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ORIGINAL PAPER

Matching a snail's pace: successful use of environmental DNA techniques to detect early stages of invasion by the destructive New Zealand mud snail

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Abstract Early detection of invasive species allows for a more rapid and effective response. Restoration of the native ecosystem after an invasive population has established is expensive and difficult but more likely to succeed when invasions are detected early in the invasion process. Containment efforts to prevent the spread of known invasions also benefit from earlier knowledge of invaded sites. Environmental DNA (eDNA) techniques have emerged as a tool that can identify invasive species at a distinctly earlier time point than traditional methods of detection. Here, we focus on whether eDNA techniques can be successfully applied to detect new invasions by the destructive New Zealand Mud Snail *Potamopyrgus antipodarum* (NZMS). It is an opportune time to apply eDNA-based detection in *P. antipodarum*, which is currently expanding its invasive range across eastern North America. We collected water samples from eight sites in central Pennsylvania that prior evidence indicated were not yet invaded by the NZMS but were part of the same watershed as other previously documented invaded sites. We found evidence for NZMS invasion at five of the eight sites, with subsequent physical confirmation of mud snails at one of these sites. This study is the first example of successful application of eDNA to detect a previously unidentified invasive population of NZMS, setting the stage for further monitoring of at-risk sites to detect and control new invasions of this destructive snail. This study also shows potential opportunities for invasion monitoring offered by using low-cost efforts and methods that are adaptable for citizen science.

Keywords Invasive species · Invasion · eDNA · Early detection · New zealand mud snail

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Introduction

Halting initial introductions has been identified as the most efficient and cost-effective method of invasion mitigation (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; Rejmánek and Pitcairn 2002). Accordingly, 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 period 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 and Pitcairn 2002, Anderson 2005; Simberloff 2014, U.S. Department of the Interior 2016). Decreasing this lag time associated with invasive species containment by focusing on early detection of invasions should thus be a key priority for invasive species management by conservation programs.

Early detection using environmental DNA

Traditional methods of detecting new invasive populations often fail to catch invasions during the early stages of invasion when the ability to contain or eradicate these populations is maximized (U.S. Department of the Interior 2016). Environmental DNA (eDNA)-based approaches have emerged as a promising means to monitor ecosystems for the introduction of invasive species during the establishment process (Jerde et al. 2011; Taberlet et al. 2012; Comtet et al. 2015; Lawson Handley 2015; Thomsen and Willerslev 2015; Brown et al. 2016; Ricciardi et al. 2017; Jerde 2021; Sepulveda et al. 2020), as well as to inform management strategies and evaluate success during eradication of invasive species (Carim et al. 2020). Sources of eDNA include soil, water, sediment, and organic media (e.g., spider webs, Xu et al. 2015; sea sponges, Mariani et al. 2019) 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 inorganic components. This DNA is then used to characterize the taxa present within the community and/or to identify particular species within that community (Comtet et al. 2015). 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), and mammals (Andersen et al. 2012), and more assays are continually being developed (Thomsen and Willerslev 2015, see Washington State University's eDNA toolbox: https://labs.wsu.edu/edna/edna-assays/).

For invasion management, eDNA has been used to detect 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 at low cost and used in conjunction with a species-specific probe in order to identify the presence or absence of an invader in ecosystems of concern. Using eDNA to identify specific species allows for higher catch per unit effort when compared to electrofishing (Jerde et al. 2011), for detection of invaders at more sites than traditional field surveys (Dejean et al. 2012; Takahara et al. 2013), and more sensitive detection of small populations compared to trapping (Valentin et al. 2018). Because eDNA rapidly degrades in a dynamic external environment, identifying DNA from an organism in an environment means that organism has recently been present in that ecosystem—though DNA preserved in sediment, permafrost, or ice (Pedersen et al. 2015) and transport of DNA via predators (Merkes et al. 2014) are important exceptions to this understanding. 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 and Altermatt 2014). Calibrating quantitative PCR (qPCR) probe fluorescence with organismal properties like population density, biomass, and allometrically scaled mass along with critical water body parameters like river discharge or water volume also makes it possible to estimate invasive population densities in aquatic environments (Goldberg et al. 2013; Yates et al. 2020). Here, we describe the first successful application of eDNA to detect a previously unknown population of the destructive invasive New Zealand snail *P. antipodarum*. We also provide an example of the efficacy of eDNA monitoring as compared to traditional surveys for identifying invasive species in at-risk areas.

