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Validation of a nutria (*Myocastor coypus*) environmental DNA assay highlights considerations for sampling methodology

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METHOD

Validation of a nutria (*Myocastor coypus*) environmental DNA assay highlights considerations for sampling methodology

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Abstract

Nutria (*Myocastor coypus*) is a semiaquatic rodent species that is invasive across multiple regions within the United States. Here, we evaluated a qPCR assay previously described for use in Japan for application across invasive populations in the United States. We also compared two environmental DNA sampling methodologies for this assay: field filtration of large volumes of water passed through filters versus direct sampling of small volumes of water. We validated assay specificity, generality, and sensitivity, compared assay performance between two independent laboratories, and successfully tested the assay in situ on a known wild population. The filtration method required fewer samples for environmental DNA detection than direct sampling, but the choice of methods should be assessed based on specific field conditions and time and budget considerations. Our extensive assay validation and comparison across laboratories suggest that the assay is ready to be applied in environmental DNA monitoring of nutria throughout the United States.

KEYWORDS
coypu, direct sampling, eDNA, filtration, invasive species, monitoring, qPCR

1 | INTRODUCTION

Nutria (*Myocastor coypus*), also known as coypu, are large, semiaquatic rodents native to South America. They were introduced to the United States in 1899 and again in the early 1930s for fur farming, and animals that escaped or were intentionally released established feral populations (Ashbrook, 1948; Carter & Leonard, 2002). They are now found in areas throughout the United States, with established populations in 18 states and occurrences documented in 29 states, and their range is predicted to expand with climate change (Hilts et al., 2019; Procopio, 2022; U.S. Geological Survey, 2021). Particularly large populations of nutria are found in four general

regions of the United States: along the Gulf Coast, the East Coast, in the Pacific Northwest, and in central California (Figure 1). Mitigation efforts have been successful in some areas (e.g., the Delmarva Peninsula; Williams, 2018) but in other areas, populations have been able to reestablish their foothold (e.g., California; California Department of Fish and Wildlife, 2022). Similar patterns of reemergence despite removal efforts have been recorded around the world (e.g., Italy; Bertolino et al., 2012), suggesting that management challenges are ubiquitous for this species.

Invasive nutria populations can have considerable economic and ecological impacts. For example, they are known to transform marsh habitat into open water, displace native species, damage crops, depredate aquatic bird nests, increase soil erosion and riverbank collapse, and damage agriculture and levee systems (Bertolino et al., 2012; Hilts et al., 2019; Jojola et al., 2009). Effective management and control are needed to reduce the damage inflicted on ecosystems and agriculture. While complete eradication in established areas has proven to be difficult and expensive, the prompt eradication of isolated and newly colonized areas can be a solid, proactive approach for managing this invasive rodent (Bertolino et al., 2012). This proactive strategy relies on the ability to monitor and detect individuals on an invasion front or a remaining few in an area following an eradication. Effective methods for timely detection are important in these cases because very few nutria need to be present for a population to establish and they are hard to detect at low densities.

Environmental DNA (eDNA) sampling can be a valuable tool for monitoring invasive or endangered species, particularly when the target species is rare or elusive, making them difficult to detect with other methods (Piaggio, 2021). Additionally, advances in eDNA monitoring continue to improve accuracy and sensitivity which may lower costs compared to traditional survey methods (Darling, 2019; McKelvey et al., 2016; Pochardt et al., 2020; Wilcox et al., 2016). Organisms shed cells into the environment (e.g., water, soil, and air) and these traces of DNA can be collected, concentrated, isolated, and amplified to detect taxa of interest without the need to capture the animals themselves. Environmental DNA can be applied to monitoring species of conservation concern as well as invasive species in support of management objectives (Piaggio, 2021; Taberlet et al., 2018). Water samples are often not only utilized for monitoring in aquatic systems (e.g., brook trout [*Salvelinus fontinalis*]; Wilcox et al., 2016) but have also been successfully applied to terrestrial mammals (e.g., invasive wild pig [*Sus scrofa*]; Williams et al., 2018) and semiaquatic species (e.g., Burmese python [*Python bivittatus*], river otter [*Lontra canadensis*], and Japanese water shrew [*Chimarrogale platycephalus*]; Padgett-Stewart et al., 2016; Piaggio et al., 2014; Yonezawa et al., 2020). In freshwater habitats, filtration via handheld or backpack pumps is the most common approach for eDNA sampling (e.g., Carim, McKelvey, et al., 2016) but direct sampling (i.e., scooping water into a plastic bottle) followed by centrifugation, chemical precipitation, or mechanical concentration is also common (Bockrath et al., 2022; Piaggio, 2021).

An assay for eDNA detection of nutria was originally described by Akamatsu et al. (2018) for application in Japan. They used the sequences of Yamanaka et al. (unpublished) to amplify nutria DNA and to calculate a limit of detection for their assay. The goal of their study was to use eDNA to delimit the distribution of nutria in the Yamaguchi Prefecture (Akamatsu et al., 2018). For our study, the goal was to validate the primers and probe reported by Akamatsu et al. (2018) for use in invasive populations in the United States. Several authors have recently advocated for stringent quality standards applied to eDNA assays (e.g., Langlois et al., 2021; Thalinger et al., 2021) suggesting that assays ought to be revalidated prior to use in new geographic areas with potentially different haplotypes of the target species and a different assemblage of cooccurring, closely related nontarget species. Therefore, a thorough validation was needed to ensure the primers and probe are both general (i.e., detecting all target haplotypes) and specific (i.e., detecting only the target species) in the United States. Our aims in this methods study were to validate the assay described by Akamatsu et al. (2018) for application in the United States and to evaluate the performance of two common eDNA sampling approaches when nutria are the target species: field filtration of large volumes of water passed through collection filters versus direct sampling of small volumes of water.

