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Development and Validation of Rapid eDNA Detection Methods for Bog Turtle (Glyptemys muhlenbergii)

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by

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Abstract

Bog turtles (*Glyptemys muhlenbergii*) are listed endangered species in the United States. Multi-state efforts are underway to better characterize extant populations of the species and prioritize restoration efforts. Traditional sampling methods for bog turtles can be ineffective due to their wetland habitat, small size, and burrowing nature. New molecular methods, such as qPCR, provide the ability to overcome this challenge by effectively quantifying minute amounts of turtle DNA left behind in its environment (eDNA). Developing such methods for bog turtles has proved difficult partly because of the high sequence similarity between bog turtles and closely-related, cohabitating species, such as wood turtles (*Glyptemys insculpta*). Additionally, substrates containing bog turtle eDNA are often rich in organics or other substances that frequently inhibit both DNA extraction and qPCR amplification. Our first goal was to develop a qPCR assay that could correctly identify blood collected from seven species of turtle over a wide geographic range. The eDNA detection method was primarily validated using contrived positive samples from the environment. Furthermore, methods employing a genetically modified strain of *C. elegans* as a full-process internal control which was used in method optimization and to determine DNA recovery from field sample.

Introduction

The bog turtle (*Glyptemys muhlenbergii*) is a critically endangered species (IUCN, 2012) and listed as threatened by the Endangered Species Act (USFWS 2001). Due to significant urban growth and new construction of energy and transportation infrastructure, there is an urgent need to survey and prioritize specific wildlife populations for protection and management. However, due to their small size and burrowing nature, available methods to detect and enumerate bog turtles through trapping and probing are highly resource intensive and, in some cases, ineffective. Alternative methods that reliably detect bog turtles are critical to properly assess the status of this declining species.

Turtles shed small amounts of DNA into the environment (eDNA) through feces, skin cells and other secretions (Bronnenhuber & Wilson, 2013). Sampling eDNA could allow detection of cryptic species that are difficult and resource intensive to detect using traditional survey methods. eDNA methods have been used previously to determine the presence of a wide range of aquatic species (Mahon et. al., 2013, Olson et. al., 2012). This strategy is proven to be effective and 2-10x more cost-efficient than traditional surveying methods in the detection of freshwater turtles (Davy et. al., 2015). Furthermore while studies using metagenomics were unable to detect turtles, targeted PCR methods were successful (Kelly et. al., 2014, Davy *et al.*, 2015). The detection of bog turtle eDNA poses a unique combination of challenges: 1) individuals are small and are often found in small, sparse populations reducing the amounts of eDNA expected to be released into the surrounding environment, 2) the bog habitat is rich in sediments and organics that can interfere with DNA extraction and PCR amplification, and 3) the wood turtle (*Glyptemys insculpta*), a closely related species that share significant homology with the bog turtle at the genetic level and often co-habitats with bog turtles.

High genetic variability among bog turtle populations is expected due to their life history traits and given the fragmented and isolated distribution of their current habitat (Nunney,

1991). However, recent studies demonstrate that the genetic variability in the mitochondrial genes is surprisingly low in the species (Rosenbaum et. al., 2007). Previous studies targeted mitochondrial cytochrome c oxidase 1 (COI) for similar applications (Vrijenhoek, 1994, Davy et. al., 2015).

In this study, our goal was to develop a method that could reliably detect the presence of bog turtles in their native environment. We designed species-specific oligonucleotides targeting the bog turtle COI gene which were validated using a blood and tissue DNA library. We also developed a control method targeting a genetically modified GFP gene in *C. elegans* to be used as an internal control to indicate poor DNA extraction from bog samples. Finally, the qPCR assays were validated in controlled field conditions.