New Zealand mud snail invasion

Potamopyrgus antipodarum, commonly called the New Zealand Mud Snail (NZMS) in invaded regions, is native to freshwater lakes and streams in New Zealand. NZMS was first observed in the River Thames in the 1850s (Smith 1889) and spread across central Europe by the 1970s (Städler et al. 2005). Invasion of North America by NZMS was discovered in 1987 in the Snake River in Idaho (Bowler 1991). The 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 and Drown 2011). New Zealand mud snails have also invaded the Great Lakes (Levri and Jacoby 2008; Levri et al. 2007, 2008, 2012)—likely a secondary invasion from European invasive populations (Dybdahl and Drown 2011; Donne et al. 2020)—and other watersheds in the Eastern US in New York (R. Hood, pers comm.), Pennsylvania (R. Morgan, pers comm., Levri et al. 2020), and Maryland (J. Kilian, pers. comm.). Additional documented invasions around the world include the Black Sea (Son 2008), Italy (Gaino et al. 2008), Japan (Ogata et al. 2010), South America (Collado and Fuentealba 2020), Spain and Portugal (Alonso et al. 2019), and Turkey (Odabaşi et al. 2019).

Multiple studies have provided important insights into the consequences of NZMS invasion. First, invasive NZMS populations can grow to extremely high densities, exceeding 500,000 individuals m^{-2} (Hall et al. 2006). This physical density can translate into the loss, via competitive exclusion, of other species that colonize or dwell along the substrate (Alonso and Castro-Díez 2012). For example, 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 and Baker 2008), and that NZMS are a poorer food source than other gastropods for tench (*Tinca tinca*) (Butkus and Višinskienė 2020). The implications are that NZMS invasions have serious potential consequences that could affect multiple trophic levels in invaded ecosystems. The New Zealand Mud Snail has had demonstrably negative effects on ecosystems where they have successfully established. Hall et al. (2003) found 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. Moore et al. (2012) found that invasive NZMS 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.

While the range of the western 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 (Levri et al. 2020). 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 and Drown 2011). These are

likely separate invasions (Donne et al. 2020): the US1 haplotype matches haplotype 37 (Genbank AY570216, Neiman and Lively 2004) found on the North Island of New Zealand, while the US2 haplotype matches haplotype 22 (Genbank AY570201, Neiman and Lively 2004), which is also found in the southern South Island of New Zealand (Neiman and Lively 2004; Neiman et al. 2011). The US2 haplotype also matches the invasive European A mitochondrial haplotype (Dybdahl and Drown 2011), indicating a possible secondary invasion originating with the successful invasive population in Europe (also see Donne et al. 2020). The invasive population found in Spring Creek is composed entirely of a clone with the US1 mitochondrial haplotype (M. Dybdahl, pers. comm.), matching the dominant clone in the western US rather than the US2 haplotype of the geographically nearer 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 (also see Donne et al. 2020).

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 and Drown 2011; McKenzie et al. 2013). The possibility of recreational transport of NZMS poses a threat to the local trout population and raises the potential for NZMS to be accidentally 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. Goldberg et al. (2013) developed eDNA protocols for *P. antipodarum* and demonstrated that these protocols were effective at detecting even the recent presence of a single snail. They also showed that they could apply these protocols successfully in a field site already known to harbor invasive *P. antipodarum*. Here, we build and expand on these results in the first application of eDNA-based early detection of NZMS of which we are aware. This study presents an important step forward in demonstrating that eDNA can be successfully applied to detect new *P. antipodarum* invasions and will allow us to more accurately track and potentially halt ongoing range expansion of this destructive invasive species.

