Field validation of an eDNA early detection and monitoring tool for Northern Snakehead (*Channa argus*) in Arkansas

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Introduction

The Northern Snakehead, *Channa argus*, is an invasive fish species in North America with populations initially established in mid-Atlantic water bodies. Its presence was first recorded in the United States in 1997. The species was listed as a highly injurious species in 2002 under the Lacey Act. Since those earliest captures, additional observations of this species have been found in waterbodies of Georgia, Louisiana, Mississippi, Missouri, and Arkansas (USGS 2024). Establishment of Northern Snakehead in the United States is largely due to species behaviors and characteristics: tolerance to a wide range of water temperatures, a swim bladder that can act as a primitive lung allowing the species to survive in low oxygenated waters or no water for multiple days, high fecundity, and its aggressive feeding habits. These characteristics adversely affect native aquatic species' ability to compete for food and habitat (Fuller et al., 2019).

The U.S. Fish and Wildlife Service (USFWS) has monitored populations of Northern Snakehead in a variety of locations of the mid-Atlantic United States using eDNA samples since 2019. Expansion of observed Northern Snakehead populations into portions of the Mississippi River watershed in recent years has created a need to explore eDNA monitoring methods for Northern Snakehead in new areas of the U.S.

The USFWS Whitney Genetics Laboratory (WGL) and Northeast Fishery Center have each completed laboratory validations of two Northern Snakehead eDNA assays and successfully established assay repeatability across laboratories and platforms. The goal of the present study was field validation of two Northern Snakehead eDNA assays using environmental water samples collected from presumed snakehead occupied and negative sites in Arkansas.

Field Sampling

Collection of water samples

Environmental water samples (Table 1) were collected in Arkansas during the week of October 17, 2022. Water was collected from targeted sites where an attempt was also made to confirm Northern Snakehead presence through direct observation or physical capture. Samples were collected using a Smith-Root eDNA sampler (SKU: 12061, Smith Root, Inc) equipped with a sampling trident attachment (SKU 11203, Smith Root, Inc.) and 5µm PES (polyethersulfone) self-preserving filters (SKU: 11580, Smith-Root, Inc) on each channel of the trident pole. The eDNA crew consisted of a single member responsible for environmental sample filtration and data collection including records of sample collection times, sample collection coordinates, water temperature, filtered sample volume, and pertinent site characteristics.

Water samples were collected from the shoreline at approximately equal-spaced intervals. At each sampling point, the eDNA collector changed into a clean pair of nitrile gloves prior to unwrapping a sterile filter and attaching it to the rubber tubing of each channel of the Smith-Root trident pole. Target filtration volume for each site (set of triplicate samples) was 3 liters. Sample water was filtered until sample volume reached ~3L or until PES filter clogging occurred. If less than the target filtration volume was reached, the volume filtered was recorded at each site. After each sample finished with filtration, the filter/tubing was held upright to pull air through the filter to ensure sufficient drying for sample preservation. Once dry, the filter was placed back into its original pre-labeled packaging and sealed. At each eDNA sampling location (waterbody), the first and last sample was predetermined as a field blank

consisting of 1 L of distilled water. Field blank water was filtered directly using the same methods as eDNA sample filtration.

Once all samples at a location were collected and bagged, filters were stored in coolers (with or without ice) and grouped by site. Filters were stored at room temperature until February of 2023 when they were transported to the WGL. After transport to the lab, the filters were stored at -20°C until DNA extraction occurred.

Visual and/or Physical Capture Confirmation

A second field crew attempted to confirm presence of Northern Snakehead at each eDNA collection site following eDNA sample collection. Northern Snakehead presence was determined by either physical capture of one or more individuals or visual confirmation of the species by expert biologists with Arkansas Game and Fish Commission. When visually confirming Northern Snakehead presence, photographic evidence of individuals was also considered. When Northern Snakehead were physically captured at a site, the sex and total length of individuals was recorded.

Physical capture or visual confirmation of Northern Snakeheads was established at five of the seven sites targeted for eDNA sampling in this study: Big Creek, Bonner Farm, Clarendon City Pond, Mallard Lane Ditch, and New Lake. Based on sightings or capture records, Clarendon City Pond was characterized as a presumed 'low density site', Mallard Lane Ditch as a presumed 'moderate density site', and Bonner Farm as an established population with known presence for over 10 years. The other two positive sites (Big Creek and New Lake) were not characterized in terms of density. The other two sampling sites, Austell Lake and Dunn Lake, were characterized as presumed negative sites for Northern Snakehead.

Laboratory Methods

DNA Extractions

Environmental DNA captured on filters was extracted using a modified protocol of the Qiagen DNeasy Blood and Tissue Kit (Catalog number 69506, Qiagen Corp.). All steps in the modified extraction protocol followed manufacturer recommendations with the several modifications. Filters were carefully removed from Smith Root filter housings with clean gloves and folded securely for placement in a Qiagen Lyse and Spin Basket (Catalog Number 19597, Qiagen Corp.) to carry out DNA digestions. Once filters were transferred to Lyse and Spin Baskets containing 370 µl Buffer ATL and 25 µl Proteinase K, digestions were incubated overnight at 56 °C in an incubator shaker with gentle shaking. After digestion, lysates were centrifuged at 6000 x g for 1 minute. Lyse and Spin Baskets containing the filters were removed, and lysates were then mixed with 370 µl of both Buffer AL and 100% ethanol. The lysate mixture was then vortexed, briefly centrifuged to collect contents, and transferred in two pipetting steps (since total digestion volume was larger than an individual spin column could hold) for binding on a Qiagen DNeasy Blood and Tissue Spin Column.