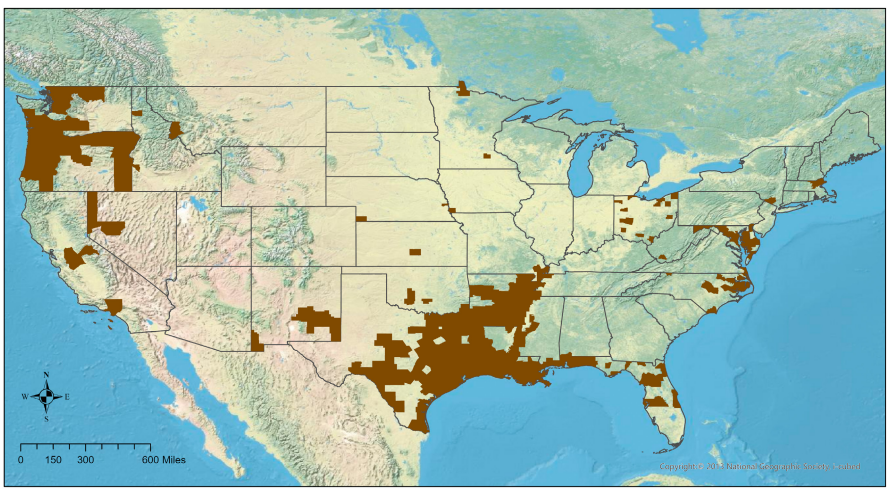

FIGURE 1 The nonnative range of nutria (*Myocastor coypus*) in the United States. Counties with nutria occurrences are shaded in dark brown. County-level occurrence data were downloaded from the Nonindigenous Aquatic Species database (Procopio, 2022) and mapped with ArcMap (ESRI, 2020). The Gulf Coast, East Coast, Pacific Northwest, and central California have particularly large populations of nutria.

2 | MATERIALS AND METHODS

2.1 | Assay testing

To evaluate assay sensitivity in silico, we aligned the primer and probe sequences presented in Akamatsu et al. (2018); forward: 5′-CACTACAACAGCTTTTTCATCAATCAC-3′, reverse: 5′-TTCCT CGTCCAATGTGGAAGT-3′, probe: 5′-FAM-TGATTAATCCGTTATAT ACACGCT-NFQ-MGB-3′ with all nine previously published nutria *cytochrome b* sequences available at the time of validation (August 2021) on the National Center for Biotechnology Information (NCBI) GenBank database (https://www.ncbi.nlm.nih.gov/genbank/; accession numbers: AF422919 [Uruguay], EU544663 [Brazil], MH182628 [Brazil], JF318985 [Germany], KU892780 [France], LC257678 [Japan], LC257679 [Japan], MF325938 [Korea], and NC_035866 [Korea]). Nutria are the only living member of the Myocastoridae family (Bertolino et al., 2012). Members of the parvorder Caviomorpha are almost entirely natively restricted to South America and the Caribbean. However, in addition to nutria, it includes guinea pigs (*Cavia porcellus*) and long-tailed chinchillas (*Chinchilla lanigera*), which are common pets, as well as New World porcupines (Erethizontidae), which are native to the United States (Lacher et al., 2016). Nutria are only distantly related to the phenotypically similar muskrat (*Ondatra zibethicus*), mountain beaver (*Aplodontia rufa*), and American beaver (*Castor canadensis*; estimated divergence 73 million years ago, Kumar et al., 2017). We evaluated assay specificity in silico by identifying the number of mismatches between oligonucleotides and six related nontarget sequences, including guinea pig, long-tailed chinchilla, North American porcupine (*Erethizon dorsatum*), muskrat, mountain beaver, and North American beaver. To ensure that the assay does not amplify more distantly related taxa, we also conducted a Primer-BLAST search of the GenBank database using default settings (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

We evaluated assay specificity in vitro by testing DNA extracted from tissue, blood, or scat samples from one individual of each of these nontarget species. Tissue samples from North American porcupine, muskrat, mountain beaver, and North American beaver were sourced from collections at the National Genomics Center for Wildlife and Fish Conservation (NGC; Missoula, MT). These samples were originally collected by state and federal partners from wildlife mortalities in accordance with appropriate sampling permits and wildlife handling approvals. Scat samples from guinea pig and long-tailed chinchilla were collected from animal pens at a local pet store and did not require any animal handling. DNA from all samples was extracted via DNeasy Blood and Tissue Kits (QIAGEN) and diluted to 0.1 ng/μL, as determined using a Qubit 2.0 fluorometer (Thermo Fisher Scientific) prior to quantitative polymerase chain reaction (qPCR). Each qPCR was 15 μL total, composed of 7.5 μL of 2× TaqMan Environmental Mastermix 2.0 (Life Technologies), 0.75 μL of 20× assay mix (600 nM of each primer and 250 nM of probe), 4 μL of template DNA, and 2.75 μL of laboratory-grade sterile water. Primer concentrations in the above reaction were chosen by testing all 16 possible combinations of forward and reverse primers at a concentration of 100, 300, 600, and 900 nM; the optimal concentration combination (600:600) was the concentration producing the lowest quantification cycle (Cq) value and highest end point fluorescence with a target synthetic gene. Thermocycling conditions were 95°C 10 min, (95°C 15 s, 60°C 60 s) × 45 cycles, as outlined by Akamatsu et al. (2018). On each qPCR plate, we included a negative control well and nutria tissue extracts as positive control wells to ensure that the assay performed as expected. We considered a positive as linear amplification, determined by expert technician judgment, prior to 45 cycles with typical curve morphology. We considered a positive in any qPCR well as a detection.

