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Environmental DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*)

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Abstract. Early detection of aquatic invasive species is a critical task for management of aquatic ecosystems. This task is hindered by the difficulty and cost of surveying aquatic systems thoroughly. The New Zealand mudsnail (*Potamopyrgus antipodarum*) is a small, invasive parthenogenic mollusk that can reach very high population densities and severely affects ecosystem functioning. To assist in the early detection of this invasive species, we developed and validated a highly sensitive environmental deoxyribonucleic acid (eDNA) assay. We used a dose–response laboratory experiment to investigate the relationship between New Zealand mudsnail density and eDNA detected through time. We documented that as few as 1 individual in 1.5 L of water for 2 d could be detected with this method, and that eDNA from this species may remain detectable for 21 to 44 d after mudsnail removal. We used the eDNA method to confirm the presence of New Zealand mudsnail eDNA at densities as low as 11 to 144 snails/m^2 in a eutrophic 5th-order river. Combined, these results demonstrate the high potential for eDNA surveys to assist with early detection of a widely distributed invasive aquatic invertebrate.

Key words: environmental DNA, eDNA, early detection, New Zealand mudsnail, *Potamopyrgus antipodarum*, qPCR.

Early detection of invasive species increases the probability that control and eradication efforts will be successful (Anderson 2005). In addition, the costs associated with early detection and subsequent rapid response efforts are far less than those of long-term management programs for populations that have already become established and spread (Vander Zanden et al. 2010). However, we lack effective tools for detecting invasive aquatic invertebrates when they are in low abundance. Current tools for the detection of aquatic invertebrates, such as Hess sampling, require tremendous effort because the probability of detecting rare species is low (e.g., Cao et al. 1998). As a result, many invasive species are not detected until they have established large populations and spread (Crooks and Soulé 1999).

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The use of environmental deoxyribonucleic acid (eDNA) shed by aquatic species can be a sensitive and effective tool for species detection (Ficetola et al. 2008, Goldberg et al. 2011, Jerde et al. 2011, Thomsen et al. 2012), and eDNA techniques may be especially helpful in early detection of invasive species. This new method is being used extensively to track the invasion front of Asian carp (*Hypophthalmichthys molitrix, Hypophthalmichthys nobilis*) in the North American Great Lakes ecosystem and has revealed that these species have spread beyond barriers that were designed to prevent their passage (Jerde et al. 2011). A field application of an eDNA test for the invasive American bullfrog (*Lithobates catesbeianus*) has demonstrated the high level of sensitivity of this method over standard field methods for detection of this species in ponds (Dejean et al. 2012).

Development of eDNA detection of macroorganisms in aquatic environments has focused principally on fish and amphibians, both of which are covered by permeable skin that can slough and contribute to eDNA presence in water samples. Invertebrates with exoskeletons or shells may be less detectable using eDNA methods because the contribution of eDNA

from individuals to the aquatic environment may be considerably less than for vertebrates. However, Thomsen et al. (2012) recently found high detection probabilities for the large white-faced darter dragonfly (*Leucorrhinia pectoralis*; 0.82) and the crustacean tadpole shrimp (*Lepidurus apus*; 1.00) using eDNA methods, thereby demonstrating the potential of eDNA for detecting arthropods. Detection of gastropods via eDNA has not yet been documented.

The New Zealand mudsnail (*Potamopyrgus antipodarum*) is a parthenogenic, live-bearing, highly fecund species found naturally throughout New Zealand and is an invasive species in freshwater ecosystems of North America, Europe, Australia, and Asia (Alonso and Castro-Díez 2008). All western US states (with the exception of New Mexico) now have confirmed populations (Benson 2008). In this region, densities in infested waters typically range from 10,000/m^2 to 40,000/m^2, but densities >400,000/m^2 have been documented (Hall et al. 2003). At high densities, New Zealand mudsnails can sequester most stream primary production, limit nutrient cycling, and dominate invertebrate stream secondary production (Hall et al. 2003, 2006). New Zealand mudsnails can competitively exclude some native primary consumers and detritivores, particularly aquatic insects and gastropods (Kerans et al. 2005, Riley et al. 2008). Unlike native aquatic insects, New Zealand mudsnails are effectively a trophic dead end. Native fish eat mudsnails, but they assimilate very little energy from eating snails and suffer reduced growth rates (Vinson and Baker 2008).

