The use of environmental DNA (eDNA) techniques for conservation of Mapleleaf Mussel (*Quadrula quadrula*) in southern Manitoba

by

Isabelle Yakmission

A thesis submitted to the Department of Biological Sciences, University of Manitoba, in partial fulfilment of the requirements for the course

BIOL 4100 (Honours Thesis)

for the degree of

Bachelor of Science (Honours)

©April, 2026

Abstract

The Mapleleaf Mussel (*Quadrula quadrula*) is a Threatened freshwater mussel species in Manitoba with a fragmented distribution that is strongly associated with its primary fish host, the Channel Catfish (*Ictalurus punctatus*). Understanding the distribution of this mussel species is important for its effective conservation and management, as it is predominantly threatened by pollution from urban, industrial, and agricultural environments, as well as impacts from invasive zebra mussels (*Dreissena polymorpha*). In this study, environmental DNA (eDNA) sampling was used to assess the distributional range of the Mapleleaf Mussel by comparing known range distributions from previous traditional (capture-based) sampling with inferred mussel presence using eDNA techniques, as well as investigating new (range extension) sites using eDNA. Field sampling, DNA extractions, and quantitative polymerase chain reaction (qPCR) analysis were used for 56 sampling sites selected based on suitable habitat. There were 21 revisited sites and 35 previously unsampled range extension sites. A previously published probe-based eDNA assay targeting a 99-bp fragment of the *COI* gene from the mussel's mitochondrial DNA, developed and tested in the Ontario portion of the species' range, was used for qPCR analysis to detect the Mapleleaf Mussel in Manitoba. The results identified six potential range extension (i.e., previously undocumented) sites in the Qu'Appelle River, Black River, Berens River, Manigotagan River, Winnipeg River, and Pigeon River, suggesting that the Mapleleaf Mussel may occupy a larger distribution in Manitoba than previously recognized. There were 13/21 positive detections at the revisited sites where the species had been previously reported, but the remaining sample sites (37/56) were non-detections, with four concerning non-detections within the species' reported range. Although follow-up sampling is required, this study shows that the

use of eDNA can provide a valuable non-invasive technique for detecting the Mapleleaf Mussel and may support future monitoring efforts aimed at reassessing its conservation in this region.

Acknowledgements

For the completion of this project, I would formally like to express my gratitude to my supervisor, Dr. Margaret Docker, for her support, and patience with providing continuous feedback and guidance throughout this project. This was truly an unforgettable experience to have as an undergraduate student. I am also grateful for the aid of my committee members, Dr. Cale Gushulak and Dr. Joy Stacey, for their support and feedback.

Within the lab setting, I would like to thank research technician Jessie Ogden for fieldwork equipment, DNA extraction, and qPCR training, as these were valuable techniques to learn and aim to master through this Honours experience. I would also like to acknowledge the many conversations, help with protocols, and curation of this project through her support. As well, my lab mate Lauren Teller was invaluably helpful through the fieldwork sampling collection process and lab support. Without her, processing a sizeable part of the DNA extractions of the samples from each field location would not have been achievable.

Many thanks to the Manitoba Métis Federation, as they conducted some of the field sampling and provided funding and supplies for this project. As well as Douglas Watkinson from the Department of Fisheries and Oceans, for providing ideas of potential range extension site locations based on the mussels' preferred habitat.

This research would not be possible without the proper funding and time dedicated to the training I received on this project. I am grateful to all who have contributed; it was a remarkably interesting, highly informative, and very influential experience. Thank you very much.

Table of Contents

Abstract i
Acknowledgements iii
Table of Contents iv
List of Figures v
1. Introduction 1
2. Materials and Methods 9
2.1 Field sampling 10
2.2 Smith-Root eDNA Citizen Scientist Sampler 12
2.3 "DIY" Peristaltic Pump eDNA Sampler 13
2.4 eDNA Extractions 14
2.5 qPCR Amplification 16
3. Results 17
3.1 qPCR Site Detections 17
3.2 Detection Frequency of Known Range Sites vs Range Extension Sites 19
4. Discussion 20
4.1 Reasons for Non-detections 22
4.2 Comparison between Historical Physical Sampling vs eDNA Techniques 25
5. Conclusion 28
References 29
Appendices 36