We evaluated assay generality in vitro by testing genomic DNA from 50 nutria collected along the Gulf Coast, the East Coast, in the Pacific Northwest, and in central California, the four most populous regions of their invasive range in the United States: California ($n = 15$), Louisiana ($n = 5$), Maryland ($n = 20$), Oregon ($n = 8$), and Virginia ($n = 2$). Additionally, a representative tissue sample from each state was Sanger sequenced in-house or via Eurofins Genomics (Appendix S1). Sequences were aligned and edited using SEQUENCHER v5.4.6 (Gene Codes) and compared to the nucleotide database on GenBank using default settings in Basic Local Alignment Search Tool (BLAST; February 2023). Tissues were collected from euthanized nutria that were sampled ancillary to population reduction for damage mitigation conducted by the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) Wildlife Services and the California Department of Fish and Wildlife. Given that tissue samples were collected secondarily to legally authorized control of nutria, sample collection was exempted from Institutional Animal Care and Use Committee review. Tissues were stored, processed, and analyzed as described above, by the NGC and the National Wildlife Research Center (NWRC; Fort Collins, CO).

2.2 | Laboratory comparison

Robust quality control and clear reporting of assay metrics ensure that results are comparable across laboratories and can be defensibly interpreted (Klymus et al., 2020). Here, we use the limit of detection (LOD) and limit of quantification (LOQ) definitions outlined by Klymus et al. (2020), where LOD is the lowest concentration of target analyte that can be amplified with a 95% detection rate and LOQ is the lowest concentration of analyte in a sample that can be quantitatively determined within a stated precision (we used a coefficient of variation [CV] of 35%). We compared LOD and LOQ across two laboratories—the NGC and the NWRC—to evaluate the consistency of assay performance. Both laboratories determined LOD and LOQ via qPCR with replicate standard curves derived from synthetic double-stranded DNA of nutria (gBlock gene fragments; Integrated DNA Technologies). Specifically, the NGC laboratory analyzed LOD and LOQ using a single plate with six replicates of each of seven dilution levels (2, 10, 50, 250, 1250, 6250, and 31,250 copies per reaction) and six no template controls on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific). The NWRC laboratory

analyzed three plates, each with 16 replicates of each of five dilution levels ($n = 48$ each at 0.4, 2, 4, 40, and 400 copies per reaction), eight positive controls, and eight no template controls ($n = 24$ each) on a CFX96 C1000 Touch Real-Time PCR Detection System (BioRad). We analyzed results using the curve fitting methods described by Klymus et al. (2020) to determine LOD and LOQ. We used the option "Best" in the corresponding R code to automatically select the best fitting model choice for LOD and the model with the lowest residual standard error for LOQ (Klymus et al., 2020; Merkes et al., 2019; R Core Team, 2021).

2.3 | Field validation

We tested the assay in situ using environmental (i.e., water) samples collected in Oregon, USA, from areas with known presence of nutria ($n = 9$ waterbodies; nutria observed or fresh sign reported by the collector; collected from Marion County in November 2021). Sites were selected based on local knowledge of nutria distribution of an author (IHP) and fellow Wildlife Services personnel. Samples were collected at locations where we could access the bank safely and without entering the water to avoid cross-contamination. We compared two commonly used DNA collection and extraction methods using known-present samples (Table 1). The first method, hereafter referred to as the filtration method, followed the protocol of Carim, McKelvey, et al. (2016). Briefly, we passed up to 5 L of water through a 1.5 μm pore size, 47 mm diameter glass microfiber filter with an electronic peristaltic pump. The collector stood on the shore and reached out approximately 1 m to submerge the filter cone approximately 10 cm under water. We collected a single sample at each site; samples were comprised of up to two filters if the first filter became clogged during the sample collection process (Table S1). We preserved the filter in silica desiccant prior to receipt at the NGC laboratory, where samples were stored at −20°C prior to analysis. To isolate DNA from the filters, we cut each filter in half; one half of the filter was archived at −20°C for future analyses and the other half was extracted using a modified DNeasy Blood and Tissue Kit (QIAGEN) protocol. We followed the manufacturer's protocol for extraction from animal tissue with the modifications described in Carim, Dysthe, et al. (2016). In cases where a sample consisted of multiple filters, we extracted each filter separately and then spun them through the same silica spin column (see Carim, Dysthe, et al., 2016). We stored DNA extractions at −20°C until analysis. Samples from areas with known absence of nutria ($n = 7$; site elevation is outside the range of nutria cold tolerance; collected in Union County, Oregon in October 2017) were also collected and extracted using this filtration method.

The second DNA collection and extraction method, hereafter referred to as the direct sampling method, followed the optimized protocol of Williams et al. (2017). Briefly, 45 mL of water were collected directly from approximately 10 cm below the surface in a 60 mL plastic bottle and 15 mL of Longmire's buffer (AquaPhoenix; 100 mM Tris, 100 mM EDTA, 10 mM NaCl, 0.5% SDS, and 0.2% sodium azide) was added for preservation (Williams et al., 2016). The collector stood on shore and reached out approximately 1 m to collect three water samples from each site. We also collected two negative controls (i.e., bottled water with Longmire's buffer) to evaluate potential contamination. We stored water samples at −20°C prior to receipt at the NWRC laboratory where they were stored at −80°C prior to analysis. For DNA capture, two subsamples (15 mL each) were centrifuged at 9000*g* for 15 min and DNA was isolated from each pellet with the DNeasy mericon Food Kit (QIAGEN) following the manufacturer's standard protocol (200 mg). DNA extracts and the remaining 30 mL of each water sample were stored at −80°C.

To analyze environmental samples, we used the qPCR conditions described in Akamatsu et al. (2018) and outlined above with

TABLE 1 Field sampling methodology.

