Using environmental DNA to determine wood turtle (*Glyptemys insculpta)* presence in New Brunswick, Canada rivers

by

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ABSTRACT

Worldwide turtle and tortoise populations are declining, including the semi-aquatic wood turtle (*Glyptemys insculpta*) of eastern North America. With a limited survey season, a more efficient method of detection was needed to monitor this cryptic species. DNA barcoding with environmental DNA (eDNA) has been shown to be an effective method of monitoring cryptic, rare, and threatened species. Water samples were collected in October 2017 and 2018 from an upstream and downstream site on fourteen rivers in New Brunswick, Canada. In our eDNA lab, the samples were filtered, extracted and, with the use of species-specific primers and probe, amplified using quantitative polymerase chain reaction (qPCR). With the use of qPCR, wood turtle eDNA was detected in five of six rivers known to inhabit the species. This method is a promising tool that can be used for the conservation and monitoring of *G. insculpta*.

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Biological replicate – water sample

Cq – Quantification cycle

DNA – Deoxyribonucleic acid

eDNA – Environmental DNA

PCR – Polymerase chain reaction

qPCR – Quantitative polymerase chain reaction

Site – Sample location in a river

Technical replicate – qPCR replicate

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Introduction

Worldwide turtle and tortoise populations are in decline with nearly 42% of species listed as either Vulnerable, Endangered or Critically Endangered, which is likely to increase as more data are collected and we update the status of data-deficient species (Buhlmann et al., 2009; Turtle Conservation Coalition, 2018). Humans are the biggest threat to turtles and tortoises as they are collected for food (the animal and the eggs), traditional medicine, and for the pet trade (Turtle Conservation Coalition, 2011). Their habitat is also being destroyed by development of roads and housing, agriculture, pollution, and recreation (COSEWIC, 2007; Turtle Conservation Coalition, 2011, 2018).

Wood turtle (*Glyptemys insculpta*) populations are among those in decline and the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) has designated the Wood Turtle threatened in Canada (COSEWIC, 2007). In addition, it is listed as endangered under the Ontario Endangered Species Act, vulnerable in Quebec (COSEWIC, 2007) and the International Union for Conservation of Nature has assessed the species as globally endangered (van Dijk & Harding, 2011).

This semiaquatic species (McCoard, Billings, & Anderson, 2016) resides in a discontinuous range from south-central Ontario to Nova Scotia in Canada and from Minnesota to Maine and south to Virginia in the northeastern United States (COSEWIC, 2007; Flanagan, Roy-McDougall, Forbes, & Forbes, 2013). They hibernate in rivers and

streams during the coldest months and spend the spring and part of the summer (until about July) in and around the water until the temperature increases and mating/nesting have concluded, at which time they become more terrestrial (Arvisais et al., 2002; Flanagan et al., 2013). Wood turtles have been found to travel up to 600 meters (m) from waterways, however, they usually stay within 300 m, with females travelling farther (Arvisais et al., 2002; Flanagan et al., 2013) and spending less time in water than males (Flanagan et al., 2013). As the ambient temperature decreases in the fall, the turtles will stay closer to the waterways and spend nights in the water until they hibernate in late October or early November (Arvisais et al., 2002; COSEWIC, 2007).

Traditional surveys of wood turtles are typically conducted on sunny spring days before the turtles (1) are no longer easy to spot basking due to vegetation growth and (2) they have dispersed from areas adjacent to waterways (Flanagan et al., 2013; Lacoursière-Roussel, Dubois, Normandeau, Bernatchez, & Adamowicz, 2016). Survey protocols vary among researchers but are generally either observational (basking or road-kill) or trap captures (Flanagan et al., 2013). Because the distribution and population size of this species in New Brunswick is not well known (COSEWIC, 2007) and there is only a limited period when detection probabilities are high, we propose a new method of detection.

A new field in genetic analyses has emerged that uses DNA collected from the environment to detect the target species without it being immediately present (Lodge et al., 2012; Rees, Maddison, Middleditch, Patmore, & Gough, 2014; Taberlet, Coissac, Hajibabaei, & Rieseberg, 2012; Turner et al., 2014; Wilcox et al., 2013). Environmental DNA (eDNA) is DNA that has been shed by organisms in the environment (Barnes &

Turner, 2016; Barnes et al., 2014; Evans et al., 2016; Ficetola, Miaud, Pompanon, & Taberlet, 2008; Jerde, Mahon, Chadderton, & Lodge, 2011; Lacoursière-Roussel et al., 2016; Taberlet et al., 2012; Turner et al., 2014). The DNA in these samples can be cellular, from shed skin, feces, urine, saliva, gametes, mucous, etc., or extracellular, DNA that is not in cells due to cell death or damage to the structure (Barnes & Turner, 2016; Ficetola et al., 2008; Rees et al., 2014; Taberlet et al., 2012). eDNA is a mixture from all the organisms found in the region (Barnes et al., 2014; Jerde et al., 2011; Taberlet et al., 2012; Thomsen & Willerslev, 2015; Turner et al., 2014). Samples can be taken from a variety of environmental media such as water (freshwater or salt water), soil, air, ice, etc. (Rees et al., 2014; Taberlet et al., 2012; Turner et al., 2014). The amount of time that DNA persists depends on the environment. DNA persists in lakes and ponds longer than stream and marine environments, weeks compared to hours or days (Collins et al., 2018; Dejean et al., 2011; Thomsen, Kielgast, Iversen, Møller, et al., 2012; Thomsen, Kielgast, Iversen, Wiuf, et al., 2012). Thus, a species detected in a water sample is likely to be present or have left the site very recently. Aquatic eDNA is susceptible to decay due to a variety of abiotic (UV radiation, temperature, salinity, substrates) and biotic (microbial community) factors, which limits persistence time in the environment (Barnes et al., 2014; Pilliod, Goldberg, Arkle, & Waits, 2014; Strickler, Fremier, & Goldberg, 2015). It can also be transported out of the system through horizontal or vertical transport, particularly in stream and marine environments (Buxton, Groombridge, & Griffiths, 2018; Pilliod et al., 2014). By contrast, soil and ice samples can contain DNA from hundreds of thousands of years ago, making it a good source of ancient DNA (Thomsen & Willerslev, 2015).

Recently, eDNA use has increased, especially in conservation and monitoring, as it is a cost- and time-efficient method of noninvasively sampling species (Akre et al., 2019; Barnes & Turner, 2016; Davy, Kidd, & Wilson, 2015; Lacoursière-Roussel et al., 2016; Pilliod, Goldberg, Arkle, & Waits, 2013; Takahashi et al., 2018). Monitoring of aquatic species is usually done with traps, nets, electrofishing, etc. which can be stressful for target and non-target species (Snyder, 2003) and can also be destructive to the environment. The eDNA method has been successful in detecting invasive vertebrate species, such as American bullfrogs (*Rana catesbeiana*) in France (Ficetola et al., 2008), invasive carp (*Hypophthalmichthys molitrix* and *H. nobilis*) near the Great Lakes (Jerde et al., 2011; Mahon et al., 2013), and Burmese pythons (*Python bivittatus*) in south Florida (Hunter et al., 2015; Piaggio et al., 2014). It has also been effective in detecting threatened and cryptic species including wood turtle (*Glyptemys insculpta*) in Virginia, Ontario and Quebec (Akre et al., 2019; Davy et al., 2015; Lacoursière-Roussel et al., 2016), spotted gar (*Lepisosteus oculatus*) in Ontario (Boothroyd, Mandrak, Fox, & Wilson, 2016), and the Hula painted frog (*Latonia nigriventer*) presumed extinct in Israel (Renan et al., 2017).