Materials and Methods

Reference Sample Collection

Blood samples throughout the known bog turtle range were analyzed for genetic markers. Samples were collected from bog turtles (n=156; Table 1) and other related species (n = 5 species; 48 individuals; Table 1). Either tail or subcarapacial blood samples were collected from healthy turtles weighing more than 100 g using a 27 gauge sterile needle (BD #305136, Franklin Lakes, NJ) and a 1 ml sterile syringe (BD #309628). Individual blood samples totaling no more than 400 µl blood per 100g body weight were immediately dispensed into 1ml Longmire buffer (100 mM Tris-HCl pH 8.0, 100 mM EDTA, 10 mM NaCl, 0.5% SDS) pre-filled into 2ml cryotubes for preservation. Samples collected were stored at -20C within 24 hours of collection. DNA from 20µl of the buffered blood samples was extracted using Qiagen Blood and Tissue Kit. Blood DNA extracts from GA, TN, and VA were extracted during a previous project (2014). Blood DNA extract quantity was assessed on a Qubit 3.0 (Thermo Fisher, Wilmington, DE).

Table 1. Taxonomic and regional distribution of blood samples analyzed

	Clemmys guttata	*Emydoidea blandingii*	*Glyptemys insculpta*	*Glyptemys muhlenbergii*	*Terrapene Carolina*	*Chelydra serpentina*	Total
Connecticut	1	-	1	4	2	-	8
Georgia	-	-	-	10	-	-	10
Massachusetts	-	10	7	-	-	-	17
Maine	-	-	6	-	-	-	6
New Hampshire	-	-	5	-	-	-	5
New Jersey	-	-	-	43	-	-	43
New York	3	-	2	29	-	-	34
Pennsylvania	3	-	5	49	1	1	59
Tennessee	-	-	-	9	-	-	10
Virginia	-	-	-	10	-	-	10
Total	7	10	26	154	3	1	201

Oligonucleotide Design and Preparation.

Representative cytochrome oxidase sequences of related turtle species were obtained from NCBI GenBank and aligned with MAFFT to indicate site heterogeneities between sequences (Figure 2). Oligonucleotides were designed (Table 2) to target mismatches

between bog turtle and r closely related and sympatric turtle species such as wood turtle (*Glyptemys insculpta*), blanding's turtle (*Emydoidea blandingii*) and spotted turtle (*Clemmys guttata*). Oligonucleotides targeting the cytochrome b gene were tested but amplified Blanding's turtle as well as bog turtle.

Primer concentration 1400 nM each and Taqman® probe concentration on 100nM was used in the qPCR reaction. qPCR conditions consisted of A two-step reaction cycle with a denaturation at 95˚C followed by annealing and extension step at 60˚C, repeated 40 times in a 96 well plate with a reaction volume of 25 µl per well was run on a QuantStudio3 (ABI). For TaqMan® qPCR, the 25 µl reaction mixture consisted of 12.5 µl of 2x TaqMan® environmental master mix (version 2.0), 3 µl primer-probe mix(add primer and probe concentration here) , 2µl template DNA. Molecular grade water was added to bring volume up to 25 µl. In addition to our probe based assay a SYBR Green qPCR was set up with similar conditions expect, the probe was excluded and, 0.25 µl of 10x SYBR Green was added to reach a final concentration of 0.1x SYBR Green. The data were initially viewed in Quantstudio™ Software V1.3. The quantification cycle (Cq) values with 0.03 threshold were exported for further analysis. Samples in which all three replicate Cq values were less than the Cq value of the gBlock Limit of Quantification (LOQ) (10 copies/reaction) were considered within the range of quantification and converted to copy number using the assay-specific gBlock standard curve equation (Table 3). Samples in which any of the replicates did not amplify sufficiently to cross the threshold were considered below the Limit of detection (LOD). Samples in which a) any of the three replicate Cq values were less than the Cq value of the gBlock LOQ and b) all replicate Cq values were £ 40 were considered within the LOD, but not the LOQ.

Figure 2: Alignment showing the placement of BT3 primers and probe on the cytochrome oxidase gene of bog turtle and related species.

Table 2. Oligonucleotide sequences designed in this study.