Detection probabilities of New Zealand mudsnails with current methods (e.g., Hess samples) are not known, but they are thought to be low when these mudsnails occur at low densities (Levri et al. 2007, Trebitz et al. 2010). Development of new techniques for detecting low-density New Zealand mudsnail populations is a top priority for the management of this invasive species (Proctor et al. 2007). In addition, the intergovernmental Aquatic Nuisance Species Task Force (http://www.anstaskforce.gov/default.php) recommends that early detection should be a core component of state management plans for New Zealand mudsnails. To meet this need, we developed a quantitative polymerase chain reaction (qPCR) assay for New Zealand mudsnails and tested its sensitivity and specificity through time and in relation to density with laboratory experiments and field observations. New Zealand mudsnails are a good candidate for eDNA detection from water samples because no life stage of this species is hidden from detection (i.e., occurs out of water or is carried exclusively by a host). Validations of our eDNA test show that it is highly accurate and ready for broad-scale implementation for early detection of this invasive species. The success of this project also indicates the potential for detecting other small, cryptic invertebrate species using eDNA techniques.

Methods

We investigated the potential for eDNA tests to provide an efficient and temporally sensitive tool for early detection of New Zealand mudsnails in a 2-phase project. In the 1st phase, we conducted a dose–response laboratory experiment to examine the sensitivity of the test to presence and removal of this invasive species. In the 2nd phase, we evaluated the effectiveness of this technique in the field by comparing eDNA results to results from standard Hess sampling of the same river reaches.

Dose–response laboratory experiment

We conducted a laboratory experiment to estimate the lower limit of eDNA detection of New Zealand mudsnails and the relationship between New Zealand mudsnail density and eDNA concentration. We acquired New Zealand mudsnails via express mail from infested springs at Hagerman National Fish Hatchery in Hagerman, Idaho (USA), to the biosecure facility at the University of Idaho. This facility is held at a constant air temperature of 15°C. This source population is dominated by individuals with the single mitochondrial DNA haplotype found across western North America (Dybdahl and Drown 2011). Upon receipt, we placed individuals in concentrations of ~300/1.5 L of fresh, dechlorinated water and allowed them to acclimate for a minimum of 24 h with food ad libitum. This species releases neonates en masse upon reaching a stable environment after shipping, so we reduced the chance of bias caused by this unusual influx of DNA sources by removing individual snails after acclimation and placing them in clean, 1.8-L plastic containers with 1.5 L fresh water in desired densities. We used 5 density treatments with 3 replicates of each density: 1, 10, 50, 100, and 200 New Zealand mudsnails. To test if nonspecific DNA affected assay success, we included 50 pebblesnails (*Fluminicola hindsii*; a common co-occurring species in the USA) in the 50-mudsnail-density treatment. A negative control treatment (3 replicates) with no snails was sampled on day 0 and on each sampling occasion during the experiment. To minimize the chance of accidental release of individuals and reduce evaporation, we covered all containers loosely with plastic wrap and placed them in larger secondary aquaria during the experiment.