DNA barcoding was developed to rapidly identify species, regardless of sex and life stage, which, even for an expert, can be difficult to do morphologically (Che et al., 2012; Hebert, Ratnasingham, & deWaard, 2003). There are two definitions of barcoding: *sensu stricto*, where a single standardized DNA fragment is used to identify the unknown sample to species level and *sensu lato,* where any DNA fragment can be used to identify the sample to any taxonomic level (Valentini, Pompanon, & Taberlet, 2009). Hebert et al. (2003a) proposed the mitochondrial gene cytochrome *c* oxidase subunit 1 (COI) as the

barcoding gene of animals. This short gene sequence (600-700 base pairs (bp)) (Che et al., 2012) was chosen because (1) mitochondrial genes are a better option for barcoding than nuclear genes because they lack introns (noncoding DNA that has a higher rate of mutation), (2) are inherited from the mother, (3) haploid (making them more conducive to Sanger sequencing), and (4) evolve faster than nuclear DNA creating a faster divergence between closely related species (Hebert, Cywinska, Ball, & deWaard, 2003; Hebert, Ratnasingham, et al., 2003). This creates the optimal amount of intraspecies sequence variation with greater interspecies variation, which increases the strength of universal primers (Che et al., 2012; Hebert, Cywinska, et al., 2003; Hebert, Ratnasingham, et al., 2003).

DNA barcoding begins by extracting DNA from a specimen, then use universal primers, that are created to amplify specific taxonomic groups, and polymerase chain reaction (PCR) to amplifying the target gene sequence enabling you to identify the species through sequencing (Thomsen & Willerslev, 2015). Primers are short (18-27 bp) sequences designed to anneal to either end of the target gene sequence and direct replication of the sequence. A similar process is also used in eDNA studies; however, species-specific primers can be used to determine if your target species' DNA is present in the DNA extracted from the environmental sample (Taberlet et al., 2012). eDNA studies also use a shorter portion of the target gene sequence (can be less than 100 bp) as DNA degrades faster in the environment causing intact sequences to be shorter (Taberlet et al., 2012). Because it can be difficult to design primers for a shorter section of the COI gene, alternative genes such as 12S and 16S (mitochondrial ribosomal genes) or cytochrome B (protein-coding genes) may be used for eDNA (Deiner et al., 2017).

More sensitive laboratory techniques have facilitated eDNA studies to move beyond simple presence/absence to semiquantitative abundance (Lacoursière-Roussel et al., 2016). Previous studies have shown that population abundance (number of individuals at a site) cannot yet be determined for freshwater turtles through eDNA samples; however, a relative abundance can be implied, typically from results of quantitative PCR (qPCR; Lacoursière-Roussel et al., 2016). The more sites along a river that are positive with qPCR the higher the abundance. This is based on the assumption that eDNA will be more homogeneously mixed in the waterway when there are more individuals present, increasing detection probability (Lacoursière-Roussel et al., 2016). qPCR is often used with animal eDNA as it has increased sensitivity to sequence mismatches and it is not measuring the concentration of DNA in the sample but a relative amount that can be compared to other samples (Lacoursière-Roussel et al., 2016).

Quantitative PCR assays replicate the target sequence of DNA with the use of two primers, which are designed to anneal to each end of the sequence (Wilcox et al., 2013). A third oligonucleotide, the probe, with a fluorescent dye attached to one end and a quencher on the other, is designed to attach to the PCR product (between the primers) (Wilcox et al., 2013). When attached to the probe, the quencher molecule absorbs the fluorescence from the dye preventing the qPCR camera from detecting it (Wilcox et al., 2013). As the sequence is amplified, the polymerase cleaves the probe, separating the fluorescent dye from the quencher molecule allowing the dye to fluoresce (Wilcox et al., 2013). As elongation cycles continue, more fluorescence is released; however, it is still below the detection threshold (Osman, Olineka, Hodzic, Golino, & Rowhani, 2012). More fluorescence accumulates with every cycle (exponential phase) and the cycle that

the qPCR camera can detect the fluorescence is called the quantification cycle (Cq; previously referred to as threshold cycle, Ct or crossing point, Cp; Bustin et al., 2009). A sample's Cq value is proportional to its amount of DNA, as samples with more DNA amplify faster and have its fluorescence detected at an earlier cycle than a sample with less DNA (Bustin et al., 2009; Wilcox et al., 2013). Cq values allow you to compare the amount of DNA among samples – samples with lower Cq values have more target species DNA.

The aim of this study is to use a state-of-the-art genomic tool to improve the conservation and monitoring of wood turtles (*Glyptemys insculpta*), an endangered species whose population is in decline. Aqueous environmental DNA (eDNA) was collected to use with species-specific primers and probe and amplified with qPCR to determine their recent presence in fourteen rivers in New Brunswick, Canada. The results of this study can improve estimates of the distribution and abundance of wood turtles in New Brunswick.

Methods

Laboratory creation

Sample filtration, extraction, PCR preparation, and post PCR work were all conducted in separate rooms as environmental DNA projects are very susceptible to contamination because the amount of target DNA in each sample is minute. Each lab room had its own lab coat and once I worked with DNA from tissue or amplified DNA, I did not re-enter the clean labs until I had changed my clothes. Prior to beginning the study, laboratory spaces for filtration, extraction and PCR preparation had the walls,

counters, cupboards, etc. cleaned with a 33% bleach solution (1-part bleach (6% Lavo Pro™ 6): 2 parts distilled water) and then wiped with 70% ethanol.

The set-up of the filtration apparatus required connecting the dry vacuum pump (Welch, Mount Prospect, IL, USA, model 2014) to the carboy (where the water would be moved to) and the carboy to the 3-place filter funnel manifold (Pall® Life Sciences, Ann Arbor, MI, USA; Figure 1). This closed loop allowed the water to be pumped through the filters at the bottom of the 500 mL Sentino™ Magnetic Filter Funnels (Pall® Life Sciences, Ann Arbor, MI, USA), resting in the filter funnel manifold, and into the carboy to be disposed of. Once it was tested with tap water to ensure it worked properly the filter funnels and the manifold were rinsed with 10% bleach (1-part bleach (6% Lavo Pro™ 6): 9 parts distilled water) three to four times and distilled water six times. Before using the MY-PCR Prep Workstation (Mystaire®, Creedmoor, NC, USA) the first time, the inside was cleaned with the 33% bleach solution followed by RNase Away and finally wiped with 70% ethanol. The ultraviolet (UV) light that the PCR workstation is equipped with was run for half an hour before cleaned pipettes and boxes of filter tips were placed inside. The UV light was then run for another half an hour. The Fisherbrand™ accuSpin™ Micro 17 Microcentrifuge and Fisherbrand™ Analog Vortex Mixer (Thermo Fisher Scientific, Waltham, MA, USA) used for extractions were also cleaned with 33% bleach, RNase Away and wiped down with 70% ethanol before any extractions took place.

Before starting any work, bench tops were cleaned with 33% bleach solution and RNase Away and pipettes were wiped with RNase Away followed by 70% ethanol. Filter

tips were used for all aspects of the project. Nitrile gloves were always worn and replaced between samples and after touching anything that was not decontaminated.