Oligonucleotide name	Sequence (5'-3')	Final reaction concentration (nM)
BT3F	GGAGTCGAAGCAGGAGCG	1400
BT3R	CCGGCGTGGGCCAG	1400
BT3P	[FAM]ACA GGC TGA[ZEN]ACT GTA TAC CCT CCA CTA GCC G[IBFQ]	100
CG4F	CGA AAG ATC CCA ACG AAA AGA GAG	1400
CG4R	CCA TGT GTA ATC CCA GCA GCT	1400
CG4P	[VIC]ACA TGG TCC TTC TTG AGT TTG TAA C[MGB]	100

Primers pairs were initially tested using SYBR green qPCR to more easily identify non-target amplification using melt curve analysis. Melt curve analysis showedtwo separate melt peaks when tested on bog turtle blood samples (Figure S1) and non-specific amplification. The CG4 assay displayed one melt peak indicating the amplification of a sample amplicon. Non-target detection was avoided by the use of TaqMan® probes.

To improve specificity we developed a TaqMan® probe. The assay was optimized using a 10 to 10^6 copies per reaction gBlock (IDT, Coralville, IA) standard curve to consistently obtain 95-100% efficiency and R^2>0.99. Efficiency *E* was calculated using the formula- $E=10^{(slope/-1)}-1$

Field Sample Collection and Processing

Three sites in NY and PA, with known bog turtle populations, were chosen to validate and optimize eDNA detection methods. For optimization purposes, some samples were collected in the vicinity (within 12 inches) of a bog turtle. Samples were also collected from a site in southern NY where bog turtles have not been observed, despite multiple surveys over 20 years. Samples of 1L containing water and sediment from the wetlands were collected, brought back to the lab and processed as described below within 8 hours of sampling.

Sample Processing Control

A genetically modified strain of *C. elegans* (SH52) whose genome contains a high copy number of a genetically modified *GFP* was used to estimate both DNA extraction recovery and qPCR inhibition. During the initial stages of the study, it was also used for optimization of sampling processing methods (Figure S2 and S4). While other organisms have been used to control for DNA loss and PCR inhibition, we chose *C. elegans* to serve as an internal control because 1) it is a model organism that can be grown, stored, observed, and genetically manipulated easily using standard protocols, 2) it's eukaryotic membrane may mimic DNA extraction from bog turtle cells more closely than bacterial (Green et. al., 2014) or viral (Bae and Wuertz, 2009) internal controls, and 3) it is eutelic which facilitates the collection and processing of a known number of cells. Briefly, ~50 adult hermaphrodite worms were added to a 2 ml tube with 1.4mm ceramic beads (VWR #10158-610, Radnor, PA) and 1ml of Elution buffer from GeneRite (DNA-EZ RW02)

kit. Worms were homogenized for 40 seconds at $6ms^{-1}$ to break apart worms to provide a lysate of free DNA and cell matter. The lysate was dispensed into 50µl aliquots, and 5µl used to spike each sample falcon tube. One aliquot was reserved for DNA recovery calculations.

Sample Processing and DNA Extraction

Initial trials showed that direct filtration methods were ineffective due to the high concentration of suspended sediment and organics that quickly clogged filter membranes. Pre-filtration in 12um pore size filters did not significantly affect the filtrate column or time until clogging (data not shown). In order to avoid filtration which would likely be a significant source of variability between sites, we then assessed the ability to recover both bog turtle eDNA and spiked *C. elegans* DNA from collected sediments in all future sampling efforts.

Five hundred milliliters of each sample was distributed between ten 50 ml Falcon tubes while being stirred continuously using magnetic stir bars. Five microliters of freshly prepared *C. elegans* lysate representing approximately $1.5*10^5$ - $2.5*10^5$ gfp gene copies were added to each of the ten falcon tubes. The 50 ml tubes were shaken by hand for ten seconds and centrifuged at 2,000 g for 10 minutes. Without disturbing the pellet, the supernatant from all ten tubes was filtered thought 0.4 um pore polycarbonate filters until water from all ten tubes were filtered or until three filters (per sample) were clogged. In case of the later, the filtered amount was noted. Filters were placed directly in GeneRite bead tubes and stored at -80 ˚C until extraction. The soil pellet was also stored at -80°C until DNA extraction. Although initial extraction efficiency trials showed a higher *C. elegans* DNA recovery from the sediment that the supernatant water (Figure S4

) both types of samples were analyzed for bog turtle eDNA during this study.