	Filtration Akamatsu et al. (2018)	Filtration Carim, Dysthe, et al. (2016), Carim, McKelvey, et al. (2016)	Direct sampling Williams et al. (2017)
Volume	1 L	up to 5 L	45 mL
Field replicates	1	1	3
Pore size	0.7 μm	1.5 μm	N/A
Filter type	Glass microfiber	Glass microfiber	N/A
Preservation	1 mL Benzalkonium chloride (10% w/v) followed by −20°C	Silica followed by −20°C	15 mL Longmire's buffer followed by −20 and −80°C
Extraction subsample	1 filter	1/2 filter	(2) 15 mL aliquots

Note: The filtration method (Carim, Dysthe, et al., 2016; Carim, McKelvey, et al., 2016) and direct sampling method (Williams et al., 2017) for water collection were compared directly in this study at sites with known nutria presence. The methods used by Akamatsu et al. (2018) were not directly compared in this study but are shown for reference.

the addition of an internal positive control to test for the presence of PCR inhibitors, defined as an internal positive control in the sample being shifted ≥1 Cq higher than that in the negative control. We multiplexed the assay with an internal positive control (TaqMan Exogenous Internal Positive Control; Life Technologies) by replacing 1.8 μL of water with 1.5 μL of 10× exogenous IPC assay and 0.3 μL of 50× exogenous IPC DNA per reaction. We analyzed each extraction with three qPCR replicates and on each qPCR plate, we included three negative control wells and a dilution series of synthetic gene fragments serving as a standard curve and as positive controls. We then calculated the detection rate for each method based on the percent of known-present sites with positive qPCR amplifications. Given the detection rate, we modeled the cumulative probability of detection over a range of sampling effort (i.e., 1–30 samples) as outlined in Davis et al. (2018) to estimate the sample size required to reach an acceptable probability of detection. Additionally, because sites in our field validation study intentionally had higher densities of nutria than we anticipate biologists would encounter in a typical eDNA monitoring effort (i.e., nutria were visually detected during sampling or heavy sign was present), we conducted an informal sensitivity analysis to determine probability of detection at low and medium densities of nutria in addition to the higher density reflected in our field study. We used the model outlined in Wilcox et al. (2018) to estimate the probability of eDNA sampling detection for our two collection and extraction protocols at low (100 copies/L), medium (500 copies/L), and high (1314 copies/L; the mean number of copies/L across all sites as quantified via filtration) concentrations of nutria DNA. We incorporated the volume of water sampled in the field, the volume of water sample analyzed in the laboratory, and the single-copy detection probability estimated from a standard curve experiment (Furlan et al., 2016) as variables in the model (Wilcox et al., 2018).

3 | RESULTS

3.1 | Assay testing

We performed an in silico analysis of the nutria eDNA primer and probe sequences (Akamatsu et al., 2018) with nine previously published GenBank nutria DNA sequences and only a single base pair mismatch was observed on the 5′ terminus of the forward primer in one sequence (EU544663). A single transversion mismatch in this position typically has very little, if any, impact on amplification efficiency (Sharma et al., 2013; Stadhouders et al., 2010; Wright et al., 2014). This evaluation of the nine GenBank sequences demonstrates that the locus targeted by this assay is highly conserved across samples from Japan, Korea, Brazil, Uruguay, Germany, and France.

We identified ≥16 total mismatches between assay oligonucleotides and sequences from co-occurring nontarget vertebrate species. This ought to be more than enough mismatches to preclude amplification (Kronenberger et al., 2022), suggesting that the assay is highly nutria specific. Furthermore, we did not identify any additional potential nontarget taxa that occur within the United States with our Primer-BLAST search of the GenBank database. Finally, we did not observe any amplification for tissue or scat samples from the six nontarget species evaluated, confirming assay specificity.

To ensure assay generality across the United States distribution of nutria, we tested the assay on DNA from nutria harvested across the four largest regions of their invaded range in the continental United States. We observed amplification for all 50 tissue samples collected in California, Louisiana, Maryland, Oregon, and Virginia. Sanger sequences of qPCR product from representative tissue samples in these five states resulted amplicon length of 131 base pairs (bp), as in Akamatsu et al. (2018), with a final amplicon length of 83 bp once primer binding regions were trimmed (CCACATTTGTC GAGATGTAAATTACGGCTGATTAATCCGTTATATACACGCTAATGG AGCATCCTTATTTTTTATTTTCCTTT). BLAST search results identified only nutria sequences to have 100% query coverage and 100% identity to all nine NCBI GenBank sequences.

3.2 | Laboratory comparison

Results from the curve fitting method (i.e., besting fitting model = probit model) determined the 95% LOD was 5.3 copies per reaction in the NGC laboratory and 6.8 copies per reaction in the NWRC laboratory. Only one standard (i.e., two copies per reaction) from the NGC test was detected in the informative range (i.e., not 0% or 100% detection), therefore, the LOD model result is less reliable although still informative (Merkes et al., 2019). The best fitting model for NGC LOQ calculations was the fifth-order exponential decay model, which resulted in a 35% CV threshold of 22 copies per reaction (Figure 2a). The best fitting model for NWRC LOQ calculations was the second-order exponential decay model, which resulted in a 35% CV threshold of 186 copies per reaction (Figure 2b).

3.3 | Field validation

The detection rate at sites with known nutria presence was 100% for the filtration method and 44% for the direct sampling method (Table 2). The mean copy number detected from filtration samples was two orders of magnitude higher than that of the direct sampling method with a range of 21.7–232.0 copies versus 0.5–2.3 copies, respectively. Detections below the assay LOD are valid positive detections but represent concentrations where detection may be inconsistent across repeated analyses (Klymus et al., 2020). For subsequent management action, there may be some additional threshold detection rate or DNA concentration, which depend on the context-specific management goals and risk tolerances (Morisette et al., 2021; Sepulveda et al., 2020). One of the samples (Site 5; Table S1) collected with the filtration method contained PCR inhibitors and was excluded from this comparison, however, a second sample collected at this same site was not inhibited and is reported in