Figure 1. Filtration set up with 500 mL filter funnels (a) on a 3-place filter funnel manifold (b) which is attached to a dry vacuum pump (behind manifold) (c) and carboy (d) to pump the sample water through filters that are secured at the bottom of the filter funnels and catch DNA and sediment larger than 1.2 µm.

Field sampling

To prepare for field sampling, amber glass bottles, that were purchased for this project, were sterilized by rinsing with 10% bleach solution, shaking them and pouring out the solution. This was repeated three or four times per bottle. After bleaching bottles were thoroughly rinsed with distilled water.

Water samples were collected from six rivers (Rivers 1-4, 9, and 10) in central to northern New Brunswick, Canada between October 3 and 17, 2017. Fall sampling was chosen because as ambient temperatures decrease the turtles will spend more time in and around the warmer water before hibernation, increasing detection probability. Samples were also collected between October 2 and 16, 2018 from four rivers sampled the previous year (Rivers 3, 4, 9, and 10) as well as eight others (Rivers 5-8, 11-14) in central and northeastern New Brunswick. Six of these rivers (Rivers 9-14) have known wood turtle populations, two are not expected to have populations (Rivers 1 and 2) and for the remaining six rivers it is not known if wood turtles are present (Rivers 3-8). Rivers and sampling locations were chosen by Maureen Toner and Hubert Askanas, from the forest planning and stewardship branch of the New Brunswick government and Deanna McCullum, a biologist with the 5th Canadian Division Support Base Gagetown. The status of the sampled rivers is based on surveys conducted by these agencies. Wood turtles are assumed absent from Rivers 1 and 2 because there are no records of occurrence and wood turtles are not known to reside at such high elevations. There are no records of wood turtles at the unknown sites, but they are at altitudes and habitats suitable for wood turtles. We were able to locate a wood turtle that had been tagged with an external VHF (very high frequency) transmitter (Holohil Systems Ltd., Carp, ON,

Canada) during late spring 2017 in River 9 and take samples downstream to use as a positive to optimize the assay.

Each river had two separate sampling sites, one above head of tide and the other at suitable wood turtle habitat farther upstream except River 4 where the two sites were not on the same branch of the river, resulting in no upstream or downstream site. In 2018 we could only sample River 4 at one site due to access restrictions that weren't in place in 2017. We were unable to find a suitable new sampling location in time for the 2018 sampling season. The two sites within each river were approximately 2-34 km apart, except for River 12, where the sites were less than 100 m apart due to limited access to the water. Above head of tide was chosen as a sampling location so that I was able to sample as far downstream as possible to try to maximize the chance of detecting DNA as all water and DNA from the river would flow past this point without having the influence of the tide, which could cause disturbance of sediment and resuspend old DNA or cause DNA to be brought upstream. Sites selected for sampling were suitable wood turtle habitat: (1) areas in a clear stream with sandy or rocky bottom with sand/gravel areas that can be used for nesting, (2) surrounded by grassy fields, meadows, or pastures for basking, and (3) a forested area (Compton, Rhymer, & McCollough, 2002). Exact sampling site locations will not be provided in this document as recommended by COSEWIC to protect the threatened wood turtles against illegal collection and trade. Locations can be obtained from the New Brunswick Museum.

In 2017 three 1 L water samples were taken at each sampling site, resulting in a 1 L and a 2 L replicate (Figure 2). This was increased to four 1 L water samples (for two 2 L biological replicates; Figure 2) in 2018 after the 2017 results revealed that DNA could

not successfully be isolated when 1 L of water was pumped through the filter. All bottles were labelled with river, site and sample number on-site at the time of sampling. The first sample at each site was taken furthest downstream and all subsequent samples were taken upstream of previous samples to avoid disturbed sediment in the samples. A new pair of gloves was used for each sample. These water samples were taken by submerging a sterilized amber glass bottle halfway into the water column until full. Thus, we took samples between approximately four and forty centimeters below the surface depending on the depth of each river. A control bottle of ultra purified water (Milli-Q) was opened and closed at each sample station. In 2017 there was one 1 L control bottle per site, which was increased to two 1 L bottles in 2018 to keep the amount of water per filter consistent across the samples. Once samples are taken, each bottle was placed in a cooler containing ice packs to remain cool and dark, to slow down the decay of eDNA. All samples were filtered within 24 hours of collection in the lab at the University of New Brunswick Saint John.

Sample filtration

Following the protocol by Lacoursière-Roussel et al. (2016), samples were filtered through a 1.2 µm glass microfiber filter (grade GF/C, Whatman International GE Healthcare, Maidstone, United Kingdom) using a dry vacuum pump (Welch, Mount Prospect, IL, USA, model 2014) and 500 mL Sentino™ Magnetic Filter Funnels (Pall® Life Sciences, Ann Arbor, MI, USA) placed on a 3-place filter funnel manifold (Pall® Life Sciences, Ann Arbor, MI, USA) that allowed three samples to be filtered simultaneously (Figure 1). Samples from 2017 were either filtered as 1 L or 2 L samples

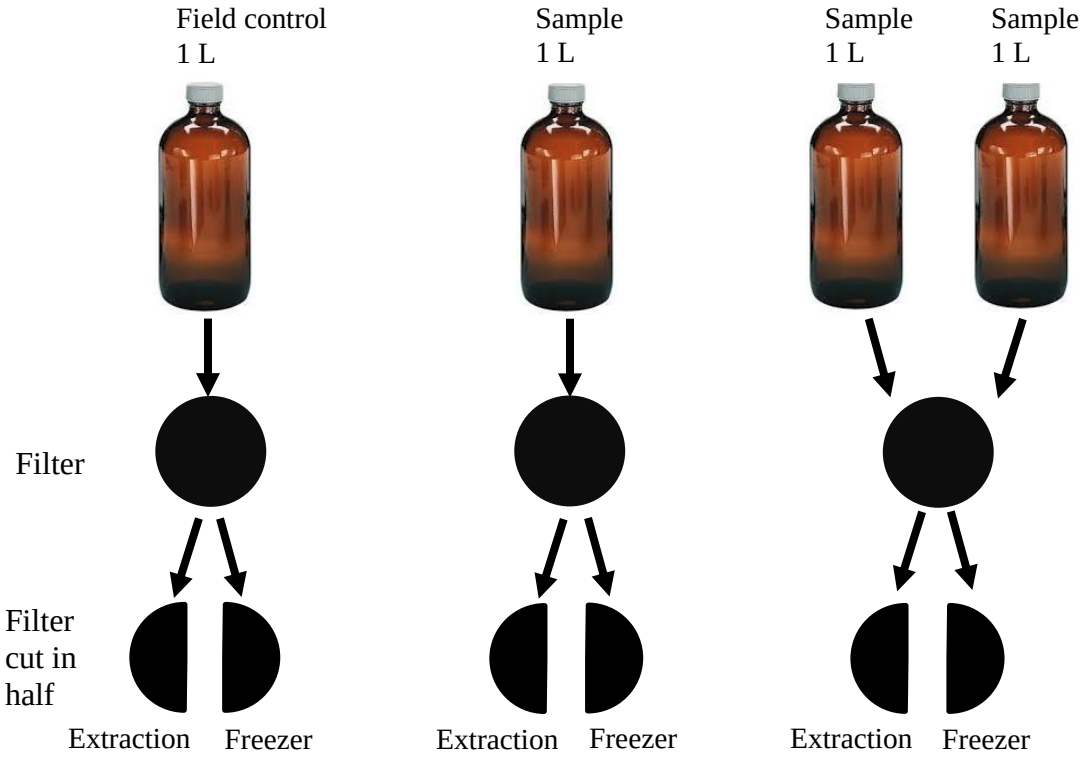
(Figure 2). To increase the chance of detecting DNA, 2 L of water (two sample bottles) were filtered through each filter in 2018 (Figure 2), unless the filter clogged, which was dependent on sample turbidity. For samples that clogged after only 1 L, a second filter was used for the second litre. If at least 1.5 L filtered before the filter was clogged only one filter was used. This was done for 2 L samples from both years. Sites were most often filtered in the order of collection, with samples from the same site being filtered at the same time to ensure samples were filtered within 24 h of collection. Filtration equipment was sterilized between each sample using 10% bleach solution and rinsed with distilled water. Filters were folded and placed in 1.7 mL microcentrifuge tubes and frozen at -80° C until extractions could be completed.