During the first part of the experiment, we collected 250 mL of water from each replicate after 2 d. To ensure that our 2-d sampling did not affect subsequent sampling through reduced water volume or addition of new water, we moved mudsnails to fresh dechlorinated water (1.5 L) and collected water samples 4 d later. After collecting day-4 samples, we poured the remaining treatment water through a disinfected 90-µm-mesh filter to remove all mudsnails and potential neonates, and collected water samples 3, 21, and 45 days after snail removal. Estimating the persistence of New Zealand mudsnail DNA provides knowledge about factors that may affect detection precision (Dejean et al. 2011) and estimates a maximum length of time eDNA could be detected in a system after eradication. Prior to collecting the water samples, we stirred each container gently to homogenize the sample and disturb any stratification of eDNA. Before mudsnails were removed from experimental containers, we used disposable paper cups to collect water samples from containers. We poured the water into disposable cellulose nitrate filter funnels (Whatman) with 0.45-µm pore-diameter filter paper (as in Goldberg et al. 2011) for capturing eDNA. After mudsnails were removed, we poured 250 mL of water directly from the containers into the filter funnels. We affixed filter funnels to a polypropylene vacuum flask with a rubber stopper, and actively drew water samples through the filter paper with a vacuum line. We removed each filter from the funnel with forceps, folded it in half, and then rolled and stored it in 1 mL of 95% ethanol at room temperature until DNA extraction. To reduce the chance of cross contamination, we soaked forceps in a 50% bleach solution, rinsed them with water, and allowed them to air dry between samples. We ln(x)-transformed estimated DNA quantities to homogenize variances and analyzed the data with repeated measures analysis of variance (rm ANOVA) in R (version 2.13.0; R Development Core Team, Vienna, Austria).

Field validation

We collected water filter samples from 2 field sites in the Portneuf River near Pocatello, Idaho. The Portneuf River is a 5th-order river that flows ~150 km from its headwaters to its confluence with the Snake River and drains a 3445-km^2 basin. It is a human-altered, eutrophic river with high nutrient and sediment loads (Marcarelli et al. 2009). Our upper site, Croney Road, was at river km (RK) 140 (lat 42.77428°N, long 111.99022°W) and our lowest site, Cheyenne Crossing, was at RK 35 (lat 42.82380°N, long 112.40585°W). Mean discharge measured at the mid-section (US Geological Survey [USGS] Station 13073000 at Topaz) ranges from 1.6 to 13.7 m^3/s during base flow. We collected water samples on 13–16 September 2011, when river discharge was 2.8 and 2.3 m^3/s at the RK 140 and RK 35 sites, respectively. Primary production and suspended sediment concentrations also varied among sites and were estimated in September 2011. At RK 140, macrophytes were common and biomass was estimated as 126 g/m^2. Periphytic chlorophyll *a* (chl *a*) levels were 10 mg/m^2. At RK 35, macrophytes were absent from our sampling reach and periphytic chl *a* levels were 1 mg/m^2. Optical turbidity averaged 2 NTU at the upper site and was nearly 5× higher in the downstream site (9 NTU).

We collected three 4-L water samples per site (river left, center, and right) along a transect established perpendicular to the stream channel. At each location, we completely submerged sterile 4-L containers (~10 cm) until full. After collection, we stored water samples in sealed containers on ice. We filtered all samples to collect eDNA within 12-h of sample collection. To assess the potential for false positives associated with handling, transport, and equipment contributions, we included 3 types of quality-control samples in our analyses. These included 3 field negatives (i.e., blanks)/site, 3 travel blanks/d, and 3 equipment blanks/d. Field negatives consisted of 4 L of deionized water that was transferred to a sample container at each field site and then handled and processed in a manner that was consistent with field samples. Travel blanks consisted of 4 L of deionized water that was transferred to a sample container at the start of each day. We handled, stored, transported, and processed blanks in a manner identical to field samples. Equipment blanks were used to detect contamination from sampling equipment. In this case, we transferred 4 L of deionized water into a sterile sample container at the laboratory and filtered it in a manner consistent with our field samples. To confirm field specificity of the test, we also tested eDNA samples collected by filtering 5 L of stream water through cellulose nitrate filters from 4 additional streams in central Idaho (Deadman Creek: lat 44.966°N, long 115.706°W; Reegan Creek: lat 44.949°N, long 115.587°W; Goat Creek: lat 44.759°N, long 115.684°W; and Nasty Creek: lat 44.877°N, long 115.696°W) where New Zealand mudsnails have not been detected.