Methods

Developed at the University of Idaho (Goldberg et al. 2013), eDNA and qPCR protocols for NZMS have proven effective at detecting these snails in known invasion sites and at estimating population density in streams with measured discharge. To our knowledge, these methods have not previously been applied to identifying new invasive populations of NZMS. We 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. We 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

We 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, Fig. 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 and Blum 2007), we chose to assess only presence/absence of NZMS at these selected sites rather than monitoring eDNA densities. Six of these sites had been the focus of multiple unsuccessful searches for physical evidence of NZMS invasion between 2014 and 2018 (Levri et al. 2020); two sites, at Yellow Creek (YC1) and Cedar Run (CR1), had not been searched prior to this study. Time constraints and unusually high water levels prevented thorough traditional sweep net-based visual searches for NZMS at these eight sites on the days of sampling.

Table 1 Results of qPCR from sites sampled in May of 2018

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	

All sites sampled were locations where NZMS were not detected previously. Locations of sites can be seen in Fig. 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 was considered positive if probe fluorescence reached an exponential phase during the qPCR assay

*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; Deiner and Altermatt 2014)

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

Field collections and filtration

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

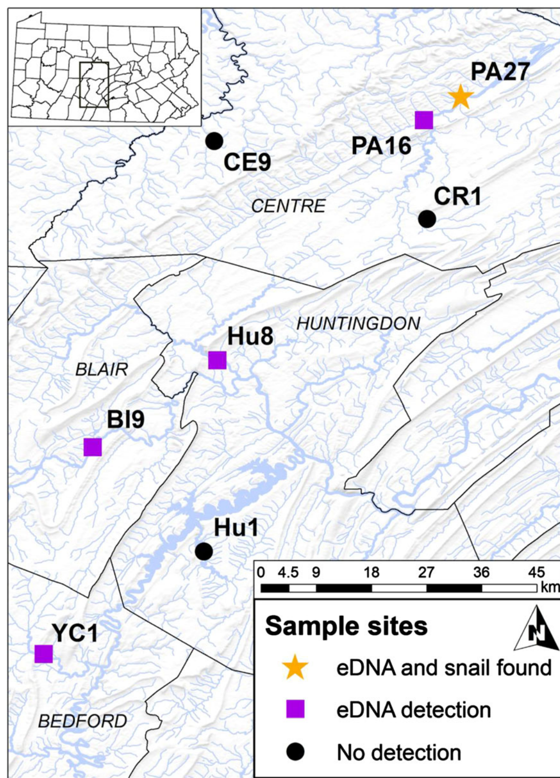

Fig. 1 Map of sampling sites in central Pennsylvania. Circles are sites where no NZMS eDNA was found. Squares indicate the presence of eDNA but without confirmation of physical presence. The star marks site PA27 on the Bald Eagle Creek where NZMS eDNA was found and the physical presence of NZMS was later confirmed through hand sampling

moving. In deeper or higher velocity streams, we 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. We filtered all water samples at the Altoona Campus of the Pennsylvania State University within 24 h of collection.