List of Figures

Figure 1. Distribution of the invasive zebra mussel presence in Manitoba as of 2021 (Manitoba Government 2023). 3
Figure 2. Overview of the lifecycle of a unionid freshwater mussel, undergoing adult reproduction, fertilization, and the development of the glochidia larval phase. The glochidia become an obligate parasite on the fish host (Channel Catfish) until juvenile settlement, concluding with adult growth and reproduction (Livingstone 1995). 5
Figure 3. Map of the 56 field site locations sampled throughout southern Manitoba in summer 2025. Sampling sites in green are the 21 "known" range sampling sites (i.e., where there are historical records from capture-based sampling), and sampling sites marked in blue are the 35 newly sampled (potential range extension) sites. This map was created in ArcGIS Online. 11
Figure 4. The Smith-Root eDNA Citizen Scientist Sampler used by Lauren Teller at sampling site CC-A, Cooks Creek, summer 2025 (photo: Isabelle Yakmission). 12
Figure 5. The DIY peristaltic pump in use at Whitemud River (site code: WMDR-A), summer 2025. A) using a cordless drill to power the pump in the field. B) filter paper inside the filter apparatus; the filter paper is then folded (DNA side in) for preservation on-site (photo: Isabelle Yakmission). 14
Figure 6. Analysis of the qPCR detections of the 56 sampled sites in southern Manitoba (summer 2025) from eDNA of the Mapleleaf Mussel. Circle size indicates the proportion of qPCR detections in the nine replicates (with nine being the largest and zero being the smallest, indicating a non-detection). Green points indicate the 21 revisited known (historical) range sites, and blue shows the 35 new (potential range extension) sites. Sites outlined in red were non-detections. Significant sites are labelled with their site code (see Table S1 for the remaining sites' information). This map was created in ArcGIS Online. 18
Figure 7. Detection frequency of the Mapleleaf Mussel at revisited (known/historical range) and potential range extension (new) sites based on qPCR analysis of the 56 sites from eDNA sampling (2025). Detection frequency represents the proportion of sites with at least one positive detection (qPCR replicate 1 > 9) (see Table S1 for qPCR replicate values). Bars represent the proportions of detections from 0 to 1 (0 = non-detection, 1 = detection) within each site type category. 19

1. Introduction

Species decline and biodiversity loss are recurring issues across global ecosystems, driven by anthropogenic disturbances (Butchart et al. 2010). Freshwater environments are particularly vulnerable to habitat degradation, pollution, and the introduction of invasive species, which impact native species, their trophic interactions, and ecosystem stability (Ardura et al. 2015; Gómez-Márquez 2025; Schlaepfer et al. 2018). Species at risk, such as freshwater mussels (family: Unionidae), are especially sensitive to these environmental changes and may experience population declines or, in extreme cases, extinction as pressures persist (COSEWIC 2016; Fordham 2024; Pip 2000; Vaughn 2018). Monitoring ecosystem health is essential to understand the status of species endangerment, especially species that are acutely sensitive to ecosystem changes, such as freshwater mussels, as they contribute to water filtration and nutrient cycling, making them influential bioindicators of ecosystem health (Vaughn 2018). Fortunately, there are effective techniques that are useful to support conservation efforts for freshwater mussels.

This study was focused on the Mapleleaf Mussel (*Quadrula quadrula*), a species of conservation concern in Manitoba, using environmental DNA (eDNA) to assess range distribution. The Mapleleaf Mussel is an ecologically important filter feeder that contributes to the purification of freshwater systems and modifies nutrient availability by consuming phytoplankton, protozoa, and bacteria (COSEWIC 2016; Vaugh 2018). However, this feeding strategy also makes the species vulnerable to environmental contamination, as pollutants such as heavy metals from industrial sources can accumulate in the mussel's tissue (Pip 1995). In addition, pollution of water systems from agricultural runoff within Manitoba may be a point of concern, as eutrophication events in nearby waterways may increase physiological stress on the