To filter water samples, we used a peristaltic pump with sterile tubing and an in-line filter apparatus fitted with mixed cellulose ester membranes with 0.45-µm pore size. We stored used filters in 95%

ethanol at room temperature after processing and rinsed tubing and the filter apparatus in a 50% bleach solution and then with 8 L of deionized water after each use. We used multiple filters when necessary, usually when filters became clogged with particulate matter before the entire 4-L sample was processed.

To confirm the presence of New Zealand mudsnails in benthic substrates, we collected Hess samples at 3 cross-sectional transects from each site immediately after water sampling. We collected 2-min Hess samples (500-μm mesh, 0.09-m^2 sampling area) from equally spaced locations corresponding to the left, center, and middle of each transect and composited the 3 Hess samples into 1 larger sample. The upstream transect at each site was identical to the transect used for eDNA sampling. We preserved macroinvertebrate samples in 95% ethanol and counted the number of New Zealand mudsnails/composite sample in the laboratory.

Genetic analysis

We designed a quantitative PCR test for New Zealand mudsnails using previously published cytochrome *b* sequence data (Neiman et al. 2005, Dybdahl and Drown 2011), obtained through GenBank (http://www.ncbi.nlm.nih.gov/nucleotide/; AY570182–AY570226, HQ680431).

We aligned 431 base pairs (bp) of all 46 sequences with Sequencher 4.7 (Gene Codes Corporation, Ann Arbor, Michigan) and exported the inclusive consensus sequence. We then used Primer Express software (version 3.0; Applied Biosystems, Carlsbad, California) to design a primer-probe set with a maximum of 1 ambiguous base on each primer and 0 on the probe. We confirmed the specificity of this assay in silico using the BLAST algorithm in GenBank. Additional sequences released in GenBank after this design revealed 2 additional haplotypes (61 and 68) from the north island of New Zealand that each differed from the primer/probe set by 1 bp (Neiman et al. 2011). Haplotype 61 was found in 1 individual from Tarawera and differs from the probe design at the 2nd base from the end, whereas haplotype 68 was found in 2 individuals from Okareka and differs from the reverse primer at the 5′ end.

We extracted the DNA from 5 individual New Zealand mudsnails from Hagerman, Idaho, and 5 individuals each of all haplotypes ($n = 4$) that have been detected in North America (haplotypes US1, 2, and 3, and unknown obtained from colonies; Dybdahl and Drown 2011) and 2 each of a set of nontarget co-occurring species (*Fossaria bulimoides, Fluminicola hindsii, Physella acuta, Physella gyrina, Stagnicola caperata, Stagnicola elodes*) using the DNeasy Blood and Tissue DNA extraction kit (Qiagen, Inc., Germantown, Maryland). We used these samples and DNA from both newly discovered New Zealand haplotypes (haplotypes 61, 68; provided by M. Neiman, University of Iowa) to test the sensitivity and specificity of the primer/probe set (Table 1) in the quantitative PCR (qPCR) we designed. We used the QuantiTect Multiplex PCR Mix (Qiagen, Inc.) with recommended duplexing concentrations (1× QuantiTect Multiplex PCR mix, 0.4 μM of each primer, and 0.2 μM of each probe) on an Applied Biosystems 7500 Fast Real-Time PCR System, downsized to 10-μL reactions after pilot testing. Cycling began with 15 min at 95°C followed by 50 cycles of 94°C for 60 s and 60°C for 60 s. We included an exogenous internal positive control (Applied Biosystems) in each well. If the internal positive control indicated inhibition, we diluted the DNA sample 1:10 in water and reanalyzed.

TABLE 1. Primer and probe sequences developed and validated for New Zealand mudsnail assay.