DNA extraction

DNA from 2 L filters was extracted with the use of DNeasy Blood & Tissue Kit and QIAshredder (Qiagen, Inc., Hilden, Germany) using the method from Lacoursière-Roussel et al. (2016). DNA was extracted from half a filter for all samples (Figure 2). To isolate DNA, 450 µL of ATL buffer and 50 µL of Proteinase K were added to each filter-containing tube. Samples were vortexed (mixed to create a homogeneous mixture) three times, with 20 to 30 minutes separating each 15 s vortex, incubated overnight at 56 °C, and then vortexed once or twice more. Samples were then centrifuged (to push all tube contents towards the bottom) at 13 000 rpm for about 15 s to remove any condensation from the lid to allow for a cleaner opening of the tubes. Filters were transferred to QIAshredder columns and centrifuged at 13 000 rpm for 1 min. The lysis buffer was then transferred to the QIAshredder column containing the corresponding filter and spun again

at 13 000 rpm for 1 min. The filtrate was then equally aliquoted to two new microcentrifuge tubes where 400 µL of AL buffer was added to each. Tubes were then vortexed and incubated at 70 °C for 10 min. After incubation, tubes were again centrifuged at 13 000 rpm for about 15 s and 400 µL of 95% ethanol was added. Once vortexed, the mixture was transferred to a DNeasy Mini spin column and centrifuged at 13 000 rpm for 1 min. Since the solution volume in the microcentrifuge tubes was greater than the capacity of the spin columns, this was done in three steps with the flow-through discarded after each. This step causes any DNA to bind with the spin column membrane as impurities pass through and are discarded. Once this was complete, the spin column was placed in a new 2 mL collection tube. The spin column filter was then washed with 500 µL of AW1 buffer to help remove remaining contaminants and centrifuged at 13 000 rpm for 1 min. The flow-through and collection tube were both discarded. The DNeasy Mini spin column was placed in a new collection tube, 500 µL of AW2 wash buffer was added and the tubes were centrifuged at 13 000 rpm for 1 min, flow through was discarded and then columns were spun at 13 000 rpm for another two minutes to dry the membrane. The flow-through and collection tubes were discarded, and the spin columns were transferred to new microcentrifuge tubes for elution. Twenty microlitres of ultra - pure water was pipetted into each column and left to incubate at room temperature for five minutes to allow the DNA to unbind from the spin column membrane before centrifuging at 13 000 rpm for 1 min. This step was repeated before eluted DNA was placed in the freezer to remain until amplification.

2017: 1 site



2018: 1 site

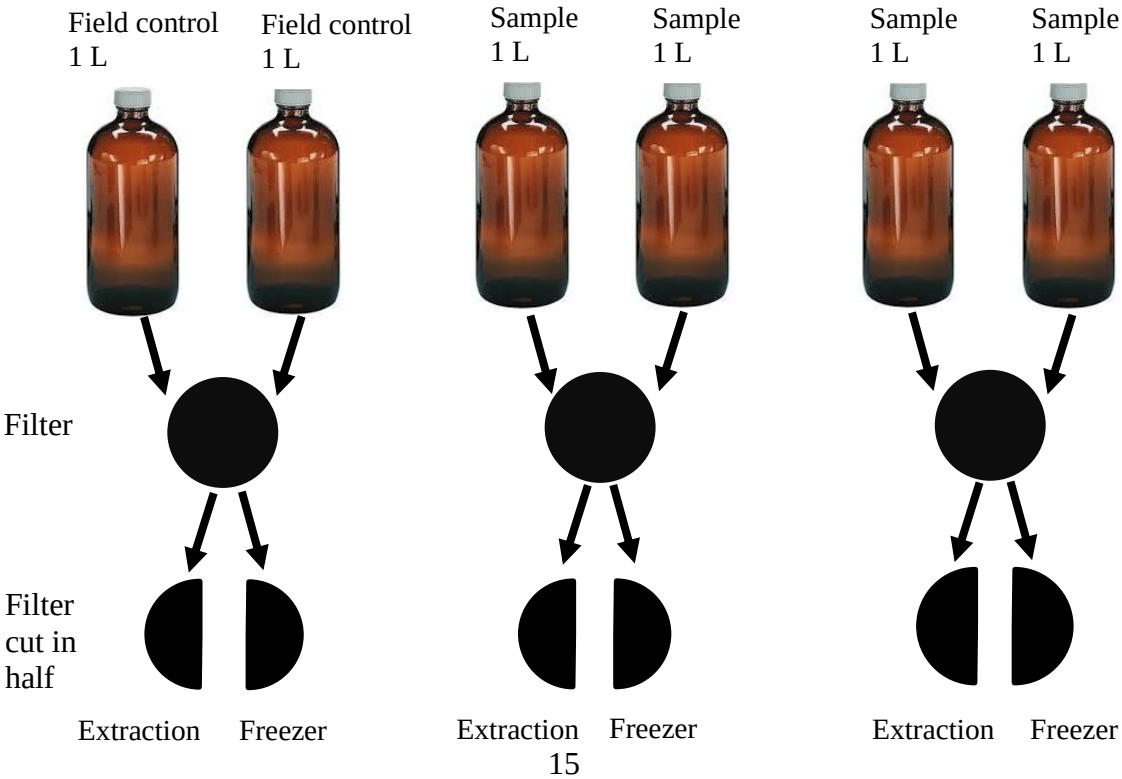


Figure 2. The amount of water pumped through a filter per site from 2017 and 2018 sampling. The amount was increased to 2 L for all replicates in 2018 once it was determined DNA could not be isolated from 1 L replicates. In both years, extractions were only conducted on half of the filter with the other half returning to the freezer to serve as a backup if needed.

DNA amplification

Eluted DNA, focusing on the samples taken downstream of the turtle located with the VHF transmitter, was initially amplified with PCR to optimize the assay. The optimized assay was a 20 µL reaction volume which contained 1.8 µL of each primer (10 µmol/L; Table 1), 4 µL of DNA, 2.4 µL of ultra-pure water and 10 µL of Applied Biosystems™ TaqMan™ Environmental Master Mix 2.0. This preparation (as well as qPCR prep) was conducted in a MY-PCR Prep Workstation. Equipment and surfaces were cleaned with RNase Away and 70% ethanol before being exposed to an hour of UV light prior to PCR and qPCR set-up. Thermocycler conditions (Agilent SureCycler 8800 (Aligent Technologies, Santa Clara, CA, USA)) of 95° C for 10 min, 40 cycles of 95° C for 15 s, 60° C for 1 min and 72° C for 1 min, and followed by a final elongation step of 72° C for 8 min were carried out. The results were visualized on a 1% agarose gel, stained with GelRed (Biotium, Inc., Fremont, CA, USA) run at 90 volts for 40 min and visualized on an ultraviolet light platform (ChemiDoc™ XRS+, Bio-Rad Laboratories, Inc., Hercules, CA, USA) to check amplification success. Once DNA was able to be consistently isolated using PCR, qPCR amplification could take place.