The final elution step for the triplicate filters in this study included a deviation from the usual Qiagen protocol used at Whitney Genetics Laboratory. Triplicate filter samples were extracted individually up until the elution step. To increase the chance for detection of NS eDNA, a sequential elution step was used in which the elution of the first filter of a triplicate set of samples was eluted in 200 µl of Buffer AE. After the first sample was eluted, the resulting 200 µl eluate was applied to the second filter extraction of the corresponding triplicate and eluted. Finally, the resulting eluate from the second filter was applied to the spin column of the third filter extraction of the corresponding sample set and eluted. This final

elution of the series contained 200 µl of eDNA and reflected the pooled result of sequential elution through triplicate DNA extractions from filters collected at the same site. This pool corresponded directly with the total filtered volume of the triplicate. After all elutions were completed, final storage of eDNA extracts was –20°C until qPCR testing.

Quantitative PCR

The Northern Snakehead primers used to test field samples were designed by Dr. James Casey (*NSCor*, Cornell University, https://fishtracker.vet.cornell.edu/) and the U.S. Fish and Wildlife Service (*CAcytB*, USFWS Northeast Fishery Center (NEFC), and targeted a 63 base pair fragment of the cytochrome *c* oxidase subunit I (*COI*) gene, and a 121 base pair fragment of the cytochrome b gene (*cytb*), respectively. Samples analyzed with the *NSCor* assay were tested in 10 µl TaqMan Environmental Master Mix 2.0, 3.0 µl template (eDNA sample), 4.0 µl deionized H_2O, 1.0 µl 10µM forward/reverse primers, and 1.0 µl 2.5 µM probe. Samples analyzed with the *CAcytB* assay were tested in 10 µl TaqMan Environmental Master Mix 2.0, 3 µl DNA template, 2.2 µl deionized H_2O, 1.8 µl 10 µM forward primer, 1.0 µl 10 µM reverse primer, and 2 µl 2.5 µM probe. All qPCR plates contained 4 replicates of 250 copy gBlock standard to serve as qPCR positive controls, and four replicates of sterile, deionized H_2O, to serve as negative controls. The thermocycling conditions for the *NSCor* and *CAcytB* assays included 3 steps: 10 minutes of denaturation at 95°C, 45 cycles of 15 seconds of denaturation at 95°C, and 1 minute of annealing and extension at 60°C. All eDNA samples were run in octet (eight molecular replicates) for both markers. Detections were called when one or more of the eight replicate reactions for a given marker crossed the threshold line and produced a *Cq* value of 45 or less.

Standard Curve Analysis

Synthetic double-stranded DNA of the complete target amplicon of both assays was used to create an 8-point standard curve consisting of 12 replicate reactions of 31250, 6250, 1250, 250, 50, 10, 2, and 0.4 copies reaction^{-1}. Standard curves were then generated by plotting the resulting *Cq* value of each PCR replicate (y) against Log starting quantity (x) and drawing a line of best fit (linear regression). Standard curve metrics were then used to determine PCR efficiency of each marker and to calculate copy per liter (CPL) estimates of any PCR replicates positive for Northern Snakehead eDNA. PCR efficiency was determined from the equation:

$$E = (-1+10(-1/slope))*100$$

where E is the PCR efficiency as a percentage and slope is the slope of the regression line obtained from each marker's corresponding standard curve.

The number of copies per liter detected of Northern Snakehead DNA from each positive sample was determined by first calculating the number of copies detected per PCR reaction from the equation:

$$CPR = 10^{(Cq - Intercept)/(Slope)}$$

where CPR is the copies detected in a single positive PCR reaction, *Cq* is the cycle threshold value, intercept is the y-intercept of the regression line of the corresponding marker's standard curve, and slope is the slope of the regression line obtained from each markers' corresponding standard curve.

The number of copies per liter was then determined through the following equation:

$$CPL = CPR \times ((a/b)/c)$$

where CPL is the copies per liter detected, CPR is the copies detected in a single positive PCR reaction, a is the elution volume of the DNA sample in microliters, b is the template volume used in the PCR reaction in microliters, a/b is the number of equivalent proportions of sample assayed in the extracted eDNA sample, and c is the filtration volume of the corresponding filter that the DNA sample was extracted from. The elution volume (a) and template volume (b) are used in the equation to estimate the total amount of eDNA extracted from the entire filtered sample.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection (LOD) was defined as the lowest concentration of DNA detected in 95% of replicates, and LOQ was defined as the lowest concentration of DNA with a coefficient of variation below 35% (Klymus et al. 2020). LOD and LOQ were determined for each qPCR marker using two distinct algorithms (R scripts), Lesperance (Lesperance et al. 2021) and Klymus (Klymus et al. 2020). Data obtained during generation of standard curves was used as a basis for LOD and LOQ determination.