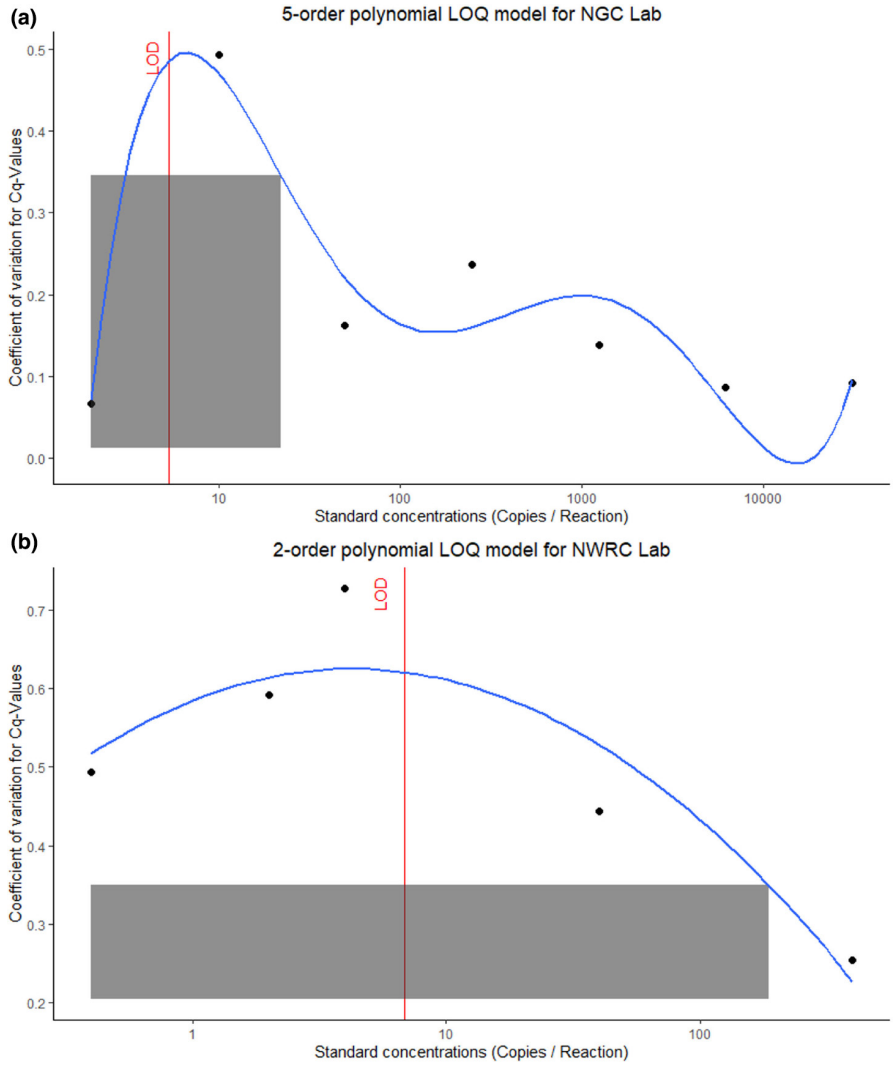

FIGURE 2 Limit of detection (LOD)/Limit of quantification (LOQ) results for the nutria (*Myocastor coypus*) eDNA assay tested in the National Genomics Center for Wildlife and Fish Conservation (NGC) and National Wildlife Research Center (NWRC) laboratories. The coefficient of variation (CV) for the quantification cycle (Cq value) is shown for the tested standard concentrations in copies per reaction. (a) LOD for the NGC laboratory is displayed with a red vertical line at 5.3 copies per reaction as determined by probit modeling. LOQ for NGC is visualized where the gray box—delimiting the 35% CV—intercepts the blue fifth-order polynomial exponential decay model line, at 22 copies per reaction. Black dots depict the seven dilution levels (2, 10, 50, 250, 1250, 6250, and 31,250 copies per reaction) tested by the NGC laboratory. (b) LOD for the NWRC assay validation is displayed with a red vertical line at 6.8 copies per reaction as determined by probit modeling. The 35% CV LOQ and is visualized where the gray box intercepts the blue second-order polynomial exponential decay model line, at 186 copies per reaction. Black dots depict the five dilution levels (0.4, 2, 4, 40, and 400 copies per reaction) tested by the NWRC laboratory.

Table 2. Sites with known absence of nutria were sampled and DNA captured and isolated using the filtration method and all seven were negative for nutria in both laboratories' qPCR analyses. Laboratory generated negative controls also did not amplify at either laboratory.

Using the filtration method, a single sample and three laboratory replicates per site were enough to detect nutria without error at the known-present sites we sampled. In contrast, using the direct sampling method, three samples analyzed with 18 laboratory replicates per site did not always detect nutria presence. We therefore modeled the cumulative probability of detection at different levels of sampling effort. Our results demonstrate that a high probability of detection (e.g., 95%) for nutria at our known-present sites could have been achieved by collecting and analyzing at least nine water samples with the direct sampling method (Figure 3). Because this cumulative probability of detection stems from the high nutria densities and subsequently high DNA concentrations sampled in this study, we also conducted an informal sensitivity analysis to estimate detection based on low, medium, and high DNA concentrations. At a high concentration (i.e., 1314 copies/L; the mean number of copies/L across all sites as quantified via filtration) our results were similar to that of the cumulative detection probability, suggesting six direct samples would be required for 95% probability of detection (Figure 4). At a medium DNA concentration (500 copies/L)—that is, when fewer nutria are present in an area—we estimated that 12 direct samples would be needed to detect nutria DNA accurately, and at a low concentration (100 copies/μL), 30 direct samples would be needed to reach 80% detection probability (Figure 4). A single filtration sample had a 100% detection probability for the high, medium, and low densities of nutria we modeled (Figure 4).

4 | DISCUSSION

In this study, we tested the assay reported by Akamatsu et al. (2018) to (1) validate the assay specificity, generality, and sensitivity across the invaded range of nutria in the United States, (2) compare the assay performance between two independent laboratories, and (3) report results from a small field study using two DNA collection and extraction methods at sites with known presence or absence of nutria. Results from both laboratories suggest that this assay is ready to be applied in eDNA monitoring of nutria for invasive populations throughout the United States. We also discovered variable

detection probabilities between the two sampling and DNA extraction methods that merits discussion in the context of management applications.

The filtration method outperformed the direct sampling method in our field validation study, detecting nutria DNA at all nine sites with known nutria presence and capturing more DNA copies (Table 2). Additionally, this high detection rate and copy number quantification was attained with minimal laboratory burden (i.e., one extraction and three qPCR replicates with filtration vs six extractions and 18 qPCR replicates with direct sampling). Therefore, when feasible, this filtration method is preferred for eDNA monitoring of nutria.