Primer/Probe	Sequence
NZMSF	TGTTTCAAGTGTGCTGGTTTAYA
NZMSProbe	6FAMCCTCGACCAATATGTAAAT–MGB
NZMSR	CAAATGGRGCTAGTTGATTCTTT

We extracted DNA from filters collected in the field and during the laboratory experiments using the QIAshredder/DNeasy Blood and Tissue DNA extraction kit method described in Goldberg et al. (2011) in a room where no high-quality DNA extracts or PCR products had been handled and where researchers are required to shower and change clothing before entering if they had previously been in a room with PCR product. An extraction negative was created with each set of extractions and an additional PCR negative was run with each plate of qPCR. We used 2.5 μL of DNA extract in each reaction and ran all reactions in triplicate. If any reaction showed incomplete evidence for presence of New Zealand mudsnail DNA (tested positive for 1 or 2 wells), the original sample was reanalyzed in triplicate. If any of the wells amplified during a 2nd round, we considered the sample positive and the quantitation amount was averaged over replicates testing positive. We assumed in this calculation that nonreaction of a positive sample reflected the stochastic nature of the PCR process rather than an estimate of 0 DNA in the well. If 0 wells amplified on the 2nd round after 1 amplified on the 1st, we considered the sample negative. If 0 wells amplified on the 2nd round after 2 amplified on the 1st, we required a 3rd round to confirm the negative result. Testing negative was indicated by no

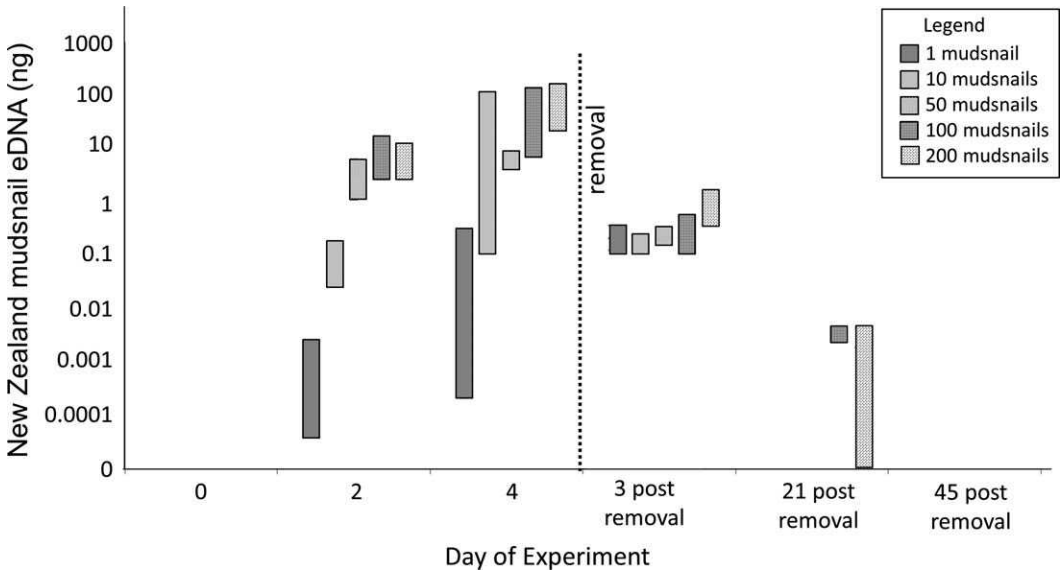

FIG. 1. Ranges ($n = 3$) of New Zealand mudsnail environmental deoxyribonucleic acid (eDNA) in the aquarium experiment. Treatments consisted of various numbers of New Zealand mudsnails in 1.5 L of water (250-mL samples at intervals). All mudsnails were removed on day 4 of the experiment, after samples were taken. No New Zealand mudsnail DNA was found in negative (water) controls at any point or 45 d after the removal of mudsnails.

exponential phase at any point during the 50 cycles. We used DNA extracted from a New Zealand mudsnail and quantified using a NanoDrop spectrophotometer to create a serial dilution to create the standard curve for quantification. Standard curves for all runs had $r^2 \geq 0.98$. For field samples taken across multiple filters, we added together all estimates of DNA to indicate the amount collected in the sample. To estimate daily DNA loads from field samples, we used DNA concentrations and discharge in the following equation: daily DNA load (mg/d) = DNA (mg/L) $\times$ discharge (m^3/s) $\times$ 8.64 $\times$ 10^7 ($L\,s/m^3\,d^{-1}$).

