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Field application of environmental DNA techniques to detect early stages of invasion by the destructive New Zealand mud snail

James D. Woodell
University of Iowa

FIELD APPLICATION OF ENVIRONMENTAL DNA TECHNIQUES TO DETECT EARLY STAGES OF INVASION BY THE DESTRUCTIVE NEW ZEALAND MUD SNAIL

by

James D. Woodell

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Thesis Supervisor: Associate Professor Maurine Neiman

ABSTRACT

Nonnative species that cause damage to ecosystems to which they are introduced are considered invasive. Restoration of the original ecosystem after an invasive population has established is expensive and difficult but more likely to succeed when invasions are discovered early. Containment efforts to prevent the spread of known invasions also benefit from earlier knowledge of invaded sites. Environmental DNA (eDNA) techniques are emerging as a tool that can identify invasive species at a distinctly earlier time point than traditional methods of detection. I collected water samples from eight sites not known to be invaded by the freshwater New Zealand mud snail (NZMS). After filtering these samples to collect eDNA, I used a species-specific probe with qPCR to identify NZMS eDNA. I found evidence for NZMS invasion at five of the eight sites, with later physical confirmation of mud snails at one of these sites. This study is the first example of successful application of eDNA to detect new invasions of the freshwater New Zealand mud snail, setting the stage for further monitoring of at-risk sites to detect and control new invasions of this destructive snail.

PUBLIC ABSTRACT

Species transported by humans that cause damage to ecosystems to which they are introduced are considered invasive. Restoration of the original ecosystem after an invasive population has arrived is expensive and difficult but more likely to succeed when invasions are discovered early. Containment efforts to prevent the spread of invasions also benefit from earlier knowledge of invaded sites. A new tool that can identify invasive species without needing to physically locate them is currently being developed. This tool uses DNA collected directly from the environment rather than from an organism itself, called environmental DNA (eDNA). By using a probe to identify this eDNA, we can tell whether a species has recently been present in a given location. I collected water samples from eight sites near a known invasive population of the freshwater New Zealand mud snails (NZMS). These sites plausibly could be invaded by NZMS and may lead to further spread due to their popularity with fly-fishermen. After filtering these samples to collect eDNA, I found evidence for NZMS invasion at five of the eight sites, with later physical confirmation of mud snails at one of these sites. This study is the first example of successful application of eDNA to detect new invasions of the freshwater New Zealand mud snail, setting the stage for further monitoring of at-risk sites to detect and control new invasions of this destructive snail.

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INTRODUCTION

Increased human global mobility has reduced historical geographic, temporal, and behavioral boundaries to species movement (Hulme 2009). Dispersal to new environments used to be a rare occurrence for most species, creating distinctive bioregions defined by their communities. However, accidental and intentional transport by humans, anthropogenic climate change, and environmental disturbance have created more frequent opportunities for foreign species to establish new populations in regions they otherwise might have never entered (Bradley *et al.* 2012, Catford *et al.* 2012, Diez *et al.* 2012, Ware *et al.* 2015, Early *et al.* 2016). Most introduced species are unable to establish new populations, either because the alien species is not well adapted to the new environment or because the number of colonists is too low to establish permanent residence (Keller & Taylor 2008). Rarely, however, a new population is established within the introduced locale. When these alien taxa are able to expand their new populations and create a negative impact on the surrounding ecosystem or human economic interests, they are considered invasive species. (Mooney & Cleland 2001, Strayer 2010). The expected continuing increase in human global movement will translate into more invasive species (Sakai *et al.* 2001, Levine & D'Antonio 2003, Early *et al.* 2015 Seebens *et al.* 2016).

There is a growing body of research pointing to the efficacy of using environmental DNA (eDNA) to monitor ecosystems for the introduction of invasive species (Jerde *et al.* 2011, Taberlet *et al.* 2012, Comtet *et al.* 2015, Lawson Handley 2015, Thomsen & Willerslev 2015, Brown *et al.* 2016, Ricciardi *et al.* 2017). Here, I describe the successful outcome of the first-ever application of a recently developed eDNA detection method for the invasive New Zealand snail *Potamopyrgus antipodarum* in the field. The methods and data described here serve as a proof-of-principle for an effective monitoring approach for an invasive species in at-risk areas as well as provide new evidence for expansion of an invasion of a destructive freshwater snail.

Mitigating Invasion

Halting initial introductions has been identified as the surest and most cost-effective method of invasion conservation (Finoff *et al.* 2007, Keller *et al.* 2007, NISC 2016). Challenges to the effective implementation of invasion prevention are posed by absence of sufficient policy or because the opportunity to prevent invasion has already passed (Simberloff 2014). The next priority should then be to eliminate the invader completely before greater damage and further spread can occur (Simberloff *et al.* 2013). While eradication is increasingly possible (Simberloff 2014), restoration of invaded ecosystems is time consuming, expensive, and in many cases unsuccessful (Myers *et al.* 2000, Rejmanek & Pitcairn 2002). Accordingly, current invasive species response efforts often focus on preventing spread rather than eradication (Leung *et al.* 2002). Establishing cost- and time-effective methods to first identify and then stop or slow the spread of early invasions is a critical means of preventing substantial future biological and economic damage (Lodge *et al.* 2006, McGeoch *et al.* 2015).