Isolated DNA was replicated with quantitative polymerase chain reaction (qPCR) using previously published wood turtle specific primers and probe (Lacoursière-Roussel et al., 2016) designed to target a specific section of the COI gene (Table 1). The primers direct replication of the target region similar to all other PCR reactions. Then, with the TaqMan method used in our study, a third oligo (probe) binds to a third region within the amplified sequence and fluoresces. After each qPCR cycle a camera measures the fluorescence to quantify the product. The cycle where the fluorescence is detected and

has crossed the threshold is the quantification cycle (Cq) and is directly related to the amount of DNA present before the reaction, as lower Cq values indicate more DNA. Since this method quantifies the amount of DNA in a sample, we can determine the relative quantity of different sampling sites and infer that some have higher amounts of wood turtle eDNA than others. qPCR reactions were carried out in 20 µL reaction volume containing 1.8 µL of each primer (10 µmol/L), 0.5 µL probe (10 µmol/L), 5.9 µL DNA, and 10 µL of TaqMan™ Environmental Master Mix 2.0 using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and Hard-Shell® PCR Plates (Bio-Rad Laboratories, Inc., Hercules, CA, USA, Cat. no. HSP9601). The amplification was conducted under the following conditions: 10 min at 95° C, followed by 70 cycles of 15 s at 95° C and 60 s at 60° C. qPCR protocol was modified from Lacoursière-Roussel et al. (2016) by increasing the amount of DNA, reducing the amount of water, and removing the initial warm up of 2 min at 50° C. Each sample was run in duplicate (for two technical replicates per sample) which resulted in 2017 sample sites having qPCR duplicate results but 2018 sites having quadruplicates as each site had two samples run in duplicate. A site was classified as being positive for wood turtle DNA if any qPCR replicates successfully amplified its DNA and if all the negative controls (field, extraction, and no template) were free of any amplification.

Table 1. Forward and reverse primer sequences and probe sequence for quantitative PCR amplification of the targeted 71 base pairs (bp) of the mitochondrial COI gene of wood turtle (*Glyptemys insculpta*).

Forward primer (5'-> 3')	TGCCTCTGTAGACCTAACCATCTTT
Reverse primer (5' -> 3')	AGTTGATAGCCCCTAAGATTGAAGATA
Probe (5' -> 3')	CTCTACACCTGGCCGGT

Experimental Controls

Three sets of controls were utilized throughout this experiment: field, extraction and PCR /qPCR. The field controls were composed of bottles of Milli-Q water that were brought to the sampling sites to ensure bottle handling, transportation and filtering of the samples was done in a clean way to eliminate contamination between the samples. Extraction controls were run alongside samples as though they contained DNA to guarantee equipment and reagents were free of DNA. No template controls (ultra-pure water is used in place of DNA) were run with the samples during PCR and qPCR to ensure no cross-contamination occurred during setup and should not fluoresce as there is no DNA being amplified.

Results

When attempting to amplify wood turtle DNA from the 2017 filters that only had 1 L of water pumped through it, it was found that regardless of PCR conditions or amount of template DNA used, the target DNA could not be isolated. Visualizing the amplified DNA with gel electrophoresis, there were always multiple larger products present in the samples, not just wood turtle eDNA. This was not the case with filters that

had 2 L of sample water filtered through it. Because of this, all filters in 2018 had 2 L of water pumped through them, or had water pumped through until the filter clogged due to the turbidity of the water at sampling sites. No samples from 2017 required an extra filter but in 2018 one site on Rivers 13 and 14 and both sites on Rivers 7 and 12 required extra filters. Early on while working to try to optimize the 1 L samples, contamination in the PCR negatives was occurring. I ran no template controls by themselves and discovered that the contamination appeared to be coming from the primers. After ordering a new set, there was no longer any contamination in PCR negatives. Once PCR optimization had successfully amplified and isolated wood turtle DNA, I could move on to amplifying the target DNA with qPCR.

As expected, qPCR was more sensitive than PCR when it came to amplifying target DNA. In 2017 PCR and qPCR both showed positive amplifications for River 9 upstream site and River 10 downstream site, with PCR also amplifying larger (non-target) products in samples for the downstream sites of Rivers 2, 3, and 9 that did not appear positive in qPCR results. Between the two amplification methods all the known wood turtle presence rivers sampled in 2018 (Rivers 9-14) were deemed to be positive for wood turtle DNA. There was only one river, River 13, in which qPCR did not amplify the target DNA when PCR did. The downstream site on River 13 was not positive for either method, but the upstream site was considered positive for wood turtle DNA from PCR. qPCR curves for all the wood turtle positive sites are displayed in Figure 3, with Cq values for each site in Table 2.