mussel due to reduced water quality and greater energetic demands for survival (COSEWIC 2016; Pip 1995). Other threats to the mussel include predation by various fish, mammal, and bird species, as well as parasitism by helminths and mites, although the mussel is not a main prey item for any predator (COSEWIC 2016). Furthermore, invasive zebra mussels (*Dreissena polymorpha*) represent a significant threat to unionid populations as they attach to the shells of native mussels in large numbers, impacting feeding and respiration. This may cause Mapleleaf Mussel mortality through starvation or suffocation and has even been known to extirpate local populations in Ontario (COSEWIC 2016; Manitoba Government 2023; Morris et al. 2020; Reid et al. 2014; VanTassel et al. 2020). In Manitoba, the zebra mussel was detected in 2013 within Lake Winnipeg and has rapidly spread throughout Manitoba's waterbodies, especially the Red River, and Lake Winnipeg tributaries overlapping with the Mapleleaf Mussel's distribution (Figure 1) (Gingera et al. 2017; Manitoba Government 2023). Zebra mussels reproduce at a rapid rate, may spawn once or twice per year, and may produce 1 million eggs or more per spawning season (Borcherding 1991; Manitoba Government 2023). The explosive growth of their populations will intensify their impact and threat to the Mapleleaf Mussel and other freshwater unionid species in Manitoba over time, potentially causing widespread mortality.

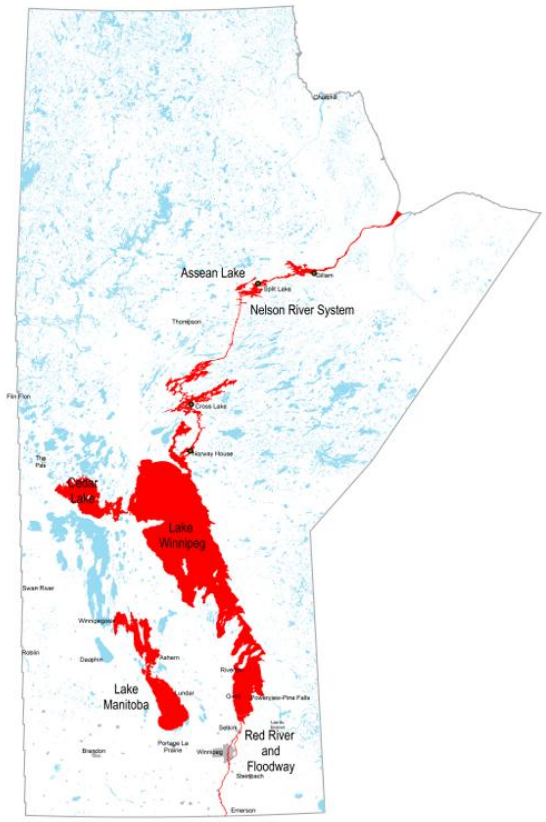


Figure 1. Distribution of the invasive zebra mussel presence in Manitoba as of 2021 (Manitoba Government 2023).

In Manitoba, the Saskatchewan - Nelson River population of Mapleleaf Mussel represents the geographic focus of this study, with the expected range primarily within the Lake Winnipeg (and tributaries), the Assiniboine River, and Red River drainages (COSEWIC 2016; Van Tassel et al. 2020). The mussel's status was assessed as Endangered as of 2006 by the Committee on the Status of Wildlife in Canada (COSEWIC). For the most recent COSEWIC assessment, increased sampling efforts were prioritized from 2006 to 2015, and the species status was reassessed to Threatened because its distribution was found to be broader than previously recognized (COSEWIC 2016). The distribution of the Mapleleaf Mussel is strongly influenced by its primary fish host, the Channel Catfish (*Ictalurus punctatus*), in its larval phase (glochidia). The mussel's reproduction occurs typically from late spring to early summer and may be influenced by water temperature, although the specific environmental triggers remain uncertain (COSEWIC 2016; Clarke 1981). Female mussels release aggregates of larval glochidia into the water column, which become an obligate parasite that attaches to the Channel Catfish's gills, where they remain temporarily before detaching and settling into the substrate below as juvenile mussels (Figure 2) (COSEWIC 2016; Hoffman et al. 2018). This host-mediated dispersal facilitates transportation, thus resulting in broader distribution of Mapleleaf Mussel populations that overlap with the Channel Catfish range of the Red, Winnipeg, and Assiniboine Rivers (Mathias et al. 2018; Stewart and Watkinson 2014; VanTassel et al. 2020). As sessile adults, Mapleleaf Mussels inhabit benthic environments within the sediments of turbid freshwater, preferring the substrate of gravel or sand. This species can live for approximately 22 years and relies on stable habitat conditions to survive and reproduce (COSEWIC 2016; COSSARO 2017; Hoffman et al. 2017). However, alterations to river systems, particularly dam construction, can disrupt host fish movement and reduce the dispersal of freshwater mussels (Butler and Wahl

2011; Hoffman et al. 2018). The monitoring of range distribution is necessary to understand the status of this mussel.