Inhibition Testing and Sample Clean-up

All environmental samples were tested for the presence of PCR inhibitors using the TaqMan Exogenous Internal Positive Control Reagents Kit (Catalog Number 4308323, ThermoFisher Scientific). Briefly, triplicate PCR reactions for each of the pooled field samples were tested following the manufacturer's recommendations in a 20 µl volume including 10 µl TaqMan Environmental Master Mix 2.0, 2 µl 10x Exo IPC Primer Mix, 0.4 µl 50x Exo IPC DNA, and 3 µl of DNA template. Cycling conditions were based on manufacturer's recommendations. Any reactions that demonstrated a delay in amplification of one cycle threshold or more (ΔCq >1) were deemed inhibited and treated with a Zymo Inhibitor Removal Column (Catalog Number D6030, Zymo Research).

Quality Assurance

Controls were included in all steps of the eDNA workflow including field sampling (negative controls), DNA extraction (both positive and negative extraction controls), and qPCR amplification (both positive and negative controls on each PCR plate). Control samples were used in the following way:

- Since field negative controls were collected at each site, any field negative control that showed amplification negated all results from any samples from the same site.
- Positive amplification in negative extraction controls negated detections from samples of the same extraction batch.
- Positive extraction controls were used to confirm that DNA extractions were successful in the event that there was no DNA amplification from filters due to a lack of target DNA collected.
- Negative PCR controls, if positive, negated detections in samples run on the same PCR plate and PCR plates were considered for re-run.
- Positive extraction and PCR controls must amplify at a consistent *Cq* value across all analyses. If a positive control failed, a rerun of that PCR plate was carried out.

Results

Visual or Physical Capture Confirmation

Of the seven Arkansas locations targeted for collection of eDNA samples in October of 2022, five were characterized as having Northern Snakehead present as determined by historical capture records, electrofishing capture near the time of eDNA sampling, or direct observation by field crews (without

physical capture). The locations where Northern Snakehead were presumed present were: Big Creek, Bonner Farm, Clarendon City Pond, Mallard Lane Ditch, and New Lake. Presumed negative locations were Austell Lake and Dunn Lake and no Northern Snakehead were collected or observed at either of these sites (Table 2).

Standard Curve Analysis

Standard curves generated for each of the two markers used in this project resulted in high PCR efficiency estimates (> 98%), coefficient of determination values (r^2) of greater than 0.99, and y-intercept values of less than 40 using serially-diluted gBlock standard DNA as template (Figures 1 and 2). These standard curve values are suggestive of qPCR assays amplifying target DNA at a high level of analytical sensitivity.

Limit of Detection and Quantification

Limit of detection and limit of quantification values were determined (Table 3) for each of the markers used in this project following the Lesperance (Lesperance et al. 2021) and Klymus (Klymus et al. 2020) methods. LOQ values were 19 copies and 6 copies for the *NSCor* and *CAcytB* assays, respectively, using the Klymus model. LOQ values using the Lesperance model were an order of magnitude lower for each marker than the Klymus model. LOD values regardless of the model or assay used were 2.67 copies or less, indicative of highly sensitive qPCR assays useful for eDNA detection of rare environmental targets such as early detection of aquatic invasive species.

Control Samples

This project included the collection of field negative controls (field blanks), DNA extraction positive and negative controls, as well as PCR positive and negative controls included on each plate of PCR analysis. All controls showed anticipated results and indicated that there was no evidence of contamination in either the field collections or laboratory processing steps.

Inhibition Testing

One environmental sample, VAL2024001013ABC (New Lake), showed slightly delayed amplification of the endogenous internal positive control DNA template (IPC) compared to other samples. This sample was treated using a Zymo Inhibitor Removal Column prior to qPCR analysis.

Northern Snakehead eDNA Detection

Northern Snakehead eDNA was detected at all sites where snakehead presence was confirmed visually or through physical capture with both qPCR markers (Table 4 and 5). All qPCR replicates analyzed for both markers (n = 8 molecular replicates for each marker) were positive for all samples collected at New Lake, Big Creek, Bonner Farm, and Mallard Lane Ditch. Clarendon City Pond was the only site where less than 100% of the samples collected (4 of the 7 samples collected) were positive with each Northern Snakehead qPCR assay, and for each of the positive samples, only 2–7 of the 8 total qPCR replicates were positive.

Copy per liter estimates also provided additional context of the various levels of Northern Snakehead eDNA detected at each of the positive sites. Clarendon City Pond had the lowest CPL detected (100–700) across all positive samples. Bonner Farm had orders of magnitude higher CPL detected (200,000–350,000) per positive sample. Other positive sites had intermediate levels of Northern Snakehead eDNA copies detected across all samples collected (Table 4, Table 5).

No visual or capture confirmation of Northern Snakehead presence was realized at Austell Lake and Dunn Lake. Likewise, no eDNA detections were found with either Northern Snakehead marker at these locations.