We used Nalgene vacuum filter flasks that were sterilized by soaking in a 50/50 bleach and deionized water solution for at least 15 min followed by a thorough rinse with deionized water before starting the filtration process for each sample. We also used a 50% bleach solution to sterilize the workspace used for the flasks as well as the forceps that we used for sample processing. As an additional means to prevent contamination, we placed fresh paper towels under the flasks during filtration for each sample. We used 0.45 um mixed-cellulose ester filter discs for filtration. Because of relatively high sediment load in the water bodies that we sampled, these filters rapidly became clogged with sediment. We 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. We 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. We used the same technique to filter 3000 mL of 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, we 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), our goal was qualitatively different and provides an important step forward from the perspective of controlling the expanding NZMS invasion: detect newly invading NZMS. Accordingly, we used qPCR-based detection of eDNA only to determine NZMS presence/absence. We extracted DNA from the filters and processed the filters for quantitative PCR (qPCR) at University of Iowa. Before DNA extraction began, we used DNAZap as well as the 50/50 bleach mixture to clean the bench space. We then used fresh paper towels on the bench and newly bleached equipment for each sample. We extracted DNA from the filters with a DNeasy Blood and 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, we processed an unused filter alongside the filters used for water samples, field and filtering negative controls, and positive control. We used a fume hood that was sterilized with 50/50 bleach and DNAZap for loading qPCR plates. The forward primer sequence was 5'-TGTTTCAAGTGTGCTGGTTTAYA-3', the reverse primer sequence was 5'-CAAATGGRGCTAGTT-GATTCTTT-3', and the probe sequence was 6FAM-CCTCGACCAATATGTAAAT. These primers were designed to amplify a polymorphic section of the mitochondrial gene cytochrome *b* (Goldberg et al. 2013). The probe was designed to have no ambiguous bases. Goldberg et al. (2013) demonstrated that these primer pairs and probes successfully detected eDNA across the range of mitochondrial cytochrome b diversity characterized in NZMS and did not result in false positives in the presence of the commonly co-occurring pebblesnail (*Fluminicola hindsii*; Goldberg et al. 2013).

We 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 15 min followed by 50 cycles of 94 °C for 60 s and 60 °C for 60 s. 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 when compared visually to probe fluorescence in control wells with known DNA densities. Wells without exponential increase in fluorescence were considered negative results. Because of varying sediment load across samples, and, accordingly, varying challenges with clogged filters, the number of filters processed per sample was not equal (N = 2–7 filters per sample). To accommodate this variable, we 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; Deiner and Altermatt 2014). We returned to the site with the highest detectability (PA27) to perform a thorough search for physical evidence of NZMS presence via hand sampling with nets. This additional line of evidence was crucial for a field demonstration of the viability of eDNA-based early detection of a new NZMS invasive population.

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; Fig. 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 but positive detectability still indicate NZMS DNA was likely present at low density. Alternatively, these results could indicate physical drifting of DNA from another site within the watershed or an error not caught by the controls. However, NZMS may be in these streams but remain undetected, and the low detectability is a consequence of low snail density rather than error. While the COVID-19 pandemic prevented planned returns to the sites, returning to these sites in the future will be the first step in determining the cause of this low detectability score. 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, we were able to return to Bald Eagle Creek at the PA27 location in November 2018 and positively identified a single individual NZMS after thorough searching.

In the absence of sequencing the eDNA amplicons, it is important to acknowledge that we cannot formally rule out the potential that our "positive" results instead represent organisms that are not *P. antipodarum*. We do believe that such false positives are unlikely in light of the fact that *P. antipodarum* is the only member of this New Zealand-native genus to ever be found in North America. We also used a nucleotide blast (NCBI's blastn) to determine that our primer pairs and probes were not represented in their entirety in the genomes of any non-*Potamopyrgus* organisms in the blastn database as of March 9, 2021.

Discussion

We were able to detect eDNA at a site, PA27, at which *P. antipodarum* had never been seen and that was later confirmed to harbor NZMS (Levri et al. 2020). We successfully applied eDNA-based methods for early detection of a previously unknown NZMS invasive population. We also demonstrated that eDNA was able to detect NZMS present at likely very low frequency where previous traditional surveys had not identified their presence. Finally, we 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. An obvious next step is to return to these sites where eDNA was detected but snails have not yet been found. Continuing to monitor these locations using eDNA will allow us to track population density increases in known populations and locate dispersal events as they occur.