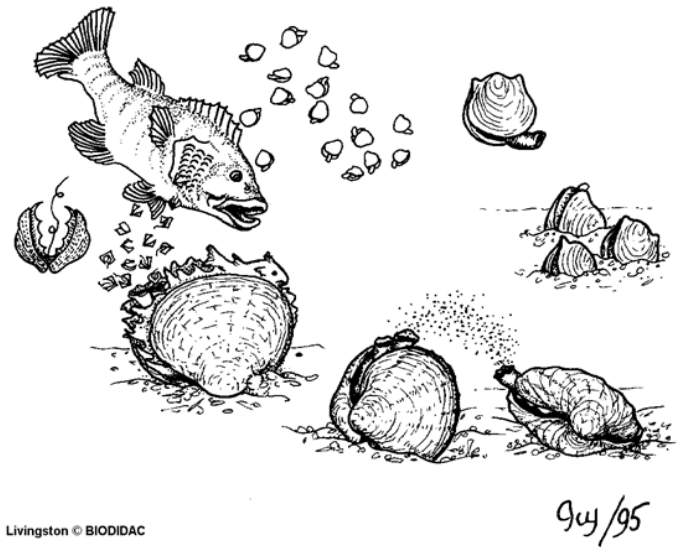


Figure 2. Overview of the lifecycle of a unionid freshwater mussel, undergoing adult reproduction, fertilization, and the development of the glochidia larval phase. The glochidia become an obligate parasite on the fish host (Channel Catfish) until juvenile settlement, concluding with adult growth and reproduction (Livingstone 1995).

eDNA techniques provide a non-invasive approach for detecting and monitoring species of conservation concerns distributional patterns without the need for physical capture or handling (Bernos et al. 2023; Biggs et al. 2015; Sigsgaard et al. 2015; Thomsen and Willerslev 2015). eDNA refers to genetic material that organisms shed into their surroundings through sources such as skin cells, mucus, blood, waste, gametes, or other biological material. These traces of DNA can be collected through sampling of water, air, or sediments, extracted, and analyzed by quantitative polymerase chain reaction (qPCR) using species-specific primers (i.e., that target unique genetic regions of the organism of interest) to detect a species of interest from their DNA

in the environment (Schmelzle and Kinzinger 2016; Taberlet et al. 2012). An organism's presence can typically be detected in lentic (e.g., lakes) or lotic (e.g., rivers and creeks) systems for approximately 1–8 weeks, indicating that the DNA within the sampling site must be from a recent target organism or it may have been carried downstream before the sampling occurred (Pilliod et al. 2014; Thomsen et al. 2012). Compared with traditional survey methods, eDNA monitoring is non-invasive, more cost-effective, reduces the risk of identification error during field sampling, and is easier to sample hard-to-access locations (Beng and Corlett 2020; Currier et al. 2018; Deiner et al. 2017; Wang et al. 2019).

eDNA methods are particularly useful for detecting freshwater mussels such as the Mapleleaf Mussel, which can be difficult to locate using traditional sampling methods. Physical sampling of mussels can be completed by quantitative excavation or qualitative visualization (Reid et al. 2014). The physical surveying often requires manual (tactile) searching or diving (snorkelling) and may be limited in deeper or turbid waterbodies, as visibility is poor (Dolson and Canada Department of Fisheries and Oceans 2023; Reid 2016). Sampling takes ample time as the mussels may be hard to locate. As a result, past sampling efforts have often been restricted to shallow shoreline habitats, potentially underestimating the true distribution of the species, and spots that are too deep or dangerous to sampled are avoided (COSEWIC 2016; Thomsen and Willerslev 2015; Reid 2016). As eDNA can detect genetic material from organisms present at low densities, it has become an effective tool for identifying rare or elusive species and refining knowledge of their distributions, especially in deeper water (Barnes and Turner 2016; Currier et al. 2018; Davison et al. 2017; Jo and Minamoto 2021; Saito and Doi 2021; Wilcox et al. 2013). As the Mapleleaf Mussel is elusive and listed as Threatened, in Manitoba, eDNA techniques are

the best method to detect the mussel in deep or turbid bodies of water and possible range extension sites.