Perhaps the most important element of a rapid response strategy is identification of a new invasion (Simberloff 2014, McGeoch *et al.* 2015). Traditional methods of surveying ecosystems for invasion requires the species to be physically located (Lawson Handley 2015), meaning labor-intensive field sampling (and a little luck). By the time a site is acknowledged as invaded, the invader is often well established and has likely spread to other sites (Simberloff 2014). This outcome is particularly likely when active searches are not regularly performed and discovery of the invasion occurs via chance encounters. This lag time between actual invasion and realization that an invasion has occurred introduces a critical time during which an unaware human population may help spread the invasive species because containment measures cannot be initiated until we are aware of the invasion. Early detection is also important because full remediation is more likely to succeed if the invasion is caught in its early stages (Rejmanek & Pitcairn 2002, Anderson 2005, Simberloff 2014, the U.S. Department of the Interior 2016). Decreasing

this lag time associated with invasive species containment by focusing on early detection of invasions should be a key priority for invasive species management by conservation programs.

Early Detection Using Environmental DNA

Environmental DNA (eDNA), defined as genetic material sampled from the environment rather than directly from organisms, is rapidly emerging as a powerful means of surveying natural biological communities (Jerde *et al.* 2011, Taberlet *et al.* 2012, Comtet *et al.* 2015, Lawson Handley 2015, Thomsen & Willerslev 2015, Brown *et al.* 2016, Ricciardi *et al.* 2017). Sources of eDNA include soil, water, sediment, or any medium that can harbor DNA from a living or recently dead organism. As an organism moves within an environment, it sloughs off skin cells or leaves behind wastes that contain its DNA. After taking samples of the environment, the sample is processed to isolate and amplify the DNA contained within the nonorganic components. This DNA is then used to describe the metagenomics of the community and/or identify species within that community (Comtet *et al.* 2015).

While initially developed for surveying microbial communities, eDNA is now used for various ecological surveys, including macro-organisms. There are now multiple species-specific assays available for use, including assays for numerous amphibians (Beauclerc *et al.* 2018), fish (Thomsen *et al.* 2012), plants (Scriver *et al.* 2015), mammals (Andersen *et al.* 2012), and more assays are continually being developed (Thomsen & Willerslev 2015, see Washington State University's eDNA toolbox: https://labs.wsu.edu/edna/edna-assays/). Environmental DNA techniques have also proven capable of detecting invasive species where traditional surveys had not (Jerde *et al.* 2011).

For invasive species conservation, the application of eDNA-based approaches allows a more efficient means of surveying potentially invaded sites for invasive species. In particular, these surveys no longer require physical location of an organism. For example, eDNA has been used to detect invasive or endangered fish, frogs, and crustaceans in aquatic ecosystems without the need to physically locate individuals at those sites (see Jerde *et al.* 2011, Dejean *et al.* 2012, Takahara *et al.* 2013). Instead, samples

of the environment can be acquired and processed cheaply and used in conjunction with a species-specific probe in order to identify the presence or absence of an invader in ecosystems of concern. Environmental DNA can therefore detect invasive species at a much earlier time point, allowing substantially more rapid management and remediation responses. Because eDNA rapidly degrades in the external environment, identifying DNA from an organism means that organism has recently been present in that ecosystem. Diffusion of eDNA, particularly in aquatic ecosystems, may also allow for detection of invaders beyond the site of deposition (*e.g.*, downstream from the invasion site; Dejean *et al.* 2011, Deiner & Altermatt 2014). Calibrating qPCR probe fluorescence with known population densities and river discharge or water volume also makes it possible to estimate invasive population densities in the aquatic environments (Goldberg *et al.* 2013). Here, I have applied eDNA techniques to perform the first field test of this approach to detect the New Zealand mud snail, a freshwater invader.

New Zealand Mud Snail Invasion

Potamopyrgus antipodarum, commonly called the New Zealand Mud Snail (NZMS), is native to freshwater lakes and (rarely) estuarine environments in New Zealand. NZMS was first observed in the River Thames in the 1850s (Smith 1889). In 1892, NZMS was recorded in Tasmania, and was found in mainland Australia in 1895 (Alonso & Castro-Díez 2008). Soon after the turn of the 20th century, NZMS was discovered in brackish waters in northern Europe (Städler *et al.* 2005). NZMS became a prominent invader later in the 20th century, establishing inland in France and Switzerland in the 1970s and then expanding across the rest of central Europe (Städler *et al.* 2005). Invasion of the US by NZMS was discovered in 1987, in the Snake River in Idaho (Bowler 1991). New Zealand mud snail has subsequently expanded along rivers and lakes of the western US, including sites in Colorado (McKenzie *et al.* 2013), Utah (Vinson 2004), Wyoming (Kerans *et al.* 2005), Washington (Davidson *et al.* 2008), and California and Oregon (Dybdahl & Drown 2011). The western invasive NZMS populations have also expanded into Canada in brackish waters along the Pacific coast, similar to the invasion of brackish waters in Northern Europe