DNA of other freshwater species has been detected via eDNA-based approaches up to 12 km downstream from its source (Deiner and Altermatt 2014). These reports are difficult to reconcile with more recent studies suggesting that eDNA does not travel further than $\sim$ 200 m (Wilcox et al. 2016; Bedwell and Goldberg 2020). Resolution is hinted at by an experimental study (Shogren et al. 2017) demonstrating that eDNA dispersion is both complex and influenced by stream properties like turbulence and substrate structure to a similar or even greater extent than invader population density and DNA release rates. Future attempts to locate and track low-density NZMS populations—and, broadly, any study applying eDNA-based detection of invasive species in an aquatic context (reviewed in Barnes and Turner 2016; Harrison et al. 2019)—will need to address how density of nearby populations and properties of the stream (e.g., morphology, substrate, and flow) may affect detectability of NZMS in the region.

A future study comparing genotypes of this new invasive population at PA27 to other nearby NZMS populations may be able to identify the source populations (Clusa et al. 2016) and address the possibility of physical drift of eDNA 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 we could not formally rule out a scenario where we had detected DNA drifting from a different site. Additional insight into whether and how we need to account for the physical drift of DNA will also come from experiments aimed at characterizing the distance and time over which drifting NZMS DNA can be detected in the environment (as suggested by Harrison et al. 2019). This information 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 our 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. We intend to return to these sites for continued traditional searches for physical evidence of the presence of NZMS. We also intend to include these sites in future eDNA surveys, and will focus on later (vs. earlier)-season efforts, which maximizes detectability in river systems (Bedwell and Goldberg 2020). We predict 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 could 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 our procedures. A subsequent negative result could also indicate the loss of an established population or failure to establish a steady population at an invasion front despite presence during our initial sampling.

Our results, along with the recent discovery of NZMS in the Bald Eagle Creek in Lock Haven (Levri et al. 2020), indicate that NZMS has expanded its invaded range in central Pennsylvania beyond the Spring Creek watershed. 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 additional discoveries of NZMS in the Mid-Atlantic region (Simberloff 2014). Once NZMS establishes in a new location it would likely take a few years for the population to become large enough to be detected via traditional approaches (Simberloff 2014, 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, NZMS was discovered in the Musconetcong River in New Jersey and Little Lehigh Creek near Allentown, PA (R. Morgan, pers. comm.), and in 2020, it was found in Codorus Creek near York, PA (C. Urban, 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.

In particular, we can use eDNA approaches to slow the rate of NZMS spread in the eastern US by making recreational water users aware of NZMS presence, implementing checkpoint procedures near invaded sites, and ideally, limiting access to invaded locations as a rapid response (Simberloff 2014, U.S. Department of the Interior 2016). We have provided a proof-of-principle for using eDNA to detect previously unknown populations of NZMS. 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; Larson et al. 2020), which have demonstrated success with respect to generating new knowledge on invasive species (Johnson et al. 2020). A relevant recent example is provided by Larson et al. (2017), who report successful application of citizen science-based collection of eDNA samples to detect invasive crayfish in North American lakes. With the cooperation of those individuals that use these ecosystems for recreation, it may be beneficial to pursue a program wherein sterile containers and basic training are supplied to fishermen, boaters, and kayakers. After sampling, the containers can be dropped off at a laboratory for quality assurance and processing. While a citizen science-oriented program could introduce more opportunities for error—Biggs et al. 2015, for example, reported in 8.7% false negatives of eDNA identification from samples collected by citizens at sites known to support the target species—ongoing assessment of potential invasion sites would allow for oversight of sampling methods and cross-referenced results. This distributed method of early detection would cast a wide net in which to catch these destructive aquatic invaders.

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Author's contributions JW planned the project, adapted eDNA techniques for the project, led sampling and analysis, and was the primary author of this manuscript. MN helped plan the project and helped write the project proposal and final manuscript. EPL led the project proposal, provided assistance with sampling, and oversaw the project and final manuscript.

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Availability of data and material Detectability numbers needed for conclusion contained within Table 1

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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