Discussion

The Northern Snakehead qPCR markers used in this project have previously been tested in USFWS labs (laboratory validation) and at field sites in Mid-Atlantic states. Standard curve and LOD/LOQ analysis (Table 3) suggested both of the qPCR markers used in this study are highly efficient and sensitive in amplifying Northern Snakehead DNA. The goal of this project was to collect samples at locations in the Mississippi River Basin where Northern Snakehead were presumed present at varying densities or presumed absent in order to validate field protocols in new watersheds.

Environmental DNA results from both the *CAcytB* and *NSCor* qPCR assays demonstrated 100% positive detections in all samples collected at each of the presumed positive locations except Clarendon City Pond. Clarendon City Pond resulted in positive detections for Northern Snakehead eDNA in 4 of the 7 samples collected and varying numbers of PCR replicates per sample. Clarendon City Pond, a presumed low-density site, also resulted in the lowest mean copy per liter estimates (CPL) of any of the Northern Snakehead positive locations for both qPCR markers. Increasing CPL estimates for each of the positive sites followed the order (for each of the markers) Clarendon City Pond (lowest, presumed low-density), New Lake, Big Creek, Mallard Lane Ditch (presumed moderate-density), and Bonner Farm (established population). Mean CPL estimates for Bonner Farm samples exceeded 200,000 copies per liter for both Northern Snakehead markers tested.

In general, the watersheds sampled in this study were characterized by high levels of turbidity. The target filtration volume was three liters for each set of three filters. However, many locations had less than three liters filtered, and less than one liter was filtered at several locations in Clarendon City Pond, Mallard Lane Ditch, and Bonner Farm. The Mallard Lane Ditch had one sample collected where no measured volume was recorded, yet we were still able to detect high levels of Northern Snakehead DNA at this site. Even though turbidity was a limiting factor for realized filtration volume, we still had eDNA detections at presumed moderate- and high-density sites (Mallard Lane Ditch and Bonner Farm) and even for a presumed low-density positive site at Clarendon City Pond. The difficulties with filtration should be explored with a goal of improving filtration volume for each filter collected in future work.

Environmental DNA data collected in this study show congruence with field confirmation of presumed positive Northern Snakehead waters in Arkansas, and non-detection of Northern Snakehead DNA in presumed negative waters. Although the mean CPL comparisons provided in this study offer additional insight into the relative detection levels of Northern Snakehead DNA at the positive locations, these data should not be interpreted as confirming low, moderate, or high-density populations but rather a loose characterization of suspected population status. DNA quantities (CPL in this study) cannot be used as a direct means of determining population abundance without measurement of or controlling for a multitude of other co-variates. Previous studies have documented a relationship between increasing organism abundance and increasing DNA concentrations in environmental samples (Yates et al. 2019, George et al. 2021, Carvalho et al. 2022), however much more work would be required to establish this relationship with Northern Snakehead. The high detectability of Northern Snakehead eDNA at positive sites in this study provided an opportunity to explore CPL comparisons since nearly all detections were above the estimated LOQ values for each marker. Future work that may characterize the Northern

Snakehead 'invasion front' using eDNA methods may result in detections that fall well below the LOQ for this pair of markers, resulting in binary data (present or absent) at a given site.

Differences were observed between the estimates obtained for LOD and LOQ of each marker using the Klymus and Lesperance models. In most cases LOD and LOQ estimates for each marker were approximately an order of magnitude (10 times) lower using the Lesperance model than the Klymus model. Similar differences in LOD and LOQ estimates between these two models have been noted in previous comparisons as well (Lesperance 2021). The main difference between the two models is that the Lesperance model relies on a Poisson-Binomial distribution that is more applicable for extremely low concentrations of DNA found in environmental samples. One recommendation given this difference would be reporting the range of values of LOD and LOQ using both the Klymus and Lesperance models. If a project is then using LOD and/or LOQ to establish cutoff thresholds for determination of detection status, a conservative approach would be to use the highest value LOD and LOQ to base any cutoff thresholds against.

Interpretation of environmental DNA detection results can be challenging at times, particularly as detections approach the established limits (LOD) of a given assay. Environmental DNA detections from samples collected do not guarantee the detections were produced by living target organisms. Vectors other than a living target organism can produce detections in collected samples such as fecal material from predators of the target species, allochthonous (long distance) transport of the target DNA, carcasses of the target species (Merkes et al. 2014), or contamination from any step during sample handling from the field through laboratory analysis. Confidence in eDNA detection results increases as the resulting cycle threshold (*Cq*) values decrease (meaning more DNA was detected in a sample), the number of positive PCR technical replicates increases, or repeated detections occur at a given site during multiple time periods. Labs engaged in eDNA analysis must follow strict decontamination protocols, adopt laboratory environmental controls (hoods, UV germicidal lamps, etc.), and establish clean sample and equipment handling protocols, all of which were a part of this effort.

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Table 1

Field sample metadata which included site sampled (Site), sampling date (Sample Date), if a sample was a field blank or not (Blank), sample ID (WGL sample number), filtration volume (Total Volume), and whether filters were eluted during DNA extractions as a single elution or as a sequential elution (DNA Elution).