DNA was extracted from filters using GeneRite (DNA-EZ 01, North Brunswick, NJ) kit whereas the soil samples were extracted using FastDNA™ SPIN Kit for Soil (MPBio, Santa Ana, CA) with Lysing matrix C. For each sample, we combined 25 µl eluate from four replicate extractions to yield 100 µl total elution volume. The extracted DNA was quantified using Qubit 3.0 fluorometer and Qubit dsDNA HS assay kit according to manufacturer's protocol.

Percent recovery for filters *Rf* and sediment *Rs* was calculated using the following formulae where *Qd* is copies/µl DNA in the sample, *Ve* is the volume of eluate, *Ql* is copies/µl of the spike, *Vs* is the volume of lysate spiked and *M* is the mass of sediment in the falcon tube.

$$1)\ Rf = \frac{(Qd * Ve * 100)}{(Ql * Vs)}$$

$$2)\ Rs = \left(\frac{M}{2}\right) * \frac{(Qd * Ve * 100)}{(Ql * Vs)}$$

Results

Assay Amplification Kinetics.

BT3 and CG4 TaqMan® assays showed a consistent efficiency of >97%, R^2 of ≥0.99, and limits of detection at ≤10 copies (Table 3) on synthetic template standard curves. Comparable performance characteristics on tissue DNA extracts suggest that the assays operate similarly within their native genomic context. In some cases, BT3 SYBR melt curves and gel electrophoresis of amplicons showed the presence of some non-specific amplification on bog turtle blood DNA (Figure 4). However, the secondary amplification did not seem to affect qPCR amplification kinetics and was observed only in bog turtle blood DNA and not in other species tested.

Table 3. BT3 and CG4 TaqMan® assay performance characteristics on synthetic template (gBlock) and tissue DNA extract dilutions. Blood and whole worms were used as starting material for tissue DNA extracts for the BT3 and CG4 assays, respectively.

	Amplicon length (bp)	gBlock				Tissue DNA extracts			
Assay		R^2	Cal. Eq.	Eff.	LOD (copies)	R^2	Cal. Eq.	Eff.	LOD (pg)
BT3	71	0.99	y=-3.327x+34.931	0.997	10	0.99	Y=-3.326x+39.002	0.998	0.095
CG4	73	0.99	y=-3.374x+39.439	0.998	10	0.99	Y=-3.370x+41.670	0.9803	0.0094

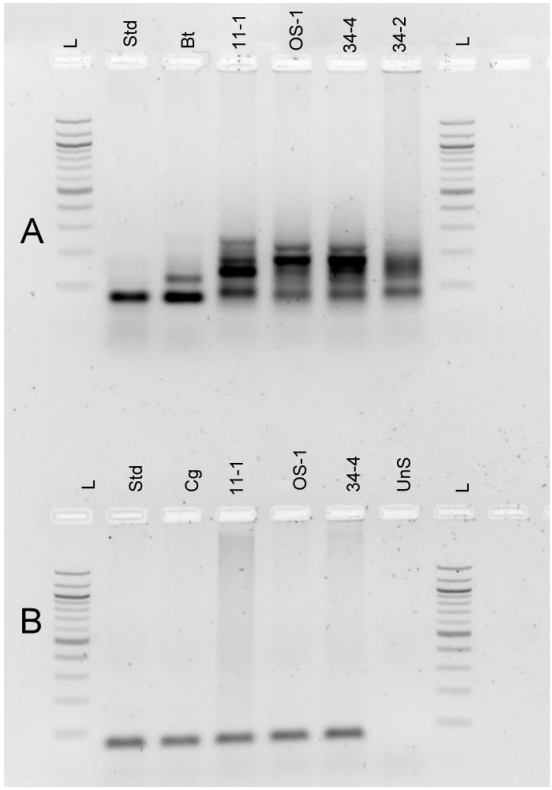

Figure 4: Gel electrophoresis of qPCR products from BT3 (A) and CG4 (B) on 2% agarose gel using 1X TBE buffer. **L.** 1kb Ladder; **Std.** gBlock standard; **Bt**. Bog turtle blood DNA; **11-1, OS-1, 34-4, 34-2.** Environmental sediment sample DNA with *C. elegans* spike; **Cg.** *C. elegans* tissue DNA; **UnS.** Sediment with no *C. elegans* spike.