For additional confirmation that positive assays reflected the presence of New Zealand mudsnail DNA, we sequenced qPCR products from 9 samples from across treatments and days of the experiment and 7 samples from across field sites. We sequenced these products in both directions using a BigDye® Terminator version 3.1 Cycle Sequence Kit (Applied Biosystems) on a 3130xl Genetic Analyzer (Applied Biosystems) and aligned the results with the consensus sequence used in the design to confirm a match.

Results

Dose–response laboratory experiment

All New Zealand mudsnail DNA samples tested positive, and all nontarget species tested negative in the assay, demonstrating the sensitivity and specificity of the test. Samples that differed by 1 bp at the 5′ end of the probe and 1 primer, respectively, also produced estimates of DNA in the range of the extracts of haplotypes with perfect matches. DNA of New Zealand mudsnails was detected in all 3 experimental 1.5-L replicates at 2 and 4 d of presence and 3 d after removal for all treatments, including the treatment consisting of only 1 mudsnail (Fig. 1; Table S1; available online from: http://dx.doi.org/10.1899/13-046.1.s1). The highest amount of DNA was estimated in a 200-mudsnail-treatment sample at 4 d (153 ng). At 21 d after removal, no DNA was detected in treatments with <100 mudsnails and in 1 of the 200-mudsnail treatment replicates. At 45 d after removal, no DNA was detected in any replicate. We sequenced 9 samples across replicates and treatment days and confirmed that products from the assay were as expected from New Zealand mudsnails (Table S1). We found strong evidence that DNA detected differed among treatments through time (treatment coefficient: 0.036, $F_{1,69} = 23.49$, $p < 0.01$), with lower-density treatments leading to lower amounts of eDNA detected. Measures for the mixed-species replicates (50 New Zealand mudsnails/50 pebblesnails) were between results for 10 and 100 New Zealand mudsnails, as expected if the addition of nonspecific DNA had no effect on assay success. Negative control replicates tested negative on all sampling occasions.