Despite the benefits of eDNA techniques, there are some limitations that are important to discuss. eDNA can degrade rapidly in aquatic ecosystems due to varying abiotic factors, including water turbulence, high water temperature, desiccation, storms, or UV radiation (Deiner and Altermatt 2014; Gingera et al. 2017; Mauvisseau et al. 2022; Saito and Doi 2021). In lotic systems, such as rivers, eDNA may also be transported downstream, potentially affecting the interpretation of species presence (Deiner and Altermatt 2014). Additionally, knowledge of the target species' habitat preferences, distribution, and life history characteristics is important when selecting specific sampling locations and the proper timing to enhance detection success. Environmental samples often contain DNA from many organisms, making the use of species-specific assays essential for accurate detection. Although eDNA techniques are extremely useful, they cannot provide information regarding abundance, biomass, sex, age of maturity, or population health. However, eDNA is still a productive tool to use for detecting species of conservation concern and can help with implementing conservation management plans by identifying the range the species occupies.

To detect a target species appropriately, a species-specific eDNA assay must be created to confirm the proper detection of the target species in the sampling location. In this study, the assay was developed by Currier et al. (2018), who designed species-specific primers and probes targeting a 99 base-pair fragment of the mitochondrial cytochrome oxidase I subunit (*COI* gene) for the Mapleleaf Mussel in the biogeographic zone of the Great Lakes – Upper St. Lawrence population of Ontario. The *COI* gene region was selected because the interspecific genetic variation among unionid mussels allows reliable discrimination between species (Currier et al.

2018). The Mapleleaf Mussel population in the Saskatchewan-Nelson Rivers biogeographic zone is the same species as the Great Lakes – Upper St. Lawrence population in Ontario but is genetically differentiated by six microsatellite loci (VanTassel et al. 2020). These two populations have their own designable units (DU) established through COSEWIC and Canada's Species at Risk Act (SARA) due to their genetic variations and habituated biogeographic zones (COSEWIC 2016; Green 2005). As identified by VanTassel et al. (2020), there are significant genetic differences between the Mapleleaf Mussel populations in Manitoba and Ontario. *In vitro* validation using tissue-derived DNA from the La Salle River in Manitoba was used to test this assay in Manitoba, which was successful for detecting the Mapleleaf Mussel in the Saskatchewan - Nelson River population. Currier et al. (2018) created multiple primers, and probe sets to target the interspecific nucleotide polymorphisms of four target species to be able to amplify short DNA fragments (between 99 and 122 base pairs) for improved detection success during eDNA sampling, as eDNA is easily degraded by environmental factors. The assay specificity was determined through *in vitro* and *in silico* testing to ensure that primers did not amplify the DNA from non-target unionid species. Cross-species amplification tests were conducted using DNA from multiple sympatric mussel species to confirm target specificity. Primer and probe sensitivity was also determined by a dilution series of standardized DNA concentrations, demonstrating the assay was able to amplify very low concentrations of the target species' DNA, as low as 10 DNA copies per reaction, and occasional detections at concentrations as low as a single DNA copy. These validation steps indicate that the assay is sensitive and species-specific to detect the Mapleleaf Mussel from eDNA sampling. The importance of having a species-specific primer-probe assay design is to avoid the risk of potential false positives or false negatives, and to efficiently detect the target species (Wilcox et al. 2013). False negatives

occur when the assay fails to detect the species if it is not abundant, and false positives refer to the amplification of the DNA of another species that is not the target (Ficetola et al. 2015).

This study will improve knowledge of the distributional patterns of the Mapleleaf Mussel to be able to contribute to future COSEWIC status reassessment and SARA listing of this mussel to aid in potential conservation management plans. This research aimed to determine whether the species occurred at new locations beyond previously documented sites to identify potential range extension. Many of the sampling locations have not been surveyed previously but were identified as suitable habitat for the Mapleleaf Mussel based on expert recommendations from Doug Watkinson from Fisheries and Oceans Canada (DFO). The objective of my study was to evaluate the distribution of the Mapleleaf Mussel in southern Manitoba using eDNA sampling and to compare detections with historical records from traditional physical sampling. I hypothesize that the Mapleleaf Mussel will be detected at sites where it has historically been detected and at some previously undocumented (range extension) sites. From this information, my prediction is that there is a wider range distribution of the Mapleleaf Mussel within southern Manitoba that may be detectable with increased sampling efforts using eDNA techniques.

2. Materials and Methods

This study entailed field sampling, DNA extractions, and qPCR amplification to test for the presence of Mapleleaf Mussel eDNA at previously reported sites and possible range extension sites. Field sampling for eDNA collection occurred in October 2024 by the Manitoba Métis Federation (MMF) and Jessie Ogden, and from May to July of 2025 by Lauren Teller, me,

and MMF. DNA extractions occurred in September – October, and qPCR amplification was completed in November – December 2025.