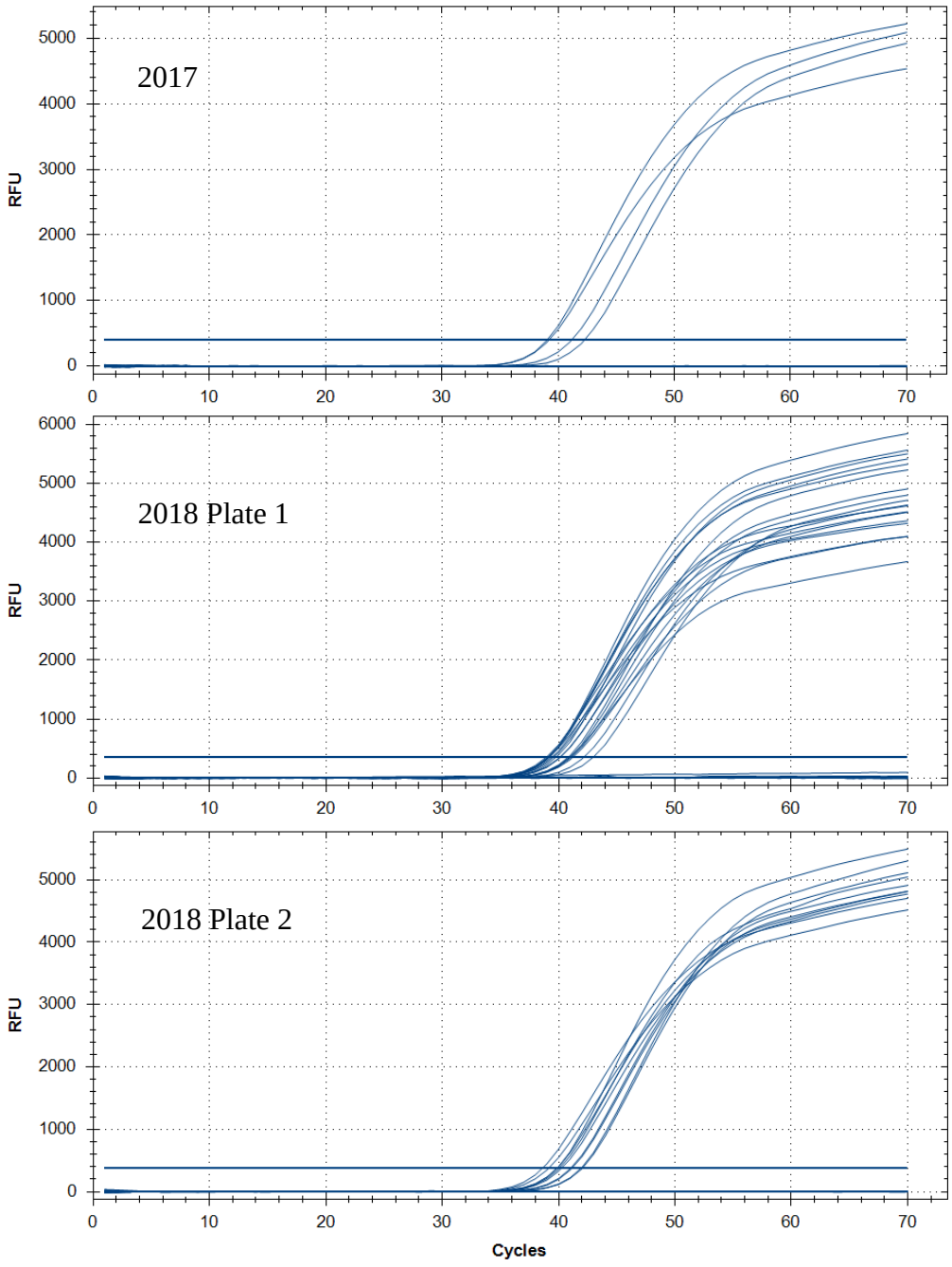


Figure 3. qPCR results for eDNA samples taken in 2017 (top) and 2018 (middle and bottom) from fourteen rivers in New Brunswick, Canada. All samples were run in duplicate. All samples that crossed the fluorescence threshold (horizontal line at 400 RFU: relative fluorescence units) were deemed positive for containing *Glyptemys insculpta* eDNA. Top to bottom: 2017- River 9 upstream

(U/S), River 10 downstream (D/S), River 10 D/S, River 9 U/S. 2018 (middle)- River 11 U/S, River 11 D/S, River 11 D/S, River 11 U/S, River 11 U/S, River 10 D/S, River 9 U/S, River 11 D/S, River 10 U/S, River 9 D/S, River 12 D/S, River 12 U/S control, River 9 D/S, River 12 D/S, River 12 D/S, River 9 D/S, River 12 U/S control, River 11 U/S. 2018 (bottom)- River 14 U/S, River 14 U/S, River 14 U/S, River 14 D/S, River 14 D/S, River 14 D/S, River 12 U/S, River 12 U/S, River 12 U/S, River 12 U/S. Note: River names and sampling locations are available from New Brunswick Museum.

Amplification success was found to differ between the two years as well as among sites. The downstream site of River 9 and the upstream site of River 10 produced positive result during the 2018 sampling after not amplifying wood turtle DNA in 2017 (Table 3). It was also found that not all sites' replicates amplified equally. A few sites, like River 9 upstream site and both River 10 sites (all in 2018), only had one of their four technical (qPCR) replicates amplify wood turtle eDNA, whereas all four technical (qPCR) replicates from River 11's upstream site were positive for wood turtle eDNA (Table 3).

There were no positive amplifications in either 2017 or 2018 for the rivers in which wood turtle presence was unknown or the rivers they were known to be absent (Rivers 1 and 2 were not sampled in 2018). All the extraction controls as well as field controls (except for one) were negative for wood turtle DNA. The field control for the upstream site on River 12 had positive amplification of the target DNA. The other half of the filter was extracted alone to try to pinpoint when contamination occurred. Since it still positively amplified wood turtle DNA, contamination either happened in the field or during filtration, causing questions about the validity of the four positive qPCR replicates from the upstream River 12 site samples and if they were contaminated as well.

Table 2. Quantification cycle (Cq) values for the five known presence rivers that successfully amplified *Glyptemys insculpta* eDNA using quantitative polymerase chain reaction (qPCR). 2017 had two qPCR replicates per site, whereas 2018 had four per site. No value indicates that *G. insculpta* eDNA was not detected in the qPCR replicate. N/A = river site was not sampled during that year.

Sample Site	2017	2018
River 9 Stream Upstream	39.05, 39.20	40.13
River 9 Stream Downstream		41.06, 41.20, 42.14
River 10 Upstream		42.95
River 10 Downstream	41.15, 42.26	40.79
River 11 Upstream	N/A	39.04, 39.10, 39.18, 40.95
River 11 Downstream	N/A	39.18, 39.73, 40.94,
River 12 Downstream	N/A	39.38, 39.52, 40.09
River 14 Upstream	N/A	39.31, 40.07, 41.96
River 14 Downstream	N/A	40.09, 41.07, 41.16

Table 3. *Glyptemys insculpta* environmental DNA (eDNA) detection results for the fourteen rivers sampled in 2017 and 2018. eDNA detection is reported as the number of positive amplifications observed from quantitative polymerase chain reaction (qPCR) site replicates. Not all rivers or sites were sampled both years which is denoted with "N/A". Samples from both years were run in duplicate, however 2017 only had one sample per site where 2018 had two samples per site.

Prior Status	Sample Site		2017	2018	
Known absence	River 1	Upstream	0/2	N/A	N/A
		Downstream	0/2	N/A	N/A
	River 2	Upstream	0/2	N/A	N/A
		Downstream	0/2	N/A	N/A
Unknown presence	River 3	Upstream	0/2	0/2	0/2
		Downstream	0/2	0/2	0/2
	River 4	Site 1	0/2	N/A	N/A
		Site 2	0/2	0/2	0/2
	River 5	Upstream	N/A	0/2	0/2
		Downstream	N/A	0/2	0/2
	River 6	Upstream	N/A	0/2	0/2
		Downstream	N/A	0/2	0/2
	River 7	Upstream	N/A	0/2	0/2
		Downstream	N/A	0/2	0/2
	River 8	Upstream	N/A	0/2	0/2
		Downstream	N/A	0/2	0/2
Known presence	River 9	Upstream	2/2	1/2	0/2
		Downstream	0/2	2/2	1/2
	River 10	Upstream	0/2	1/2	0/2
		Downstream	2/2	1/2	0/2
	River 11	Upstream	N/A	2/2	2/2
		Downstream	N/A	2/2	1/2
	River 12	Upstream	N/A	2/2*	2/2*
		Downstream	N/A	2/2	1/2
	River 13	Upstream	N/A	0/2	0/2
		Downstream	N/A	0/2	0/2
	River 14	Upstream	N/A	2/2	1/2
		Downstream	N/A	2/2	1/2

Note: * - Control also provided a positive result.

Discussion

In this study, water samples were collected from fourteen rivers in New Brunswick, Canada, brought back to the lab, filtered, eDNA extracted and amplified using PCR and qPCR. Two litre samples were required to positively detect wood turtle eDNA in five of the six known presence rivers; eDNA was not detected in rivers where the turtle was known to be absent or where presence/absence wasn't known. This study has shown that wood turtle DNA can be detected in water samples obtained from rivers within New Brunswick, Canada, the first study to demonstrate this.

