to maximize the probability of detection (Schultz and Lance 2015).

Considerations for DNA Extraction

DNA extraction using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, Ca) in combination with the QIAshredder spin columns (Qiagen, Valencia, CA) proved to be efficient at eluting eDNA. While numerous methods exist for the extraction of eDNA (Ficetola et al. 2008; Roh et al. 2006; Taberlet et al. 2012; Leff et al. 1995; Miller et al. 1999; Whitehouse and Hottel 2006), consideration of extraction efficacy is crucial. For detection of the COI gene in aquatic eukaryotes, filtration of the water samples coupled with the DNeasy Blood and Tissue kit and Animal Tissue Spin-Column protocol is recommended compared to alternatives such as the Power Water DNA Isolation Kit (MoBio) (Deiner et al. 2015). In this study, all extractions following this protocol yielded high amounts of DNA when expected. Therefore, it is highly recommended that future eDNA studies utilize the DNeasy extraction kit in combination with the QIAshredder spin columns, which increase overall DNA yield (personal correspondence, Goldberg; Goldberg 2015).

Consideration for qPCR Assays

As the primary method for detecting species-specific genetic markers, PCR is a crucial aspect of any eDNA study. When conducting PCR on environmental samples, false negatives may be attributed to the low concentration of the target marker in PCR replicates. When marker concentrations are low, the division of samples can lead to PCR replicates with sufficiently low copy numbers so as to be undetectable. For example, a target marker concentration of 10 copies/L results in 97% of PCR replicates containing no copies of the target marker, while a concentration as high as 1,000 copies/L leads to 65% of PCR replicates containing no more than three copies of the target marker (Schultz and Lance 2015). As was noted previously, qPCR is capable of detecting as little as one copy number of target marker per liter (Doi et al. 2015; Takahara et al. 2013). However, when using eDNA as a means of detecting a yet unstudied species in an aquatic environment, it is reasonable to expect that species density will be low and therefore eDNA concentrations will also be

low (Schultz and Lance 2015). As a result, the aforementioned division of samples amongst PCR replicates will inevitably result in replicates that contain no detectable amount of the target marker. Therefore, it is suggested that future qPCR assays increase the number of PCR replicates, as this can increase the probability of detection (Ficetola et al. 2015; Schultz and Lance 2015). .

Furthermore, the presence of PCR inhibitors, which tend to be present in environmental samples, can have significant effects on the sensitivity of qPCR assays (Pilliod et al. 2013)

PCR inhibitors consist of all substances that have a negative effect on the PCR reaction. These may be organic or inorganic substances that are introduced or inherent within the sample. While their mechanism of action varies depending upon the specific inhibitor, they generally interfere with some step of the PCR reaction (Schrader et al. 2012). Ultimately, PCR inhibitors can result in limited sensitivity or false-negatives. Furthermore, they may increase with distance from the eDNA source organism (jane et al. 2015). Thus, steps should be taken to remove inhibitors when possible. Schrader et al. (2012) details several approaches for their removal in a variety of applications. In the context of an aquatic eDNA study, TaqMan Environmental Master Mix 2.0 (ThermoFisher Scientific, Chardon, OH) eliminates most, if not all, inhibition from environmental samples (Jane et al. 2015). Future aquatic eDNA studies should implement this Master Mix to minimize the chances of false-negatives.

Final Remarks

While the evidence presented in this study suggests that round gobies are not present in the main stem of the French Creek watershed, observational evidence indicates otherwise. The above discussion indicates that this discrepancy is likely due to false-negatives in the molecular analysis. Of course, the possibility remains that the animal is not actually present in this portion of the watershed and has in fact migrated to other locations beyond the sites that were sampled.

However, the behavior of the round goby contradicts this conclusion. The round goby is typically sedentary with a limited home range, estimated to be 5 ± 1.2 m^2 (Kornis et al. 2012). Large scale migratory rates range from 500 m/yr (Bronnenhuber et al. 2011) up to 1-4 km/yr (Kornis and Vander Zanden 2010). The variation in migration rates can be attributed to a number of factors, including environment (the lower bound was found using data from rivers, while the upper bound was found using data from the Great Lakes and surrounding tributaries) to density-dependent factors such as intraspecific competition (Kornis et al. 2012). In general, *N. melanostoums* will remain within their home range unless competition is high such that it forces them forward on the invasion front (personal correspondence, C. Bradshaw-Wilson).

With this in mind, the well-supported conclusion is that the failure to detect round goby DNA in environmental samples is the result of false-negatives. Future studies can investigate this problem in a variety of ways, some of which have already been discussed. An interesting approach would be to conduct an eDNA survey targeting an aquatic species known to be abundant in French Creek, such as the rainbow darter or creek chub (Beggs and VanRy, n.d.). This "control study" should elucidate the strengths and weaknesses of any protocol, as the species' presence is already confirmed and should result in positive detections and accurate estimates of organism density. Additionally, a "control study" organism such as the rainbow darter or creek chub could be used as a positive control when conducting eDNA studies of species whose presence is unknown. In other words, a study that targeted a species whose presence was in question could simultaneously target a species whose presence has been confirmed in those waters. Failure to detect the control organism would indicate shortcomings within the protocol, while positive detections would lend strength to the conclusions drawn from the data generated from the experimental species assay. In addition, it would be advantageous to elucidate the dynamics of eDNA within French Creek so as to better understand the behavior of DNA within this system. Specifically, a protocol similar to that

described by Jane et al. (2015) may uncover the true upper bounds to detection, as this has yet to be conclusively established. In doing so, future sampling efforts would benefit from increased efficiency and efficacy.

Without effective surveillance methods, successful management of rare species is impossible (McDonald 2004). Given that molecular detection techniques carry with them greater detection probabilities per unit effort compared to more traditional sampling approaches, the continued investigation and application of eDNA protocols is essential to managing rare species (Hayes et al. 2005). However, it is just as important that this evidence be corroborated with evidence provided by traditional sampling techniques, as false-positives and false-negatives may still persist until protocols are reviewed and optimized. By using this study as a framework, future projects seeking to detect and quantify organisms using eDNA can be streamlined. Ultimately, this approach will increase the ability of natural resource managers to track endangered species and reduce the damages of invasive species in aquatic environments.

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