(Davidson *et al.* 2008). An additional independent invasion of the US occurred beginning in Lake Ontario and the St. Lawrence River in 1991 (Zaranko *et al.* 1997), possibly a secondary invasion from genetically identical European invasive populations (Dybdahl & Drown 2011). New Zealand mud snail has since spread across the Great Lakes (Levri *et al.* 2012) and other watersheds in the Eastern US in New York, Pennsylvania, and Maryland (Levri, pers. comm.). Additional documented invasions around the world include the Black Sea (Son 2008), Italy (Gaino *et al.* 2009), Japan (Ogata *et al.* 2010), and, most recently, South America (Collado 2014). NZMS native to New Zealand are either sexual or asexual, but invasive populations seem to be invariably asexual (Alonso & Castro-Díez 2012). While asexual NZMS harbor a great deal of genetic diversity (Jokela *et al.* 2003), only a handful of NZMS clones have been successful invaders (Städler *et al.* 2005, Alonso & Castro-Díez 2012).

Multiple studies have provided important insights into the current and potential consequences of NZMS invasion. First, NZMS populations can grow to extremely high densities, with some wild populations numbering up to 500,000 individuals•m^{-2} (Tatara *et al.* 2012). This physical density can translate into the loss, via competitive exclusion, of other species that colonize or dwell along the substrate: experimental studies demonstrate a negative effect of NZMS on macroinvertebrate colonization where NZMS populations are relatively high (Kerans *et al.* 2005). Other experiments have demonstrated that rainbow trout (*Oncorhynchus mykiss*) fed exclusively NZMS lose weight because the fish are not able to digest the snails (Vinson & Baker 2008). The implications are that NZMS invasions have serious potential consequences that could affect multiple trophic levels in invaded ecosystems, especially in light of the observation that native fish are increasingly consuming NZMS (Vinson & Baker 2008).

The NZMS invasion also affects the environmental conditions of invaded ecosystems. Hall *et al.* (2003) showed that invasive NZMS dominated nitrogen and carbon fluxes in the invaded Polecat Creek in Wyoming, finding that NZMS consumed 75% of gross primary productivity, represented two-thirds of ammonium demand, and constituted 97% of invertebrate biomass. Krist and Charles (2012) discovered

that invasive NZMS also seem to outcompete native grazers, perhaps via direct competition for food. This study also revealed that competition imposed by NZMS altered native diatom community assemblages. Diatom communities can be used as ecological indicators for environmental conditions, meaning changes in diatom community structures are often indicative of environmental changes (Keck *et al.* 2015, Pandey *et al.* 2018). Moore *et al.* (2012) found that invasive NZMS, which use their radula to scrape algae off substrate, altered algal communities via direct competition with native scraping grazers, reporting an increase in piercing-type grazers in the community from 0 individuals•m^{-2} to an average of 1500 individuals•m^{-2}. This shift from scraping to piercing-type grazers is associated with depleted stable nitrogen isotopes in native invertebrates (Moore *et al.* 2012). Community phase shifts are indicative that NZMS are dramatically altering the ecosystems they invade. As these alterations continue, predicting outcomes, mitigating damages, and restoring the native environment and community will become increasingly difficult.

While the range of the western US NZMS invasion is well characterized, the full extent of the NZMS invasion in the eastern US is less defined. NZMS was discovered in Centre County, Pennsylvania in 2013 at Spring Creek, but was well established when discovered and might have persisted undetected for years (Ed Levri, pers. comm.). Spring Creek is a popular fishing location, raising suspicions that these NZMS were transported via recreational water use. Data pointing in this direction include the genetic background of the Spring Creek population compared to other invasive populations in the US. Mitochondrial data suggest two primary invasive clones in the US: US1 in the western US and US2 in the Laurentian Great Lakes (Dybdahl & Drown 2011). These are likely separate invasions: the US1 haplotype matches haplotype 37 (genbank AY570216, Neiman & Lively 2004) found in Lake Taupo on the North Island of New Zealand, while the US2 haplotype matches haplotype 22 (genbank AY570201, Neiman & Lively 2004; 2% sequence divergence from the US1 haplotype, Dybdahl & Drown 2011) found in lakes Gunn and Te Anau at the southeastern tip of New Zealand (Neiman & Lively 2004). The US2 haplotype

also matches the invasive European A mitochondrial haplotype (Dybdahl & Drown 2011), indicating a possible secondary invasion originating with the successful invasive population in Europe. An additional mitochondrial haplotype was discovered at a single site in lower densities alongside US1, US3 (genbank HG680431, <0.2% sequence divergence from the US2 mitochondrial haplotype). These US3 snails are not currently considered invasive as they have not been identified beyond a single site and are a minority compared to the greater US1 population at that location (Dybdahl & Drown 2011). The invasive population found in Spring Creek is comprised entirely of a clone with the US1 mitochondrial haplotype (Finger *et al.* in review), matching the dominant clone in the western US rather than the US2 haplotype of the closer Great Lakes populations. Because the western US1 population has existed at least since 1987 (Bowler 1991), the invasion in Pennsylvania is likely a secondary invasion originating via human-mediated transport of individuals from the western US.