Increase from 1 to 2 L of water per sample filter increased the chance of detecting wood turtle DNA from the watercourses. Our study is consistent with recent eDNA filtration research showing that 1 L of water is sufficient for lentic systems (ponds and lakes) whereas lotic systems (rivers and streams) require 2 L of water (Feist et al., 2018; Lacoursière-Roussel et al., 2016). Rivers and streams need more intensive sampling than lakes or ponds because (1) they are shallower and thus experience greater UV degradation (2) flowing water has increased mixing thus diluting eDNA faster and (3) DNA is flushed more quickly from the system (Thomsen, Kielgast, Iversen, Wiuf, et al., 2012).

Another reason why the increase in sample volume could have helped with detection is that reptile DNA has been more difficult to detect in the environment than other groups, such as fish and amphibians (Feist et al., 2018; Lacoursière-Roussel et al., 2016; Raemy & Ursenbacher, 2018). Along with shedding skin cells, urine and feces, fish and amphibians also have an outer mucus layer and emit gametes and eggs in the water, all of which contain the organism's DNA (Lacoursière-Roussel et al., 2016). Aquatic

reptiles have scales instead of skin cells, no mucus layer, and they produce less urine and feces than fish and amphibians, resulting in a reduced DNA shedding rate, and therefore are more difficult to detect (Akre et al., 2019; Raemy & Ursenbacher, 2018). The wood turtles may have been excreting even less waste at the time of sampling than they would earlier in the summer as they were getting ready for hibernation and may have stopped eating (Ernst & Lovich, 2009). Lacoursière-Roussel et al. (2016) found that amphibian eDNA is easier to detect than reptile eDNA, however they were able to detect wood turtles in all the Québec, Canada rivers in which they observed wood turtles during their abundance surveys in a few months prior. This resulted in 80% of the sampled sites being positive for wood turtle eDNA (Lacoursière-Roussel et al., 2016). Akre et al. (2019) were able to positively detect wood turtle eDNA at 76% of the northern Virginia, USA river sites where visual surveys verified their presence plus three sites where visual surveys did not, whereas Feist et al. (2018) were only able to detect alligator snapping turtle (*Macrochelys temminckii*) at half of their river sampling sites. The results of the present study are consistent with the results of these studies that also collected two litre samples from lotic environments. I was able to positively detect wood turtle eDNA with qPCR at 50% of the sampling sites in known wood turtle inhabited rivers in 2017 and at 75% of the sites in 2018 in known presence rivers.

Quantitative polymerase chain reaction has become the gold standard for species-specific environmental DNA studies. The combination of a primer set with a probe allows for a specific sequence to be amplified and detected with increased confidence. Studies have shown that qPCR has a lower limit of detection than conventional PCR (Xia et al., 2018), making it more sensitive to low amounts of DNA as well as sequence

mismatches (Lacoursière-Roussel et al., 2016; Wilcox et al., 2013). This sensitivity leads to higher detection rates for low DNA samples, such as environmental DNA samples of low density species (Xia et al., 2018). This increased sensitivity is present even without the use of a probe (Xia et al., 2018). The results of qPCR can potentially be used to semiquantitatively determine abundance, biomass and/or density of the target organism at each site with more positive qPCR replicates per site indicating a greater abundance of your target species compared to other sites. The Cq values of sampling sites can also be used to semiquantitatively determine abundance as lower Cq values means there is more DNA in the sample, allowing it to be detected above the background sooner than a sample with less DNA. As wood turtle and other reptile DNA occurs in smaller amounts in the water than other aquatic species this method may not be possible. The best course of action to determine relative abundance may be to determine presence/absence over many sites in a watercourse. As this study only investigated whether eDNA could be used to detect wood turtles in New Brunswick rivers, only presence/absence was used. Future work of comparing abundance from visual surveys and qPCR results would be required to determine if semiquantitative abundance can be achieved in this area.

With qPCR, wood turtle eDNA was found in five of the six watercourses which are known to have wood turtles though amplification success was not equal among all the sites. The River 9 upstream site and River 10 downstream site in 2017 and River 11 upstream site in 2018 amplified DNA in all replicates (biological and technical; Table 3), while other sites only amplified DNA in one technical replicate of one biological replicate (River 9 upstream and both River 10 sites in 2018; Table 3). Lack of amplification of some sites may be due to the low amounts of DNA in the water,

degradation of the present DNA, and/or the dilution of DNA in the watercourses. The differences could also be because of varying turtle density among sites. Lacoursière-Roussel et al. (2016) found that wood turtle abundance was positively correlated with the number of positive qPCR amplifications for each of their sampled rivers.

There was also found to be a difference in amplification success between years. This could be for the same reasons as site differences or, at least for River 9, it could be because in 2017 River 9 was lower than 2018 causing the stream bed to not be completely inundated in some areas. If wood turtles are typically found around the upstream site or further upstream, the low water flow could prevent DNA from flowing downstream to the other site as it would settle to the bottom more quickly (Jane et al., 2015; Wilcox et al., 2016; Wipfli, Richardson, & Naiman, 2007). This could be why no DNA was amplified from the River 9 downstream site samples in 2017 but 75% of the technical replicates from 2018 were positive for the presence of wood turtle eDNA. Few studies have investigated the distance that eDNA can travel in a watercourse before it is no longer able to be detected (Deiner & Altermatt, 2014; Jane et al., 2015; Pilliod et al., 2014). Results have shown that DNA can travel less than 50 m from amphibians (Pilliod et al., 2014) to nearly 12 km from invertebrates (Deiner & Altermatt, 2014) and from sampling it was found that alligator snapping turtle eDNA can be detected 30 m downstream of an individual (Feist et al., 2018). Feist et al. (2018) were not investigating eDNA travel distance in their study, just reporting sampling distance from turtle cages. In the present study, each river's sampling sites are no more than approximately 14 km apart, except sites in Rivers 1 and 2 where the distance is approximately 34 km. Since River 9 sites were close to 12 km apart, the upper limit of detection for invertebrate

eDNA detection and much farther than the 30-50 m detection for reptiles and amphibians, we feel it is unlikely that eDNA from the upstream site would persist long enough in the water column to travel to the downstream site. This goes for all the watercourses, except maybe River 12, where the sites were less than 100 m apart.

In 2018 Rivers 7, 8, 13, and 14 were sampled after a period of heavy rain that raised the water levels as much as 60 cm (M. Marshall personal comm., October 16, 2018). The increase in water increased the flow as well as turbidity. The increase in flow has been found to lower the amount of eDNA detected near the source (Jane et al., 2015) as well as downstream (Akre et al., 2019), which could have resulted in the eDNA being below the limit of detection. The turbidity and amount of organic matter (fallen leaves) in the rivers also may have prevented wood turtle eDNA from being replicated during qPCR. This could be why no wood turtle eDNA was detected in samples taken from the River 13, a known presence river.

The field control from the River 12's upstream site amplified wood turtle DNA in all of its qPCR replicates. No wood turtle DNA was found in the extraction controls that were extracted at the same time as these samples, indicating cross contamination at this stage is unlikely. I also extracted the other half of the River 12 upstream site field control filter by itself and once again the extraction controls were negative for wood turtle DNA, but the field control was positive. This means that contamination occurred in the field or while filtering. Filtration contamination could have occurred because the filter funnel and possibly the filter funnel manifold were not cleaned well enough after the previous sample, though they were cleaned the same after every sample. It also could have occurred if there was any splash when pouring the samples into the filter funnel, however

I poured all samples slowly and carefully to try to eliminate this risk and did not notice it happen. The last possibility is that even though I set up the bottles to match the funnel they were to be poured into I mistakenly poured water from a sample bottle into the funnel that was for the control water. Out of the 35 field controls filtered over the two sampling seasons, this was the only one that amplified wood turtle eDNA.