TABLE 2 Results of quantitative polymerase chain reaction (qPCR) analyses of eDNA field samples.

Site #	Filter wells amplified	Filter mean copy number	Direct sample wells amplified	Direct sample mean copy number
1	3/3	204.2	5/18	2.3
2	3/3	55.0	1/18	0.5
3	3/3	23.3	0/18	N/A
4	3/3	46.9	0/18	N/A
5	3/3	232.0	9/18	1.0
6	3/3	21.7	0/18	N/A
7	3/3	53.1	0/18	N/A
8	3/3	31.9	0/18	N/A
9	3/3	144.0	12/18	2.1

Note: Nine waterbodies with known nutria (*Myocastor coypus*) presence in Marion County, Oregon, were sampled via filtration and direct sampling and processed by the National Genomics Center for Wildlife and Fish Conservation (NGC) and National Wildlife Research Center (NWRC) laboratories, respectively. Mean copy number values are reported along with the number of replicates that amplified out of the number of laboratory replicates analyzed.

Abbreviation: N/A, no amplification occurred.

While filtration is practical in a wide range of environments and sampling situations and produced more sensitive eDNA detection results in our study, direct sampling has benefits in certain circumstances. These trade-offs are further described in other publications (e.g., Morisette et al., 2021), but we briefly discuss several issues that deserve consideration when planning eDNA monitoring. Habitats with turbid water can slow down filter collection. Although the sites sample in this study had relatively clear water, most filters still clogged and required two filters to process up to 5 L of water (Table S1). In more turbid environments, filters clog more quickly and may not be able to process the volume of water required to reach the detection rate reported here. A benefit of the direct sampling method is that no filters are required and very turbid samples can be collected rapidly (Williams et al., 2016). Another consideration for deciding which method is practical is the amount of equipment one can transport into the field and the time available for eDNA sample collection (Piaggio, 2021). The NGC filtration method uses a sampling kit which includes an electronic peristaltic pump, pump battery, battery adapter, tubing with adapter, filter holder with filter, forceps, bucket, and plastic bags with desiccant (Figure 5a; Carim, McKelvey, et al., 2016). It takes approximately 5–20 min to collect one or two filters at a site, as done in this study, depending on the turbidity of the water being filtered. The direct sampling method was designed to

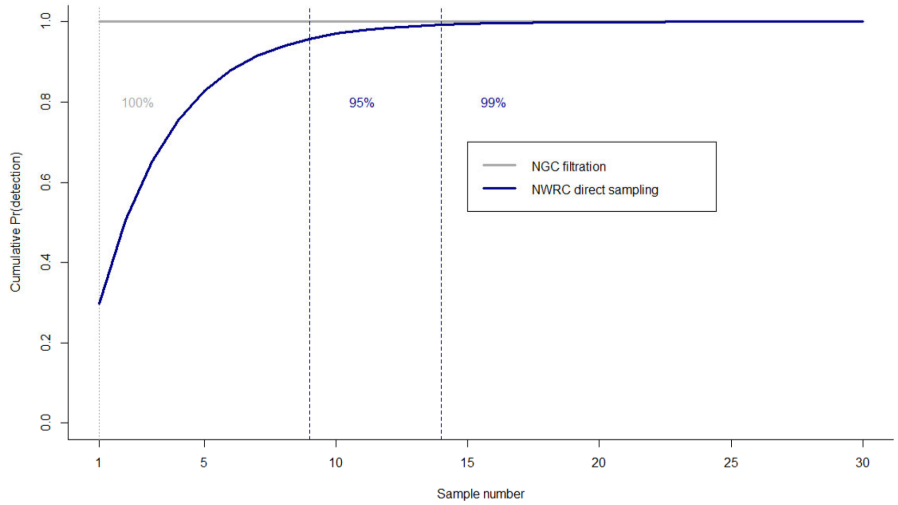

FIGURE 3 Cumulative probability of detection for filtration and direct sampling methods. Given the detection rate calculated for eDNA at sites with known nutria (*Myocastor coypus*) presence in Marion County, Oregon, with the filtration and direct sampling methods (100% and 44%, respectively), the cumulative probability of detection was modeled for 1–30 samples for each method (gray = filtration, blue = direct sampling). Filtration methods were conducted by the National Genomic Center for Wildlife and Fish Conservation (NGC) and direct sampling methods were conducted by the National Wildlife Research Center (NWRC). Dashed vertical lines indicate the number of samples required to reach detection probabilities of 100% for filtration (i.e., gray line, one sample) and 95% and 99% for direct sampling (i.e., blue lines nine and 14 samples, respectively).