Site	Sample Date	Blank	WGL sample number	Total Volume (l)	DNA Elution
Austell Lake	2022-10-17	y	VAL2024001001	1.12	Single Elution
Austell Lake	2022-10-17	n	VAL2024001002ABC	1.6	Triplicate Sequential Elution
Austell Lake	2022-10-17	y	VAL2024001003	1.09	Single Elution
Austell Lake	2022-10-17	n	VAL2024001004ABC	3.23	Triplicate Sequential Elution
Austell Lake	2022-10-17	y	VAL2024001005	1.13	Single Elution
Austell Lake	2022-10-17	n	VAL2024001006ABC	3.2	Triplicate Sequential Elution
Austell Lake	2022-10-18	y	VAL2024001007	1.04	Single Elution
New Lake	2022-10-18	y	VAL2024001008	1.16	Single Elution
New Lake	2022-10-17	n	VAL2024001009ABC	1.62	Triplicate Sequential Elution
New Lake	2022-10-17	y	VAL2024001010	1.03	Single Elution
New Lake	2022-10-17	n	VAL2024001011ABC	2.05	Triplicate Sequential Elution
New Lake	2022-10-17	y	VAL2024001012	1.1	Single Elution
New Lake	2022-10-17	n	VAL2024001013ABC	1.89	Triplicate Sequential Elution
New Lake	2022-10-17	y	VAL2024001014	1.14	Single Elution
Big Creek	2022-10-18	y	VAL2024001015	1.14	Single Elution
Big Creek	2022-10-18	n	VAL2024001016ABC	3.18	Triplicate Sequential Elution
Big Creek	2022-10-18	y	VAL2024001017	1.1	Single Elution
Big Creek	2022-10-18	n	VAL2024001018ABC	2.92	Triplicate Sequential Elution
Big Creek	2022-10-18	y	VAL2024001019	1.07	Single Elution
Big Creek	2022-10-18	n	VAL2024001020ABC	2.14	Triplicate Sequential Elution
Big Creek	2022-10-18	y	VAL2024001021	1.14	Single Elution
Bonner Farm	2022-10-18	y	VAL2024001022	1.06	Single Elution
Bonner Farm	2022-10-18	n	VAL2024001023ABC	0.35	Triplicate Sequential Elution
Bonner Farm	2022-10-18	y	VAL2024001024	1.11	Single Elution
Bonner Farm	2022-10-18	n	VAL2024001025ABC	0.26	Triplicate Sequential Elution
Bonner Farm	2022-10-18	y	VAL2024001026	1.09	Single Elution

Site	Sample Date	Blank	WGL sample number	Total Volume (l)	DNA Elution
Clarendon City Pond	2022-10-19	y	VAL2024001028	1.05	Single Elution
Clarendon City Pond	2022-10-19	n	VAL2024001029ABC	0.7	Triplicate Sequential Elution
Clarendon City Pond	2022-10-19	y	VAL2024001030	1.07	Single Elution
Clarendon City Pond	2022-10-19	n	VAL2024001031ABC	0.66	Triplicate Sequential Elution
Clarendon City Pond	2022-10-19	y	VAL2024001032	1.04	Single Elution
Clarendon City Pond	2022-10-19	n	VAL2024001033ABC	0.72	Triplicate Sequential Elution
Clarendon City Pond	2022-10-19	y	VAL2024001034	1.04	Single Elution
Clarendon City Pond	2022-10-19	n	VAL2024001035ABC	0.79	Triplicate Sequential Elution
Clarendon City Pond	2022-10-19	y	VAL2024001036	1.12	Single Elution
Clarendon City Pond	2022-10-19	n	VAL2024001037ABC	0.59	Triplicate Sequential Elution
Clarendon City Pond	2022-10-19	y	VAL2024001038	0.97	Single Elution
Clarendon City Pond	2022-10-19	n	VAL2024001039ABC	0.69	Triplicate Sequential Elution
Clarendon City Pond	2022-10-19	y	VAL2024001040	1.13	Single Elution
Mallard Lane Ditch	2022-10-19	y	VAL2024001041	1.05	Single Elution
Mallard Lane Ditch	2022-10-19	n	VAL2024001042ABC	0	Triplicate Sequential Elution
Mallard Lane Ditch	2022-10-19	y	VAL2024001043	1.15	Single Elution
Mallard Lane Ditch	2022-10-19	n	VAL2024001044ABC	0.09	Triplicate Sequential Elution
Mallard Lane Ditch	2022-10-19	y	VAL2024001045	0.97	Single Elution
Dunn Lake	2022-10-19	y	VAL2024001046	1.09	Single Elution
Dunn Lake	2022-10-19	n	VAL2024001047ABC	3.19	Triplicate Sequential Elution
Dunn Lake	2022-10-19	y	VAL2024001048	1.13	Single Elution
Dunn Lake	2022-10-19	n	VAL2024001049ABC	3.09	Triplicate Sequential Elution
Dunn Lake	2022-10-19	y	VAL2024001050	1.05	Single Elution
Dunn Lake	2022-10-19	n	VAL2024001051ABC	3.04	Triplicate Sequential Elution
Dunn Lake	2022-10-19	y	VAL2024001052	1.12	Single Elution

Table 2

Locations sampled, whether the location was characterized as presumed positive or negative for Northern Snakehead presence (based on physical evidence), and population verification information.