Assay Specificity and Sensitivity on Blood Samples.

Blood samples yielded on average 2.8±2.8 and 4.8±2.8 ng/µl DNA for GLMU and non-GLMU samples, respectively. Tail-clipping DNA extracts yielded on average 23.0±27.4 ng/µl. After screening all 201 blood and tissue extracts with the BT3 TaqMan® assay, all 156 bog turtle samples exhibited strong amplification whereas amplification did not occur with any of the 49 non-bog turtle samples. Bog turtle blood DNA and tail clipping DNA contained an average of $1.3x10^6 \pm 6.6x10^6$ and $2.9x10^2 \pm 5.4x10^2$ marker copies/ng DNA, respectively.

Detection of Bog Turtle eDNA from Confirmed Bog Turtle Sites.

Initial trials demonstrated that direct filtration methods were ineffective due to high sediment content in the wetland habitats. Hence, some level of preprocessing was required to separate the sediment from the sample. Pre-filtration using larger pore size filters did not have a high impact on the filtration volume or time (Data not showed). Centrifugation of sample at 2000g for 10min allowed ample separation and allowed filtration through 0.4-micron pore-size filters. Hence the sediment and aqueous phases of the sample were processed separately. Trials with internal control showed the sediment phase had higher DNA recovery than the aqueous phase (Figure S4).

Ct values were converted to quantities per reaction well using the linear regression obtained from the gBlock standard curve. Bog turtle eDNA was detected from two sites that had known populations of bog turtles. Bog turtle eDNA was not detected from a third site which had no reported populations of bog turtles demonstrating the specificity of the assay (Table 3). The detectable percent recovery the internal control DNA varied from 1.33% to 27% showing that the majority of the eDNA could be lost in the sample processing, inhibition or inefficient amplification.

Table 4: Detection of bog turtle eDNA in various field sites. Percent recovery indicates the recovery of *C.elegans* DNA from the samples. Samples with no detectable amplification in all three qPCR replicates are denoted by "< LOD" whereas "+" denotes samples with amplification lower than gBlock LOD.

Site location	Sample	Contrived	Bog turtle presence	Disturbed	Internal control % recovery	Marker copies/gram of sediment
NY site A	1	Yes	yes	No	5.31	< LOD
NY site A	2	Yes	yes	Yes	12.06	< LOD
NY site A	3	Yes	yes	No	27.5	< LOD
NY site A	4	Yes	yes	Yes	3.62	+
NY site A	5	Yes	yes	No	9.27	+
NY site B	2	No	No	No	8.64	< LOD
NY site B	3	No	No	Yes	20.86	< LOD
NY site B	4	No	No	No	7.03	< LOD

NY site B	5	No	No	Yes	17.8	< LOD
NY site C	1	Yes	yes	No	10.58	+
NY site C	2	Yes	yes	Yes	8.64	+
NY site C	4	Yes	yes	Yes	12.06	< LOD
PA site A	1	No	yes	No	22.07	2748.6± 253.1
PA site A	2	No	yes	No	1.33	< LOD
PA site A	3	No	yes	No	1.74	< LOD
PA site A	4	Yes	yes	No	20.04	457.7±98.2