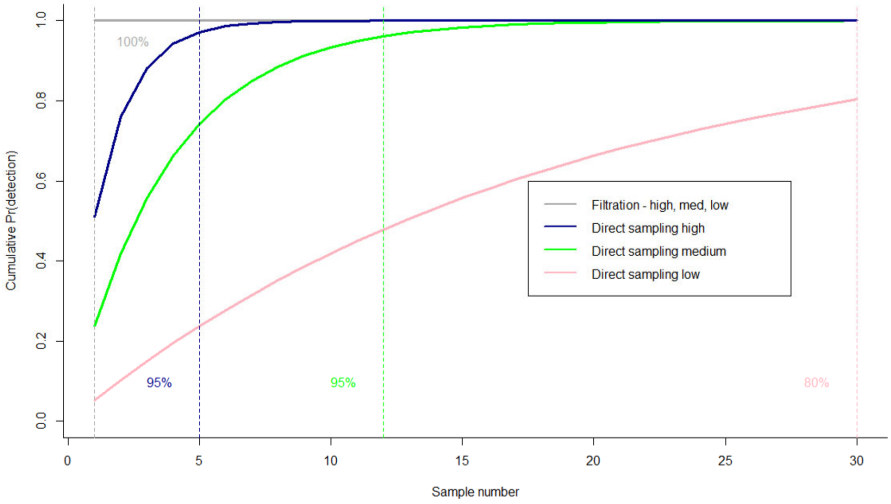

FIGURE 4 Sensitivity analysis for the predicted cumulative detection probability for filtration and direct sampling methods at high (1314 copies/L), medium (500 copies/L), and low (100 copies/L) DNA concentrations. The high DNA concentration reflects the mean number of copies per site quantified via filtration in our field study. The filtration method, in which environmental DNA (eDNA) is sampled by passing 5 L of water through a glass microfiber filter, was predicted to detect nutria (*Myocastor coypus*) with 100% accuracy using a single sample at all DNA concentrations modeled. The direct sampling method, in which 45 mL of water is collected and preserved with 15 mL of Longmire's buffer, is predicted to have 95% detection probability of nutria DNA with six samples from high density sites and 12 samples from medium density sites. At low density sites, the direct sampling method is predicted to require 30 samples for 80% probability of detection.

specifically address the need for an efficient, non-intensive sampling method with the goal of reducing the equipment and time burden on field personnel and allowing samples to be collected during the course of other field duties (Williams et al., 2016). The direct sampling method requires 60 mL bottles, a 50 mL tube and parafilm (for a field negative control), and Longmire's buffer for preservation (Figure 5b). It takes approximately 5 min to collect three 60 mL direct water samples at a site, as done in this study. Both methods require clean gloves, sampling protocols, and a GPS unit for collecting metadata. It is important to note that both methods tested in this study have eliminated the need for cold storage, which allows for more efficient collection and reduces risks associated with DNA degradation at ambient temperatures. In situations where waters are turbid, when sites are remote or difficult to access (where fewer supplies may be important to field teams), or where sampling time is limited, direct sampling may be an acceptable monitoring solution if enough samples are collected to overcome lower detection probabilities. Well-designed eDNA monitoring efforts require collaboration and good communication between field biologists, laboratory scientists, and natural resource managers with careful consideration of trade-offs regarding the difficulty of sampling (e.g., weight, time), costs, and detection probabilities (Morisette et al., 2021; Mosher et al., 2020). For example, Morisette et al. (2021) recommend conducting a cost–benefit analysis when designing eDNA sampling schemes. It is important that these trade-offs in performance and efficiency be carefully considered based on study and partner needs and capabilities.

Recognizing that filtration may not be an option in all habitats and situations, it is valuable to consider how much sampling effort is required for the direct sampling method to perform as well as the filtration method. As described above, up to ~37 times more water was collected via filtration than in direct sampling (5 L vs. 135 mL per site). It is not surprising that filtration results showed higher detection rates and higher mean copy numbers, and that our modeling demonstrated higher sample sizes of direct sampling would be required to reach a similar detection probability (Hunter et al., 2015; Figures 3 and 4). Interestingly, the direct sampling results from our cumulative detection probability models are similar to the findings of a multi-scale occupancy modeling study for eDNA monitoring with direct sampling methods for feral swine (*Sus scrofa*; Davis et al., 2018), an invasive terrestrial mammal that uses water for drinking and wallowing. Davis et al. (2018) found that collecting 23 direct samples per site maintained a high detection probability (>95%) even in the presence of variability and unfavorable site characteristics (e.g., seasonality, water source, pH, etc.). While more limited in scope, our results suggest that at sites with relatively high nutria densities (i.e., known-presence sites where animals or abundant sign such as tracks or scat where observed), processing six to nine direct water samples would offer a 95% detection probability. In contrast, areas with low nutria densities would require considerably more sampling effort and practitioners should be aware that there is a lower probability of detecting nutria when interpreting results. If the consequences of false negative results are too great when nutria densities are low, practitioners should consider increasing the sample size of direct samples or utilizing the filtration method. Given the substantial laboratory burden that processing large numbers of field samples entails, Davis et al. (2018) recommended a removal design for processing samples in which laboratory analysis can begin on fewer samples and additional samples are only analyzed if no detections were made