That the Spring Creek invasive NZMS have the US1 haplotype is concerning given the widespread invasion of this lineage, which currently ranges from California to southern Canada to Colorado (Vinson 2004, Kerans *et al.* 2005, Davidson *et al.* 2008, Dybdahl & Drown 2011, McKenzie *et al.* 2013, others). The possibility of recreational transport of NZMS poses a threat to the local trout population and raises the potential for NZMS to be accidently transported to new localities through ballast water or fishing equipment. In particular, it is very plausible that NZMS has already spread to new eastern North American sites where it has established new invasive populations but has remained undetected. Here, I attempted the first field-based application of eDNA-based early detection of NZMS using methods developed for NZMS by Goldberg *et al.* (2013). This study was performed for two reasons:

1. to determine the effectiveness of eDNA and qPCR for early detection of invasive NZMS at natural sites that contain previously undetected NZMS.
2. to more thoroughly describe the extent of NZMS spread near the known invasion in Centre County, Pennsylvania.

METHODS

Developed at the University of Idaho (Goldberg *et al.* 2013), eDNA and qPCR protocols for NZMS have proven effective at detecting the species in known invasion sites and at estimating population density in streams with measured discharge. To my knowledge, these methods have not previously been applied to identifying new invasive populations of NZMS. I successfully refined the filtering protocols from Goldberg *et al.* (2013) and then used these updated methods in a stream water survey in central PA in May 2018. I focused on applying eDNA to determine whether NZMS might be found in locations that could be plausibly invaded but where no snails had previously been reported.

Site Selection

I selected eight sites at risk of recreational aquatic activity-related transport of new colonists and that represented a more significant risk of further human-mediated spread after an invasion occurred (Table 1, Figure 1). The eight selected sites were spread across six different rivers and four counties in central Pennsylvania. The sites all are contained within the Susquehanna River watershed, which ultimately feeds into the Chesapeake Bay as part of the Mid-Atlantic watershed. Due to a lack of stream discharge measurements across these sites and the potential for inaccurate population density estimates (Darling & Blum 2007), I chose to assess only presence/absence of NZMS at these selected sites. Six of these sites had been the focus of unsuccessful searches for physical evidence of NZMS invasion; two sites, YC1 and CR1, had not been searched prior to this study. Time constraints and high water level prevented thorough searches for snails at these eight sites on the days of sampling.

Field Collections and Filtration

I collected two water samples of 3.8 liters from each of the eight sites by submerging containers approximately 10 cm under the surface of the stream until full. I soaked each container in a 50/50 bleach solution and rinsed the containers thoroughly with deionized water before use. I collected one sample from the bank and one sample from the center of the stream when the waters were relatively shallow and

slow moving. In deeper or higher velocity streams, I took two bank samples at two locations moving approximately 10 m upstream for the subsequent sample. For a negative field control, a blank sample consisting of only deionized water was transported to the field along with other containers. This deionized water was then transferred from its original container into a new container after collections were made to check for contamination of samples during transport. I filtered all water samples at Pennsylvania State University Altoona within 24 hours of collection.

I used Nalgene vacuum filter flasks that were sterilized by soaking in a 50/50 bleach and deionized water solution for at least 15 minutes followed by a thorough rinse with deionized water before starting the filtration process for each sample. I also used a 50% bleach solution to sterilize the workspace used for the flasks as well as the forceps that I used for sample processing. As an additional means to prevent contamination, I placed fresh paper towels under the flasks during filtration for each sample. I used 0.45 um mixed-cellulose ester filter discs for filtration. Because of relatively high sediment load in the water bodies that I sampled, these filters rapidly became clogged with sediment. I replaced clogged filters as needed after the filter had processed at least 300 ml of water. This minimum requirement took more processing time but ensured a minimum water sample for each filter. All filters from a sample (range = 2-7) were then processed for qPCR-based eDNA detection for each of the two individual water samples at each site. I only filtered 3000 ml from each sample, which left approximately 800 ml of water at the bottom of the flask where the heaviest sediment load settled. After filtration, filters were folded and stored in 95% ethanol to preserve the samples until DNA extraction took place. I used the same technique to filter water from a tank of laboratory-cultured NZMS as a positive control to ensure the qPCR protocols were detecting NZMS DNA. As an additional negative control to check for contamination during the filtration process, I used the same approach described above to filter deionized water in the laboratory to ensure sanitization of the filtering equipment was adequate. Any evidence of *P. antipodarum* DNA in this negative control would indicate that contamination had occurred.

DNA Extraction and qPCR

Although qPCR was used to estimate NZMS population density by Goldberg *et al.* (2013), my goal was different: detect new NZMS invasions. Accordingly, I used qPCR-based detection of eDNA only to determine NZMS presence/absence. I extracted DNA from the filters and processed the filters for quantitative PCR (qPCR) at University of Iowa. Before DNA extraction began, I used DNAZap as well as the 50/50 bleach mixture to clean the bench space, and then used fresh paper towels on the bench and newly bleached equipment for each sample. I extracted DNA from the filters with a DNeasy Blood & Tissue kit with QIAshredder following the DNA extraction protocol described at the Goldberg lab website (https://labs.wsu.edu/goldberglab/edna-assays/). To detect contamination during the extraction and qPCR procedures, I processed an unused filter alongside the filters used for water samples, field and filtering negative controls, and positive control.