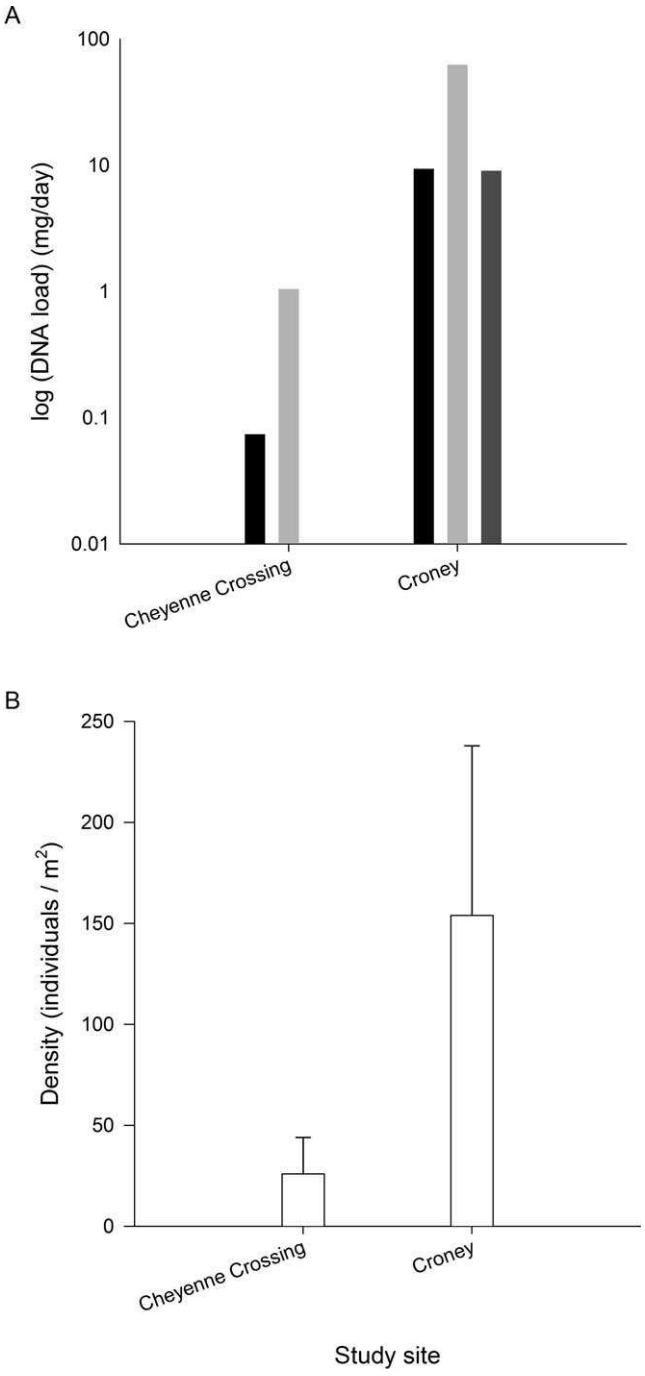

FIG. 2. Mean environmental deoxyribonucleic acid (eDNA) load (A) estimated from water samples collected in the right (black), center (light gray), and left (dark gray) of transects established perpendicular to flow in the Portneuf River and mean (±1 SE) density of New Zealand mudsnails (B) estimated from 3 composited Hess samples at each study site.

Field validation

We detected New Zealand mudsnails in all 3 water samples from the upper site and in 2 of 3 samples from the lower site. Hess sampling confirmed the presence of New Zealand mudsnails at both locations, with densities ranging from 78 to 1078 individuals (ind)/m^2 across our 3 transects at the upper site and 11 to 144 ind/m^2 across our 3 transects at our lower field site. Estimated DNA loads differed among samples collected from each site and ranged from 0.908 to 6.268 mg DNA/d for the upper site and 0 to 1.048 mg DNA/d at the lower site. At both locations, DNA concentrations were highest in samples collected from the middle of the channel when compared to the channel margins (Fig. 2A). We sequenced assay products from 7 samples from across sites and confirmed that sequences matched the alignment used in assay design (exact match with GenBank AY570216, which Dybdahl and Drown 2011 found to be widespread throughout the western USA). Field negatives, travel blanks, and equipment blanks all tested negative for New Zealand mudsnail DNA (Table S2; available online from: http://dx.doi.org/10.1899/13-046.1.s1). Field samples from additional streams in Idaho with no physical evidence of New Zealand mudsnails all tested negative.

Discussion

The ability to detect aquatic invasive species at low densities, whether it is early in an invasion, during transport, or after an eradication attempt, is critical to successful control and management (Hulme 2009). We designed and validated a specific and sensitive assay for detection of invasive New Zealand mudsnails. This method was able to detect as few as 1 individual in 1.5 L of water in a laboratory setting. This method also was able to detect New Zealand mudsnails at low densities (11–78 individuals/m^2) in a large river, indicating a high level of sensitivity and potential utility. Testing the specificity and sensitivity of this method and validating its use in the field is an important first step for managers considering eDNA as a tool for detecting invasive species like the New Zealand mudsnail.

The rapid field-collection protocol, relatively simple field equipment, and low cost make eDNA sampling for New Zealand mudsnails widely applicable to broad-scale inventory, detection, and monitoring efforts. Water samples can be collected throughout the year, even in high-flow conditions that are especially hazardous when using traditional sampling techniques. Moreover, the potential exists to increase sampling effort (e.g., triplicate samples multiple times a year) because eDNA costs (at ~$35 to 80US/sample) are much less than the costs involved with collecting, sorting, and identifying Hess samples (at >$300US/sample). Last, the potential exists to train citizen scientists and field crews that are already involved

with formal water-quality or invasive-species-monitoring programs. However, the spatial resolution of eDNA is not currently known, so, depending on the management need, positive eDNA results may still have to be followed by sampling with traditional tools.