Site	Population Status	Verification Notes
Big Creek	Positive	Several Northern Snakehead captured by field crew near the time of eDNA sampling
Bonner Farm	Positive	Northern Snakehead have been known to be present at site for greater than 10 years. No further effort into confirmation
Clarendon City Pond	Positive	One Northern Snakehead captured one week prior to eDNA sample collection. Presumed low density
Mallard Lane Ditch	Positive	One Northern Snakehead captured and several others observed. Presumed moderate density
New Lake	Positive	Northern Snakehead have been caught here multiple times historically. No further effort into confirmation
Austell Lake	Negative	
Dunn Lake	Negative	

Table 3

Limits of detection (LOD) and limits of quantification (LOQ) for both qPCR markers (*CAcytB* and *NSCor*) using the Lesperance (Lesperance et al. 2021) and Klymus models (Klymus et al. 2020).

Assay	Model	LOD	LOQ
CAcytB	Lesperance	0.2	0.9
NSCor	Lesperance	0.4	1.4
CAcytB	Klymus	2.67	6
NSCor	Klymus	1.33	19

Table 4

Quantitative PCR detection results for Northern Snakehead eDNA using the *CAcytB* marker. Displayed is the Sample ID, Site Name, Sample Type (ENV = environmental, EXTN = negative extraction control, EXTP = positive extraction control, NPCR = negative PCR control), number of positive detections ('Hits', out of a total of 8), mean *Cq*, and mean copies per liter (CPL) detected. 'ABC' after the sample name indicates that the field sample represents three filters combined into a single sample for laboratory analysis.

Sample ID	Site	Sample Type	Hits	Mean *Cq*	Mean CPL
VAL2024001002ABC	Austell Lake	ENV	0	NA	NA
VAL2024001004ABC	Austell Lake	ENV	0	NA	NA
VAL2024001006ABC	Austell Lake	ENV	0	NA	NA
VAL2024001009ABC	New Lake	ENV	8	34.9	1125.4
VAL2024001011ABC	New Lake	ENV	8	35.0	787.3
VAL2024001013ABC	New Lake	ENV	8	34.4	1278.6
VAL2024001016ABC	Big Creek	ENV	8	32.5	2633.7
VAL2024001018ABC	Big Creek	ENV	8	33.9	1103.2
VAL2024001020ABC	Big Creek	ENV	8	31.3	9168.7
VAL2024001023ABC	Bonner Farm	ENV	8	28.6	341966.0
VAL2024001025ABC	Bonner Farm	ENV	8	29.1	343636.8
VAL2024001029ABC	Clarendon City Pond	ENV	7	36.9	717.1
VAL2024001031ABC	Clarendon City Pond	ENV	7	37.6	456.5
VAL2024001033ABC	Clarendon City Pond	ENV	0	NA	NA
VAL2024001035ABC	Clarendon City Pond	ENV	4	37.5	504.2
VAL2024001037ABC	Clarendon City Pond	ENV	2	38.2	300.8
VAL2024001039ABC	Clarendon City Pond	ENV	0	NA	NA
VAL2024001042ABC	Mallard Lane Ditch	ENV	8	35.5	Inf
VAL2024001044ABC	Mallard Lane Ditch	ENV	8	34.7	21749.5
VAL2024001047ABC	Dunn Lake	ENV	0	NA	NA
VAL2024001049ABC	Dunn Lake	ENV	0	NA	NA
VAL2024001051ABC	Dunn Lake	ENV	0	NA	NA
VAL2024001001EN1_52		EXTN	0	NA	NA
VAL2024001EN29_51		EXTN	0	NA	NA
VAL2024001EN2_25		EXTN	0	NA	NA
VAL2024001001EP1_52		EXTP	0	NA	NA
VAL2024001EP29_51		EXTP	0	NA	NA
VAL2024001EP2_25		EXTP	0	NA	NA
NTC		NPCR	0	NA	NA

Table 5

Quantitative PCR detection results for Northern Snakehead eDNA using the *NSCor* marker. Displayed is the Sample ID, Site Name, Sample Type (ENV = environmental, EXTN = negative extraction control, EXTP = positive extraction control, NPCR = negative PCR control), number of positive detections ('Hits', out of a total of 8), mean *Cq*, and mean copies per liter (CPL) detected. 'ABC' after the sample name indicates that the field sample represents three filters combined into a single sample for laboratory analysis.