I used a fume hood that was sterilized with 50/50 bleach and DNAZap for loading qPCR plates. The forward primer sequence was TGTTTCAAGTGTGCTGGTTTAYA, the reverse primer sequence was CAAATGGRGCTAGTTGATTCTTT, and the probe sequence was ЬFAM-CCTCGACCAATATGTAAAT. These primers were designed to amplify a polymorphic section of cytochrome *b* (Goldberg *et al.* 2013). The probe was designed to have no ambiguous bases, and the probe sequence was shown to work for NZMS without providing false positives in the presence of the commonly co-occurring pebblesnail (*Fluminicola hindsii*; Goldberg *et al.* 2013). I used 0.4 uM of each primer and 0.2 uM of the probe along with 1X mastermix and 2.5 ul of DNA extract in 20ul reactions in a Roche LightCycler 480. Cycles began at 95°C for 15min followed by 50 cycles of 94°C for 60sec and 60°C for 60sec. Following Goldberg *et al.* (2013), amplification was considered positive, and therefore indicated detection of NZMS eDNA, if probe fluorescence reached a phase of exponential increase (Figure 2). Wells without exponential increase in fluorescence were considered negative results (Figure 3). Because of varying sediment load across sam-

ples, and, accordingly, varying challenges with clogged filters, the number of filters processed per sample were not equal (N = 2-7 filters per sample). To accommodate this variable, I compared site results by calculating the ratio of the number of DNA amplifications (exponential probe fluorescence phase observed during a given qPCR assay) over the total number of qPCR assays for that sample (N = 6-21 qPCR assays per sample). This ratio is hereafter called detectability, a commonly used metric in eDNA-based detection studies (Jerde *et al.* 2011, Dejean *et al.* 2012, Goldberg *et al.* 2013, Deneir & Altermatt 2014).

If any site previously not known to be invaded by NZMS returned a non-zero detectability, my collaborator, Dr. Ed Levri, planned to return to the site to perform a thorough search for physical evidence of NZMS presence. This additional line of evidence is crucial for a field demonstration of the viability of eDNA-based early detection of an NZMS invasion.

RESULTS

Detectability of 0.0 for all negative controls (Table 1) shows that none of the five negative control samples tested positive for *P. antipodarum* eDNA. The laboratory positive control taken during this round returned a detectability of 1.0: the one filter from the one sample tested positive for *P. antipodarum* eDNA (3/3 qPCR assays). Sites at Sixmile Run (CE9; Figure 1) and Cedar Run (CR1) both had detectability of 0.0, indicating no NZMS presence. These negative results at CE9 and CR1 were consistent with the failure to detect *P. antipodarum* in earlier physical searches for the snails. One site of Little Juniata River (Hu1) showed a detectability of 0.0, while another (Hu8) had a detectability of 0.167 (6/36 qPCR assays). Juniata River (Bl9) had a detectability of 0.030 (1/33 qPCR assays) and Yellow Creek (YC1) had a detectability of 0.056 (1/18 qPCR assays). One site on Bald Eagle Creek (PA16) had a detectability of 0.100 (3/30 qPCR assays). These sites with relatively low detectability still indicate NZMS DNA was likely present.

The highest detectability from a site where *P. antipodarum* had not previously been seen was 0.933 (14/15 qPCR assays) from a site on Bald Eagle Creek (PA27) approximately 5.5 km downstream of

where Spring Creek empties into Bald Eagle Creek. This result is strongly suggestive of the physical presence of an invasive NZMS population at PA27. While unusually high water levels prevented a follow-up search for NZMS immediately following the results of the May 2018 study, Dr. Levri was able to return to Bald Eagle Creek at the PA27 location in November 2018 and positively identified a single individual NZMS.

DISCUSSION

The goals of my project were to successfully apply eDNA-based methods for early detection of previously unknown NZMS invasions and to use this method to more thoroughly describe the invasion in central PA. I was able to detect eDNA at a site, PA27, at which *P. antipodarum* had never been seen and that was later confirmed to contain NZMS. I also found evidence that NZMS eDNA may be present at four other sites (Bl9, YC1, PA16, Hu8) at low detectability, indicating NZMS presence but without physical confirmation to date.

While the high detectability at PA27 indicates that NZMS are present at the site, especially in light of Dr. Levri's subsequent discovery of a NZMS individual, it is impossible to exclude the possibility that DNA in the water could have drifted from the known Spring Creek populations upstream increasing the detectability. Indeed, DNA of other freshwater species has been detected via eDNA-based approaches up to 12 km downstream from its source (Deiner & Altermatt 2014). A future study comparing genotypes of this new invasive population at PA27 to others nearby may be able to identify the source populations (Clusa *et al.* 2016) and address the possibility of DNA drift influencing detectability at PA27. In particular, finding that the genotypes of eDNA detected at PA27 match both the Spring Creek population upstream and the individuals found physically at PA27 would mean that I could not formally rule out a scenario where I had detected drifting DNA from a different site. Additional insight into the role of drifting DNA will also come from experiments aimed at characterizing the distance and time over which

drifting NZMS DNA specifically can be detected in the environment. This information on drifting DNA will also set the stage for the exciting possibility of identifying the presence of NZMS from sections of the watershed downstream from areas they have invaded. If so, monitoring watersheds by sampling the river they flow into near the mouth and moving upstream may be a way to rapidly survey larger regions for invaders.