2.1 Field sampling

The fieldwork occurred by filtering water samples on-site from rivers, creeks, and lakes from 56 sites throughout southern Manitoba in the summer of 2025, with six revisited sites from 2024 (Figure 3) (see Table S1 and Table S2 for full site information). We conducted *in situ* validation at historically known range sites (21/56) from physical sampling (e.g., Red, Brokenhead, Roseau, and Assiniboine Rivers) (Table S3). New sampling "range extension" sites (35/56) were also investigated for the detection of the Mapleleaf Mussel (Table S4). Two water sampling methods were used: the Smith-Root eDNA Citizen Scientist Sampler (product number: 12099; Smith-Root Inc., Vancouver, WA, USA) and a "DIY" peristaltic pump system (see Sections 2.2 and 2.3 for each sampling technique's differing description). DNase and RNase decontamination was strictly enforced between sites; ELIMINase® (product number: 0435531; Decon™ Laboratories, Inc.), a DNase and RNase decontaminant, was used to cleanse the surface of tools and wipe down the containment bins. Nitrile gloves (new at each site) were always worn to avoid potential contamination. For each sampling method in the field, there was one negative control (to test for contamination) and three replicates from each sampling site. When sampling, the negative control was filtered before the triplicate, flushing approximately 1 L of distilled water brought from the lab. After filtering each replicate, filter papers were replaced and stored appropriately to obtain three distinct replicates. Filtering occurred until the pores of the filter paper were full of debris, and water could no longer be filtered (averaging 1 L of water filtered). General information documented at each site consisted of location, latitude, longitude, air

temperature (C°), weather, and volume of water filtered (L). Additionally, turbidity (ppm), conductivity (µS/cm), and water temperature (C°) were measured by a digital water tester. Samples were kept on ice, transported back to the lab, and stored at –20 °C prior to DNA extraction.