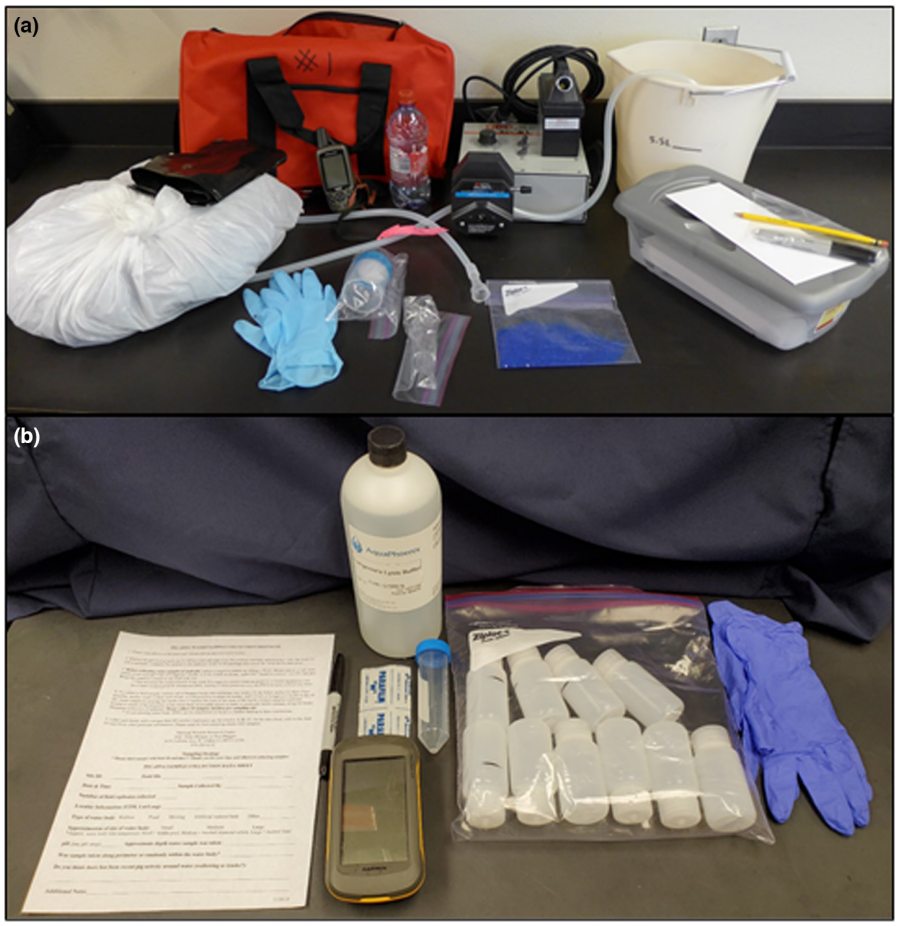

FIGURE 5 Water sampling kits for nutria (*Myocastor coypus*) eDNA collection. (a) Filtration supplies utilized in this study include: a peristaltic pump, pump battery, battery adapter, tubing with adapter, filter holder with filter, forceps, bucket, and sample bags with desiccant. Image reproduced from Carim, McKelvey, et al. (2016) with permission. (b) Direct sampling supplies utilized in this study include: 60 mL bottles, a 50 mL tube and parafilm, and Longmire's buffer. Both methods also require gloves, a GPS unit, and a sampling protocol.

from each previous extraction. Implementing this type of laboratory workflow can help balance the needs for high detection probability with finite laboratory resources.

While our laboratory and field results indicate that the assay will be useful for nutria eDNA monitoring, there are several important limitations to our study. First, detection probability is always predicated on organism density. While we did not have estimates of nutria density, we did have knowledge of nutria presence via abundant and recent sign or observation of nutria during sampling. Nutria densities in our study were likely higher than in areas where eDNA monitoring would typically be implemented as a management tool. The results of our informal sensitivity analysis are therefore helpful to predict the number of samples that would be required at sites with lower nutria densities and consequently, lower DNA concentrations. The sensitivity analysis model we applied (Wilcox et al., 2018) allows users to adjust the eDNA concentration and predict the number of samples required to reach an acceptable detection probability in a new area. Particularly at low densities, there is a strong possibility that DNA is not evenly distributed in the waterbody (Furlan et al., 2016; Shogren et al., 2017), but the Poisson distribution used for our sensitivity analysis assumes homogeneity. Thus, ideally, users should include more realistic heterogeneity in sampling design, with additional samples collected around the perimeter or randomly throughout the waterbody. Second, the scope of our inference is limited to the field testing we conducted in Marion County, Oregon. Given our additional assay testing, sequencing from four regions in the United States, and results reported previously for nutria in Japan, we believe that our results are generalizable to other invaded regions. However, monitoring efforts for management in areas with unknown nutria presence should consider sequencing an amplicon from one or more positive eDNA samples to confirm amplification of the target, although the number of amplicons to sequence is likely to be context-dependent (e.g., Campbell et al., 2022; Peixoto et al., 2021). Additionally, a pilot test should be conducted in regions of interest before relying on eDNA results including revalidation of the assay specificity and sensitivity with any changes in laboratory equipment or reagents (Klymus et al., 2020). While both laboratories produced a similar LOD (i.e., 5.3 and 6.8 copies per reaction for NGC and NWRC, respectively), the NGC laboratory demonstrated better precision for quantification (i.e., LOQ of 22 vs. 186 copies/µL). This variable result could be related to differences in the precision of laboratory technicians, use of different instruments, or differences in the number of replicates analyzed, but it is important for each laboratory to make inference within the limits identified in these tests (Klymus et al., 2020). Akamatsu et al. (2018) determined LOD in their laboratory as well. The authors amplified and sequenced a range of synthetic DNA from 1 to 5 copies per reaction with two copies per reaction being the lowest concentration that could be sequenced. Although both assay validation approaches found similar LOD (ranging from 2.0 to 6.8 copies per reaction), it is difficult to

directly compare our methods to the Akamatsu et al. (2018) article and their synthetic sequence is unpublished. For transparency and comparison between laboratories, we urge testing and reporting LOD/LOQ, as recommended by Klymus et al. (2020). Finally, the limited field component of our study did not allow for investigating site variables that influence detection probability such as pH, water depth, seasonality, etc. (Davis et al., 2018). As this assay transitions to field application, additional data on site variables and subsequent modeling would allow for refined estimates of detection probabilities under various scenarios.

While trade-offs exist between alternative field sampling and DNA extraction methods for nutria eDNA monitoring, we have demonstrated the assay's success in silico, in vitro, and in situ. Our results suggest that this assay can be confidently applied to eDNA monitoring of nutria throughout the continental United States with minimal additional field validation. Given the success of this assay in Japan (Akamatsu et al., 2018) and now in the United States, and the highly conserved sequences from this study and five other countries, we believe there is potential for this assay to monitor nutria populations more broadly, namely in Europe where nutria are included in the list of Invasive Alien species of Union Concern (European Commission, n.d.). As we have seen when eDNA monitoring is applied in other systems (e.g., feral swine, Asian carp, etc.), it is most effective when used in combination with other monitoring techniques. Complimentary methods such as trail cameras, detector dogs, or community reporting of sightings remain critical to the management of rare and elusive invasive species. Our primary goal in this study was to validate a previously described nutria assay for use throughout the continental United States, but we also offer general guidance for eDNA practitioners working in other systems to assess alternative eDNA sampling and extraction methodologies; the optimal approach is likely to be a trade-off between several factors, including sensitivity, cost, and sampling effort in the field.

AUTHOR CONTRIBUTIONS

Conception and study design: TMW and AJP; data acquisition, analysis, and interpretation: AMM, JAK, IHP, TMW, and AJP; article writing: AMM, JAK, IHP, TMW, and AJP.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Dryad at https://doi.org/10.5061/dryad.vq83bk3wr (Mangan et al., 2022).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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