While returning to the PA27 site to search for NZMS was my highest priority because of the very high detectability, sites with low detectability still require thorough searches to see if our detection of NZMS eDNA at these locations might also be linked to previously unknown invasions. Given the fact that only one individual was found at PA27, these other sites may harbor NZMS at population densities still too low to find via traditional search methods. My collaborator intends to return to these sites in the spring to search for physical evidence of the presence of NZMS. We also intend to include these sites in future eDNA surveys, with the predictions that these sites will either again test positive for *P. antipodarum* (reflecting an established and likely increasing population; in this case, detectability should increase) or will return negative results. The latter would either implicate a rare drifting DNA event and/or suggest that contamination (or mismatches with another species' DNA; Wilcox *et al.* 2013) might not have been totally eliminated from the procedures.

My results, along with the recent discovery of NZMS in the Bald Eagle Creek in Lock Haven (Ed Levri, pers. comm.), indicate that NZMS has expanded its invaded range in central Pennsylvania beyond Spring Creek. The discovery of NZMS in Spring Creek in 2013 was of great concern due to the potential for spread of the snail from that location to other streams and watersheds across the Appalachian Mountains and the eastern US because of the popularity of Spring Creek for trout fishing. Given the widespread success of NZMS in the western US, it would follow that within a few years there would be several more NZMS findings in the Mid-Atlantic region (Simberloff 2014). Once NZMS establishes in a

new location it would likely take a few years for the population size to become large enough to be detected via traditional approaches (Simberloff 2014, the U.S. Department of the Interior 2016). Now, a few years after its initial discovery at Spring Creek in Pennsylvania, there has been an increase in new findings of the snail in the eastern US. In 2017, NZMS was found in two streams in Syracuse, NY and in the Gunpowder Falls River in Maryland. In 2018, it was discovered in the Musconetcong River in New Jersey and Little Lehigh Creek near Allentown, PA (Ed Levri, pers. comm.). Unfortunately, these populations are all very well established and may already have sourced new unknown invasions elsewhere. That newly invaded sites can now be detected before NZMS populations are large will allow more rapid measures to be taken to educate the public and limit their spread.

Early detection with eDNA can provide us with the ability to identify these populations much earlier (Jerde *et al.* 2011). Doing so will enable us to slow the rate of spread in the eastern US by making recreational water users aware of NZMS presence, utilizing checkpoint procedures near invaded sites, and ideally limiting access to invaded locations as a rapid response (Simberloff 2014, The U.S. Department of the Interior 2016). This study has, for the first time, proven the possibility of detecting previously unknown populations of NZMS using eDNA methods. Future studies should focus on smaller water samples for more rapid filtration and sample more frequently and broadly across the region in order to detect establishing populations and monitor established invasive population densities (Goldberg *et al.* 2013). There is also the exciting opportunity for citizen science to contribute to these efforts (Biggs *et al.* 2015). With the cooperation of those individuals that use these ecosystems for recreation, it may be beneficial to pursue a program wherein sterile containers and instructions are supplied to fishermen, boaters, and kayakers. After sampling, the containers can be dropped off at a laboratory for processing. While such a program could introduce more chance for error, continual assessment of potential invasions sites would allow for cross-referencing results. This distributed method of early detection would cast a wide net in which to catch these destructive aquatic invaders.

TABLES AND FIGURES

Table 1. Results of qPCR from sites sampled in May of 2018. All sites sampled were locations where NZMS were not detected previously. Locations of sites can be seen in Figure 1. Positive control consisted of a sample from a lab aquarium containing NZMS. Field negative controls were DI water transported to field sites, transferred to new containers, and filtered as though they were collected samples. Extraction negative control was DI water processed alongside sample filters for DNA extraction. Amplification is considered positive if probe fluorescence reaches an exponential phase (Figure 2) in during the qPCR assay.

Site ID	Site Name	Location	Sample Number	# Filters Processed	# qPCR Reactions*	# Amplified	Sample Detectability**	Total Site Detectability***
Field Negative Controls				3	9	0	0.000	0.000
Filtration Negative Control				2	6	0	0.000	0.000
Extraction Negative Control				1	3	0	0.000	0.000
Positive Control				2	6	6	1.000	0.000
CE9	Sixmile Run	40.909102° -78.104381°	1	3	9	0	0.000	0.000
			2	2	6	0	0.000	
CR1	Cedar Run	40.795188° -77.791951°	1	2	6	0	0.000	0.000
			2	2	6	0	0.000	
Hu1	Little Juniata River	40.307000° -78.119700°	1	6	18	0	0.000	0.000
			2	3	9	0	0.000	
Bl9	Juniata River	40.459908° -78.282918°	1	6	18	0	0.000	0.030
			2	5	15	1	0.067	
YC1	Yellow Creek	40.156552° -78.354921°	1	3	6	1	0.167	0.056
			2	4	12	0	0.000	
PA16	Bald Eagle Creek	40.940094° -77.796659°	1	6	18	2	0.111	0.100
			2	4	12	1	0.083	
Hu8	Little Juniata River	40.587767° -78.099817°	1	7	21	5	0.238	0.167
			2	5	15	1	0.067	
PA27	Bald Eagle Creek	40.975180° -77.742108°	1	2	6	5	0.833	0.933
			2	3	9	9	1.000	