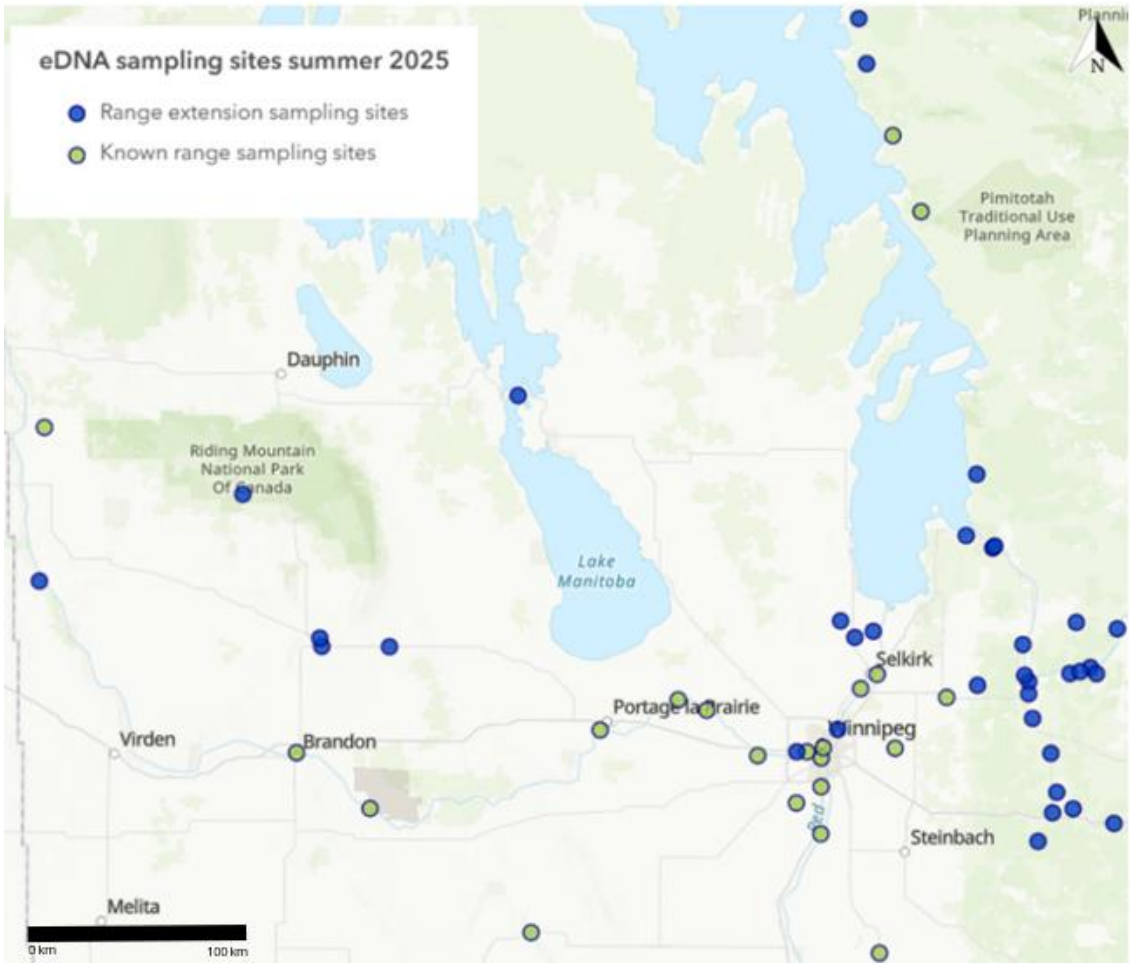


Figure 3. Map of the 56 field site locations sampled throughout southern Manitoba in summer 2025. Sampling sites in green are the 21 "known" range sampling sites (i.e., where there are historical records from capture-based sampling), and sampling sites marked in blue are the 35 newly sampled (potential range extension) sites. This map was created in ArcGIS Online.

2.2 Smith-Root eDNA Citizen Scientist Sampler

The eDNA sampling was conducted with the Smith-Root Sampler in October 2024 and May 2025. It functions by a vacuum filtration pump drawing up water into the tubing system directly after immersion in the water body and passes water through the attached filter capsule, catching eDNA on the filter paper (Figure 4). Throughout sampling, the filter was encased in a plastic self-preserving housing capsule that aids in desiccation of the filter paper and protects it from contamination.

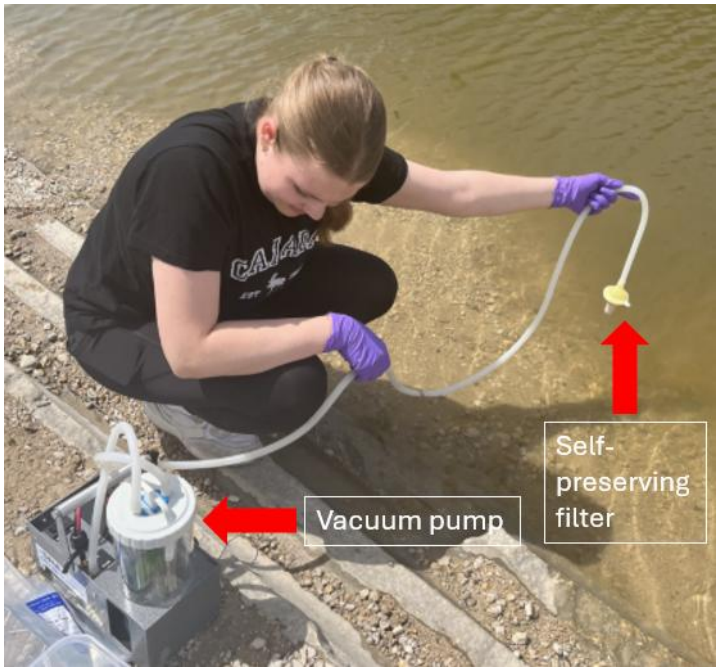


Figure 4. The Smith-Root eDNA Citizen Scientist Sampler used by Lauren Teller at sampling site CC-A, Cooks Creek, summer 2025 (photo: Isabelle Yakmission).

2.3 “DIY” Peristaltic Pump eDNA Sampler

This sampling method was used later in the sampling season during June and July 2025, due to a shortage of appropriate filters for the Smith-Root Sampler. Water was collected in 1 L Nalgene bottles directly from the water body and then sampled on-site using the “DIY” peristaltic pump. eDNA was extracted from the water through peristalsis of the tubing powered by a cordless electric drill pushing the water through the pores of the filter paper (Figure 5). Filter papers with 1.2 μm pore sizes (product number: 1827-047) were manually inserted into the pump head between replicates. Sterile tubing was treated with ELIMINase, ethanol, and UV light prior to use at each site to avoid contamination between sites. Additionally, a flush of clean distilled water, approximately 500 mL, was run through the tubing before the negative control and after all three replicate samples. After filtering, the papers were stored in small coin envelopes and then surrounded in silica gel desiccant contained in a Ziploc to absorb the moisture from the filter papers and aid in preservation.