Discussion

The development of a specific and sensitive qPCR assay for the detection of bog turtles from environmental samples may be a useful tool for monitoring the recovery of thisdeclining species. Traditional methods of bog turtle detection remain costly, time-consuming, and often, ineffective for this elusive species. In contrast, the prospect of indicating the presence of bog turtle from a two-gram sediment sample has the potential to dramatically alter surveying strategies and, ultimately, the trajectory of the declining species. While, for the time being, these methods are not replacements for traditional survey methods they may offer specific advantages that supplement or improve the effectiveness of current detection methods. eDNA methods are easily scaled up facilitating cost-effective screening prioritization of specific sites for follow-up assessment and in theory could lead to the discovery of previously unknown sites as has happened in other eDNA studies (Spear et al, 2014). Additionally, contingent on further methodological improvements and a better understanding of storage and transport of bog turtle DNA markers, these methods may help circumvent difficulties with access by sampling “downstream” of potentially inhabited sites. Additionally, the relatively short length (73 bp and 71 bp for CG4 and BT3 respectively) of qPCR amplicons increases detection ability as larger fragments are less likely to persist in the environment.

Many studies seek to make quantitative inferences about the number of individuals (Spear et al, 2015) or species biomass (Takahara et al, 2012) from a measured number of environmental markers. However, using a novel internal control we found the recovery of total DNA from sediment samples varies by over an order of magnitude and seems to significantly affect our ability to detect bog turtle eDNA. In some cases, DNA recovery differed greatly between samples taken within the same site highlighting the need for such controls as a quality measure to prevent incorrect interpretation of false negative samples.

Many eDNA studies in low sediment quantity settings have used filtration methods to concentrate cells suspended in the aqueous phase on a polycarbonate or glass fiber filters (Turner et al, 2014; Davy et al, 2015, Rees et al, 2014), which offer some advantages over DNA extraction from sediment. However, in this study direct filtration of highly turbid bog surface waters cloggedfilters reducing their ability to concentrate eDNA. DNA is known to rapidly diffuse from the source organism in previous studies in aquatic systems (Rees *et al*, 2014). Most eDNA sources including skin cells do not stay suspended in water (Turner et al, 2014) and, eDNA persists longer in sediment than in surface water (Turner *et al*, 2015). In our study, we detected eDNA only in sediment

samples validating the claims above. This suggests low diffusion from the source organims and thus requires the development of a more strategic approach to field sampling. Since a high amount of DNA was lost during DNA extraction (Figure S2; S4) of sediment samples, a sampling approach bypassing the processing loss also needs to be developed.

The BT3 assay was highly specific and sensitive, however, has some non-target amplification. Since the non-target amplification is only observed in bog turtle DNA, this does not reduce the effectiveness or the utility of the assay. Non-target amplification was also observed when the BT3 assay was run on high concentration of *C. elegans* DNA. Even though no evidence of PCR interference was observed in low and experimentally significant concentrations between ($10 – 10^8$ copies/µl). PCR amplicon sequencing could provide more information on the causes of this non-target amplification, from bog turtle and *C. elegans* DNA, and potentially mitigate them.

The preliminary field trials discussed in this thesis provide a proof of concept for rapid detection of bog turtle eDNA in sediment-rich samples. However, the relatively low eDNA recovery led to false negatives in almost half of the samples. Further optimization of DNA extraction and processing is necessary. For implementation in regular population monitoring, an effective sampling procedure is necessary. eDNA hotspots such as nesting habitat or hibernating burrows can be targeted during seasons of high activity. Our results show that eDNA was detected only in the sediment, and not in the aqueous layer. Thus, we recommend a higher emphasis on optimizing DNA extraction from soil samples. Understanding the effects of environmental factors like pH, sediment composition, salinity on bog turtle eDNA persistence would aid in data interpretation Incorporation of bog turtle behavioral ecology will make eDNA sampling methods more effective.

Future work should focus on the sequencing of PCR amplicons to observe inefficiencies and interferences in PCR amplification. Similar methods could be developed for other members of the *Emydidiae* family that face similar issues in effective monitoring and management, including wood and spotted turtles.,. A comparison of the effectiveness of the proposed method with the traditional survey and trapping methods for population analysis are underway.