*3 qPCR reactions/filter

**= # positive amplifications per sample/# qPCR reactions per sample (following the example of Jerde *et al.* 2011, Dejean *et al.* 2012, Goldberg *et al.* 2013, Deneir & Altermatt 2014)

***= # positive amplifications per site/# qPCR reactions per site

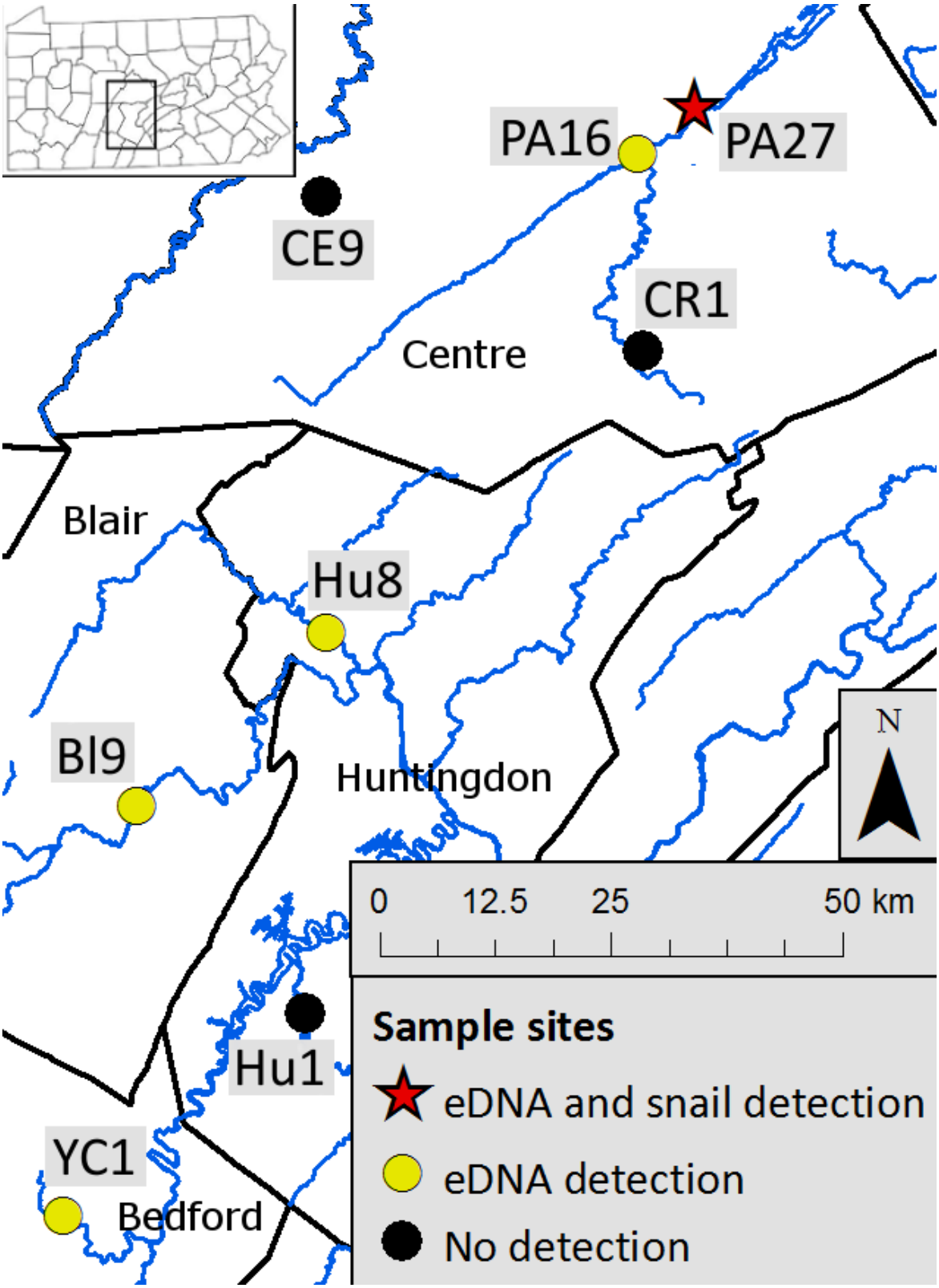

Figure 1. Map of sampling sites in central Pennsylvania. Black circles are sites where no NZMS eDNA was found. Yellow circles indicate the presence of eDNA but without confirmation of physical presence. The red star marks site PA27 on the Bald Eagle Creek where NZMS eDNA was found and the physical presence of NZMS was later confirmed.