Sample ID	Site	Sample Type	Hits	Mean *Cq*	Mean CPL
VAL2024001002ABC	Austell Lake	ENV	0	NA	NA
VAL2024001004ABC	Austell Lake	ENV	0	NA	NA
VAL2024001006ABC	Austell Lake	ENV	0	NA	NA
VAL2024001009ABC	New Lake	ENV	8	33.86983001	1345.807674
VAL2024001011ABC	New Lake	ENV	8	33.8687122	1107.535939
VAL2024001013ABC	New Lake	ENV	8	33.46834353	1555.567264
VAL2024001016ABC	Big Creek	ENV	8	32.46172079	1772.65387
VAL2024001018ABC	Big Creek	ENV	8	34.1850142	598.3457922
VAL2024001020ABC	Big Creek	ENV	8	31.25683573	6074.302932
VAL2024001023ABC	Bonner Farm	ENV	8	28.74957031	209414.057
VAL2024001025ABC	Bonner Farm	ENV	8	29.06471001	228727.6016
VAL2024001029ABC	Clarendon City Pond	ENV	5	37.66811565	245.2772126
VAL2024001031ABC	Clarendon City Pond	ENV	7	36.85586338	488.4676656
VAL2024001033ABC	Clarendon City Pond	ENV	0	NA	NA
VAL2024001035ABC	Clarendon City Pond	ENV	4	37.73321916	258.1807323
VAL2024001037ABC	Clarendon City Pond	ENV	3	38.82301066	204.7403196
VAL2024001039ABC	Clarendon City Pond	ENV	0	NA	NA
VAL2024001042ABC	Mallard Lane Ditch	ENV	8	35.21857071	Inf
VAL2024001044ABC	Mallard Lane Ditch	ENV	8	34.62094997	14909.96526
VAL2024001047ABC	Dunn Lake	ENV	0	NA	NA
VAL2024001049ABC	Dunn Lake	ENV	0	NA	NA
VAL2024001051ABC	Dunn Lake	ENV	0	NA	NA
VAL2024001001EN1_52		EXTN	0	NA	NA
VAL2024001EN29_51		EXTN	0	NA	NA
VAL2024001EN2_25		EXTN	0	NA	NA
VAL2024001001EP1_52		EXTP	0	NA	NA
VAL2024001EP29_51		EXTP	0	NA	NA
VAL2024001EP2_25		EXTP	0	NA	NA
NTC		NPCR	0	NA	NA