Using environmental DNA to detect wood turtles has now been effective in three field studies (the present study, Akre et al., 2019; Lacoursière-Roussel et al., 2016). These studies have been conducted in rivers at different locations within the species' range (Virginia, USA, Akre et al., 2019; Québec, Canada, Lacoursière-Roussel et al., 2016; and New Brunswick Canada, present study). In addition to collecting eDNA samples, the previous studies also conducted visual surveys to determine abundance at their sites. This additional information was used to determine a relationship between the number of positive qPCR replicates and abundance (Lacoursière-Roussel et al., 2016) and create a model to determine which method had a higher probability of detection (Akre et al., 2019). Lacoursière-Roussel et al. (2016) found a relationship between abundance and the number of positive qPCR replicates per site. Akre et al. (2019) determined that visual surveys had a higher probability of detection but the increase to four filter replicates per site had the same level of detection certainty as conducting two visual surveys per site.

There are a few concerns with solely using eDNA for monitoring of wood turtles. Since aquatic reptiles shed less DNA than other aquatic species, this can potentially lead to false negatives at sampling sites as the amount of present eDNA could be lower than the detection limit. Increasing the number of qPCR replicates run of each sample will

increase the chance of detection. Sampling in the Chickasawhay River, Mississippi required six technical qPCR replicates to get one positive detection of an alligator snapping turtle located 30 m upstream in a trap, compared to triplicates (technical replicates) at another site (Feist et al., 2018). As mentioned, studies examining the distance eDNA can travel and still be detected has found that eDNA from vertebrates does not travel very far (less than 50 m for amphibians to at least 240 m for fish) (Jane et al., 2015; Pilliod et al., 2014). Because of this limited travel distance, sampling should be conducted at suitable habitats of the target species, as well as coordinating sampling time with when the target species is active (de Souza, Godwin, Renshaw, & Larson, 2016). There are many species, including the wood turtle, that do not spend all their time in one environment. During the summer wood turtles are more terrestrial, lowering the probability of detection from water samples but in the spring and fall the turtles spend more of their time in the water increasing the probability of detection. This is the reason why this study was conducted in the fall and that sampling, especially of upstream sites, was performed at wood turtle suitable habitats (clear stream with sand/gravel nesting areas surrounded by fields and forest). Environmental particles in the water can cause PCR inhibition and lead to false negatives (Jane et al., 2015; Raemy & Ursenbacher, 2018). To mitigate any PCR inhibition, I used TaqMan™ Environmental Master Mix 2.0 which is designed to help detect your target DNA in the presence of inhibitors.

False positives (i.e. detecting target species DNA when it isn't there) are also possible with eDNA studies. They can be the result of contamination, either occurring in the field or laboratory with cross contamination among samples or work done previously with the target species' DNA in the lab. This can typically be mitigated with clean

sampling, handling and laboratory practices. Species can also be wrongly detected due to predators transporting the DNA as a carcass or in their feces. It can also be from boats, or other equipment transferring DNA from another location. Both false positives and negatives can negatively impact the monitoring of a species. Since I used experimental controls at every step, I am fairly certain that I did not experience false positives from contamination (other than potentially the upstream River 12 site). False negatives could have occurred for any of the unknown rivers as well as River 13 as this river is known to have wood turtles but none of the qPCR replicates had a positive detection. This could be because the amount of eDNA at the sites was below the limit of detection.

Species monitoring includes estimating the population of the target species to determine whether it is increasing or decreasing. There have been a few studies that have identified a relationship between the amount of target eDNA and abundance, biomass, or density of the target species (Lacoursière-Roussel et al., 2016; Mahon et al., 2013; Pilliod et al., 2013; Takahara, Minamoto, Yamanaka, Doi, & Kawabata, 2012; Thomsen, Kielgast, Iversen, Wiuf, et al., 2012). The majority of these studies were conducted on fish (Mahon et al., 2013; Takahara et al., 2012) and amphibians (Pilliod et al., 2013; Thomsen, Kielgast, Iversen, Wiuf, et al., 2012). Lacoursière-Roussel et al. (2016) found a correlation between relative wood turtle abundance and the number of positive qPCR amplifications for each river sampled. However, Raemy and Ursenbacher (2018) were not able to find a correlation between the estimated number of European pond turtles (*Emys orbicularis*) at each of their sample locations and the concentration of DNA. Since eDNA detection and persistence depends on stream environment (flow rate, UV radiation, temperature, inhibitors, etc.) and varies seasonally findings from previous

studies cannot be transferred to other studies unless all the conditions are the same (Goldberg, Pilliod, Arkle, & Waits, 2011; Rees et al., 2014). Since I did not conduct visual observation surveys to find abundance at my sampling sites, I am unable to use the qPCR results to find a relationship between the two that could be used in the future. The eDNA method also cannot distinguish between DNA that has been shed by a live animal or one that is deceased. If using mitochondrial DNA to identify the species, detection of hybrid species cannot be detected as mitochondrial DNA is inherited only from the mother. There has been a report of a female wood turtle mating and producing offspring with a male Blanding's turtle in captivity (Harding & Davis, 1999).

Using eDNA to determine abundance, density, and/or biomass of your target species in an area assumes that all individuals, regardless of size and age class, have the same shedding rate. However different age classes may shed DNA at different rates and spend different amounts of time in the water. There could also be differences in shedding rates among individuals of the same life stage. Pilliod et al. (2014) examined the rate of eDNA production of five Idaho giant salamanders (*Dicamptodon aterriums*) and found that one produced 4.4 times as much eDNA as the others. Strickler et al. (2015) found the starting eDNA concentration of two bullfrog (*Lithobates catesbeianus*) tadpoles in mesocosms ranged from 0.06 to 5.83 ng DNA per 250 mL. These individual differences can potentially cause an over- or under-estimation of the population/abundance/density/biomass of the target species when trying to find a correlation to qPCR results.

This study has shown that wood turtle DNA can be detected in water samples collected from New Brunswick, Canada rivers. The success rate of detection using qPCR

was similar to the two other eDNA studies on wood turtles and with other reptile eDNA studies, with 50-75% of sites on known present rivers having positive eDNA amplification with qPCR. This method can be used to determine a more complete species distribution within the province by sampling more sites along rivers that have habitat suitable for wood turtles. Previous studies have found that using eDNA to detect wood turtles has similar results to traditional visual surveys, but is faster and can be as little as half the cost (Akre et al., 2019; Lacoursière-Roussel et al., 2016). Traditional visual surveys also tend to be limited to one month in the spring whereas eDNA samples can be taken during at least three months of the year. As the eDNA method advances, determining abundance from samples may become reliable but until then the presence/absence data will allow conservation and management agencies to focus survey resources to areas where there has been positive wood turtle detection. Future work should further investigate the effects of flow on eDNA transport distances, especially with aquatic reptile species, as there are currently no studies on how far reptile DNA can travel in lotic environments. This knowledge will help determine maximum distance between sampling sites for more robust sampling to help determine a more accurate distribution of the species.

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