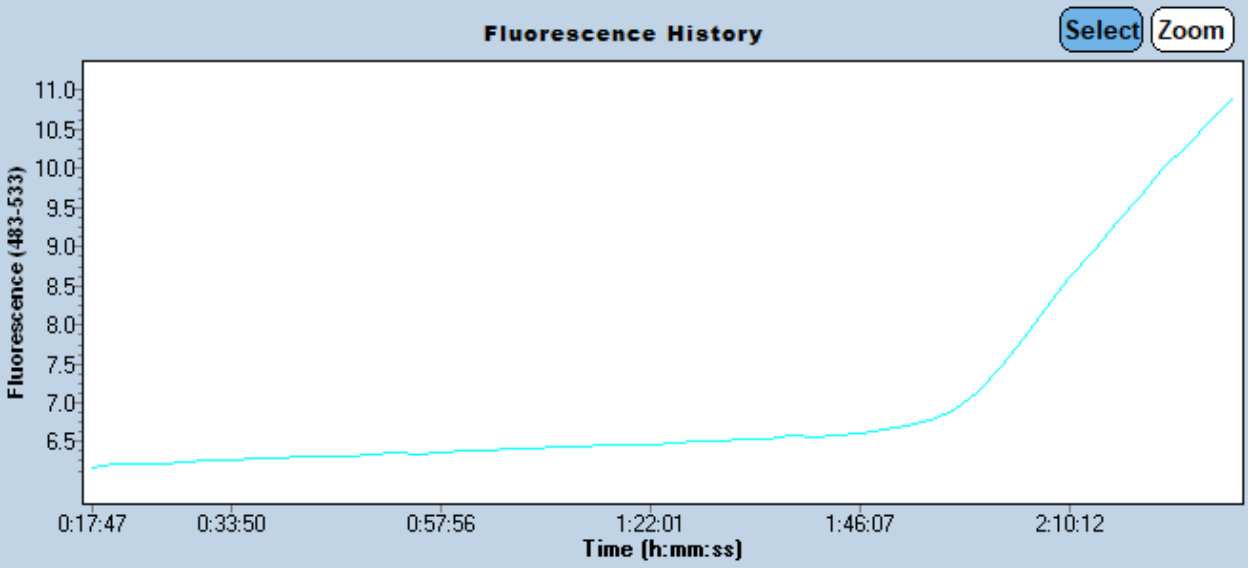

Figure 2. Example of a sample showing successful amplification during qPCR. Fluorescence increased minimally until it reached a point of exponential increase which is indicative of the probe successfully accumulating on amplified DNA.

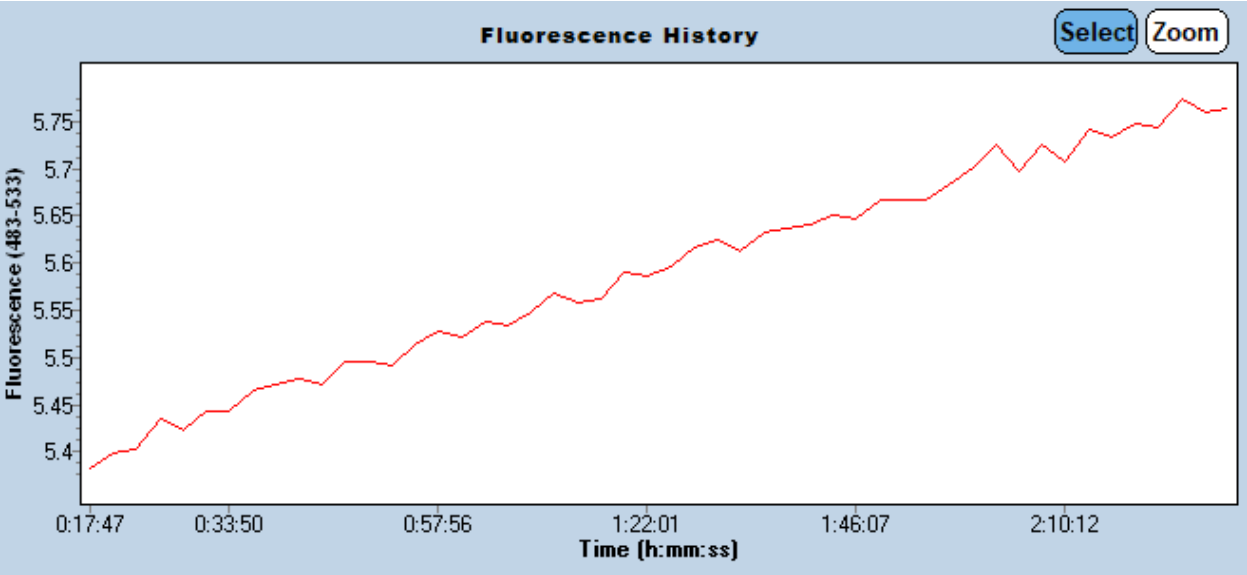

Figure 3. Example of no amplification during qPCR. Fluorescence levels remain similar throughout the qPCR cycles, and show no exponential increase.

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