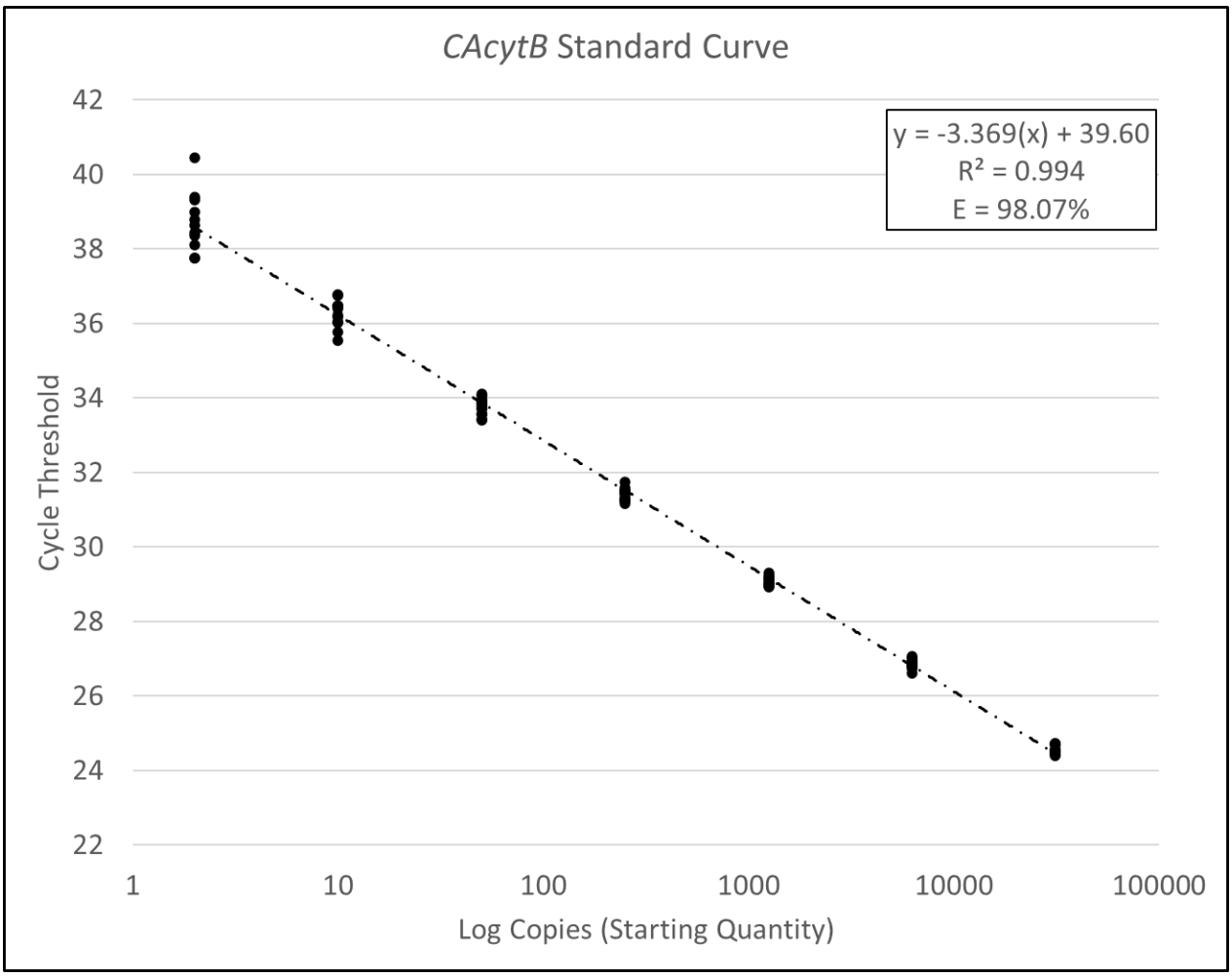

Figure 1

Standard curve plot and associated metrics used for determining copy per liter (CPL) calculations for the cytB locus (CAcytB). Linear regression was applied to amplification data resulting from an 8 point set of gBlock standard qPCR starting quantities in a 5x dilution series starting at 31250 copies per reaction and ending at 0.4 copies per reaction. The line of best fit was used to determine slope, y-intercept, r^2, and PCR efficiency (E).

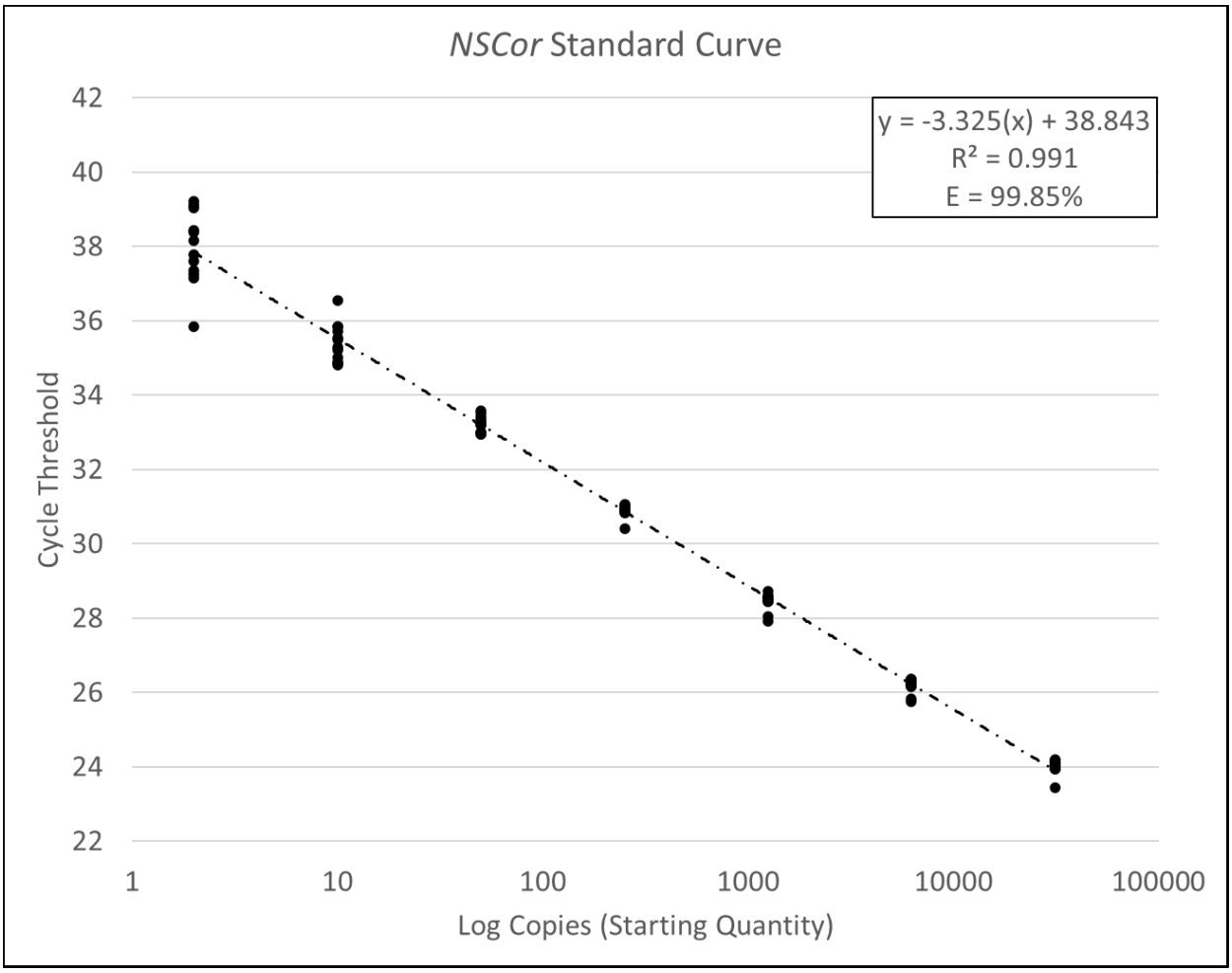

Figure 2

Standard curve plot and associated metrics used for determining copy per liter (CPL) calculations for the *COI* locus (*NSCor*). Linear regression was applied to amplification data resulting from an 8 point set of gBlock standard qPCR starting quantities in a 5x dilution series starting at 31250 copies per reaction and ending at 0.4 copies per reaction. The line of best fit was used to determine slope, y-intercept, r^2, and PCR efficiency (E).