Though initial lab experiments have shown the proposed method to be highly sensitive, specific and efficient. Methodological improvements to increase DNA recovery and a better understanding of DNA shedding, transport, storage, and decay are needed to optimize eDNA detection assays. Our inclusion of a full process internal control allowed the optimization of sampling and sample process methods. Additionally, multiplex methods can reduce the resources required for eDNA monitoring, but need further optimizations.

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Appendix

Supplementary Methods

Method Optimization using *C. elegans*

A genetically modified *C. elegans* strain was used as a full process internal control during this study. It was also used in field sampling and sample process development. Water and sediment samples collected were spiked with *C. elegans* lysate to determine the extraction efficiency and DNA recovery using CG4 qPCR assay. The sediment for the trials was collected from a field site and extracted as per FastDNA™ SPIN Kit for Soil protocol. 100 ml of field water samples were spiked and filtered through 0.4 um filters and extracted using GeneRite DNA extraction kit. Initial tests were done to quantify the DNA recovery from sediment or water samples with the method described under the Field Sample Collection and Processing Section. (Figure S4). Twelve total samples, including six sediment and six supernatant water samples, were processed in triplicates. The *C.elegans* DNA spike was constant and pre-quantified allowing the accurate estimation of detectable DNA recovery.

Another study to optimize the Lysing matrix (bead type) type was performed using *C.elegans* lysate to samples retrieved from field sites. The optimal bead beating time was also estimated using the same method, as little homogenization would leave cells unlysed and too much could damaged the eDNA. Lysing matrixes A, C, D, E, F, G, H, and I were used to test for best recovery.

Supplementary Figures

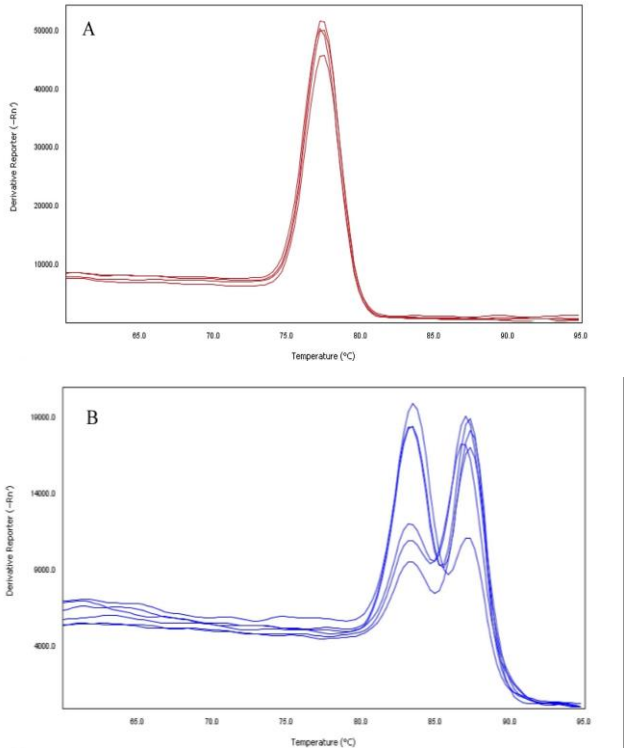

Figure S1: Melt curves using SYBR green sowing A) Single melt peak upon the amplification of C. elegans with the CG4 assay. B) Two distinct melt peaks upon amplification of bog turtle DAN from various states.

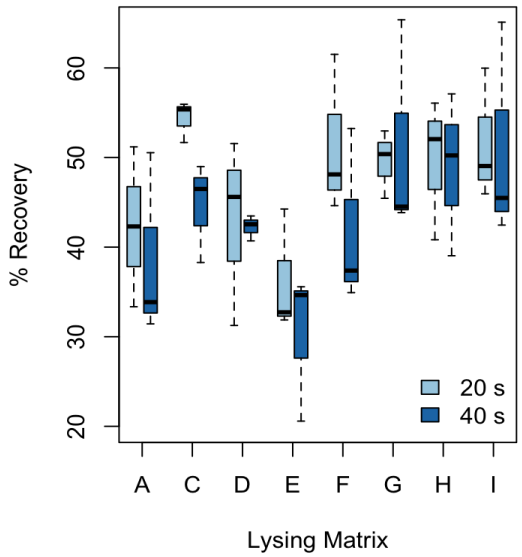

Figure S2: Lysing matrix and bead beating time optimization trials for the highest detectable DNA recovery.