Validation of eDNA detection for New Zealand mudsnails

Our test did not produce false positives when DNA from co-occurring, nontarget species was tested or with samples from streams where New Zealand mudsnails have not been documented. We found no evidence that the presence of co-occurring species in the field or the aquarium experiments prevented detection of New Zealand mudsnail with the eDNA assay. The assay was designed and validated based on the cytochrome *b* haplotypes present in North America (Dybdahl and Drown 2011) and should provide positive results for all documented haplotypes of this species, including those with new haplotypes recently discovered in New Zealand. In accordance with other recent studies (Dejean et al. 2011, Thomsen et al. 2012), we found that eDNA evidence of species presence disappeared between 1 and 6 wk after animals were removed in a lentic laboratory setting. This time probably would be lower under lotic conditions. However, additional work is needed to understand how flow interacts with or diminishes the eDNA signal. Regardless, this assay is complete and ready to apply as a tool for detection of New Zealand mudsnails in North America.

Preventing and detecting false negatives and false positives

The discovery of additional genetic variation in the mitochondrial DNA (mtDNA) region targeted by our assay highlights the need for a comprehensive sequence database to develop eDNA tests. In the case of our study, the previously unknown variation did not prohibit samples from testing positive, but had these mutations occurred at the 3′ instead of the 5′ end of the primer and probe for this assay, false negatives could have been produced. This unknown genetic variation can lead to false negative tests using eDNA in 2 ways: 1) a previously unknown variant in the population that is distinct enough not to test positive in the assay becomes the dominant genotype, or 2) population establishment by individuals with such an unknown variant. The probability of these events is low, but we recommend periodic field sampling and genetic analysis at invasion fronts in addition to eDNA methods for early detection programs to ensure the discovery of new haplotypes if they were to occur.

Conversely, genetic mutation or undetected genetic variation in closely related co-occurring species could lead to false positives using eDNA techniques. This event is less likely than the scenario described above because the probability of this mutation or unknown variation specifically matching the assay is much lower than the probability of it mutating away from the target sequence (as would occur with a false negative). However, if species are detected in unexpected locations using qPCR-based eDNA techniques, sequence analysis should be conducted to confirm species identification.

Because of the low quantities of DNA involved in detection of species through eDNA methods and the high sensitivity of the assay, forensic-type laboratory procedures must be followed to prevent sample contamination. These procedures include careful field handling and decontamination, dedicated low-quantity DNA and PCR-free laboratory spaces, and the use of negative field, extraction, and PCR controls. We found that 50% commercial bleach solution adequately decontaminated our forceps, but analysis of negative controls for future applications should be used to detect if field or laboratory decontamination procedures have been inadequate.

Population quantification using eDNA

An additional use for quantitative eDNA results would be to draw inference to the density or abundance of the target species. Data from our aquarium experiment with New Zealand mudsnails indicate a significant relationship between population density and quantity of eDNA detected, with an estimated 22 ng of DNA added per 1.5-L aquarium for each additional 100 New Zealand mudsnails. A relationship between eDNA detected and population density has also been found for amphibians and biomass of fish in aquaria and ponds (Takahara et al. 2012, Thomsen et al. 2012). To apply these relationships to estimate density in small closed systems, we must assume that the eDNA is mixed thoroughly, or spatially integrate sample collections to make sure we sample randomly from the pool of eDNA in the water. Larger wetlands and lotic systems present additional challenges because our spatial extent of inference is not known. Moreover, our field data suggest that eDNA in lotic systems may not be thoroughly mixed in that eDNA concentrations were highest in samples collected from the middle of the channel compared to the channel margins. With additional work to increase our understanding of eDNA transport in lotic systems, eDNA assays may prove useful in distinguishing areas of low from high population densities of aquatic species.

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