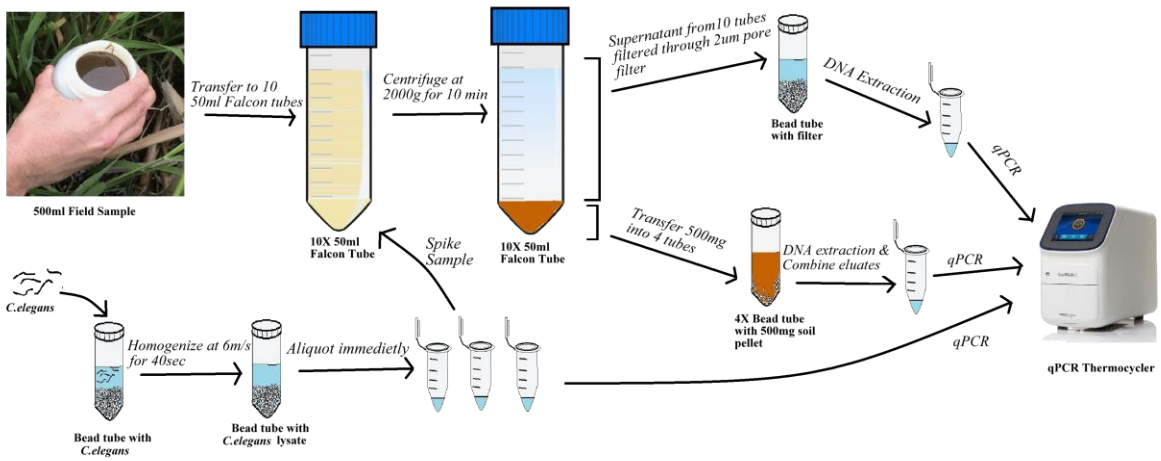

Figure S3: Workflow to determine the efficiency of eDNA detection in the sediment and the aqueous part of collected field samples using *C. elegans* internal control.

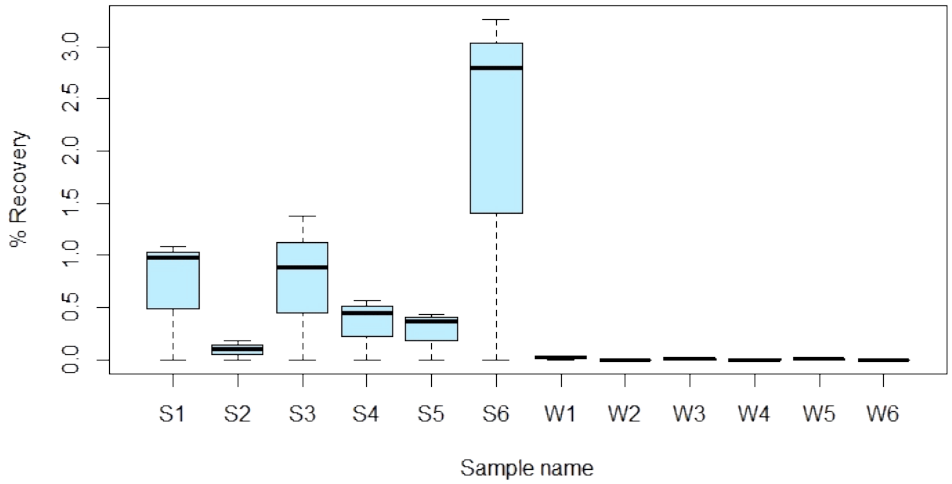

Figure S4: Detectable DNA recovery from the sediment and supernatant water from field samples during extraction efficiency trials. “S1-S6” denote sediment samples and “W1-W6” denote aqueous samples.