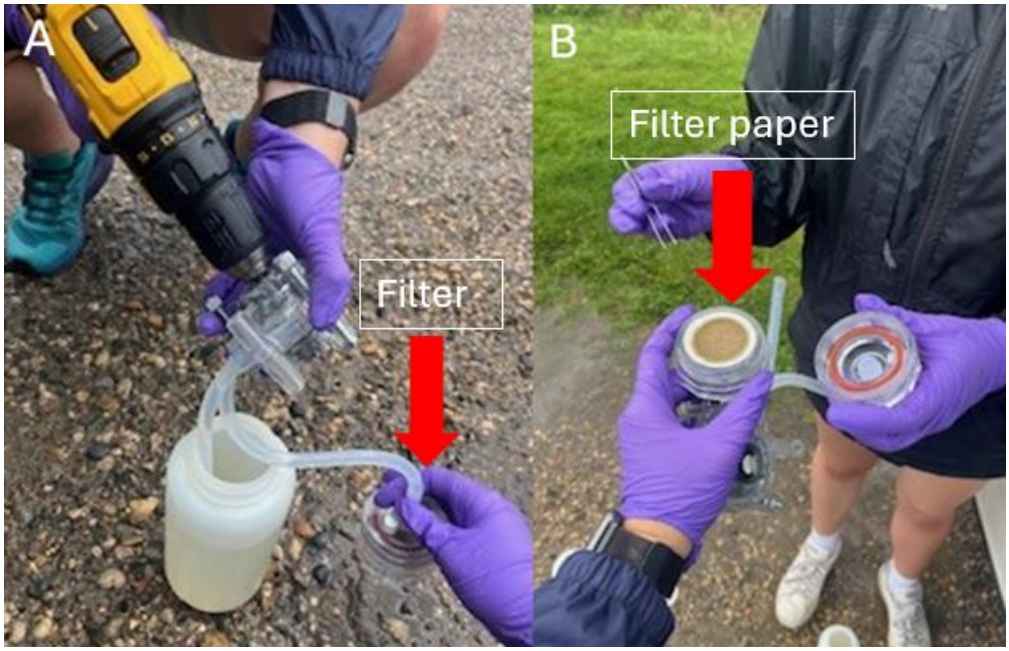


Figure 5. The DIY peristaltic pump in use at Whitemud River (site code: WMDR-A), summer 2025. A) using a cordless drill to power the pump in the field. B) filter paper inside the filter apparatus; the filter paper is then folded (DNA side in) for preservation on-site (photo: Isabelle Yakmission).

2.4 eDNA Extractions

A modified version from the Docker Lab was used for DNA extractions from the filter paper based upon the Qiagen DNeasy Blood and Tissue Extraction Kit (product number: 69504; Qiagen Inc., Valencia, CA, USA). The modifications from the Qiagen manual were used to improve DNA yield from water-filtered samples, as the manual describes in the context of blood and soft tissue pellets. Adequate precautions were taken to improve the DNA yield through wash buffers and inhibitor removal. DNA extractions for 36/56 samples were completed by Lauren Teller in summer 2025. The remaining 20 DNA extractions were processed by me over two-day intervals in September and October (2025). All eDNA extractions were performed in a

designated UV-treated hood (15-minute intervals), and ELIMINase was used to surface sterilize the area.

Each filter paper sample for the three biological replicates (1, 2, 3) was digested by 450 µL of lysis Buffer ATL (product number: 939011) and 50 µL of proteinase K (product number: 25530049) in 1.5 mL plastic tubes. A tube for each field negative control (NC), and a lab negative control (LNC) was allotted per site to observe for contamination from the field and lab settings. The filter papers were folded three times and inserted with sterile forceps into the tubes; the LNC was the only tube without a filter paper for each site. After preparation, the tubes were then vortexed for 15 seconds and placed in the thermomixer at 56°C, and agitated at 350 rpm, and incubated for 18 to 24 hours to allow for proper DNA extraction.

After cell lysis, 6 µL of RNase A (product number: EN0531**)** was pipetted into each tube to remove RNA contaminants. Tubes were spun down in the centrifuge again for 10 seconds at 13,000 rpm and then incubated in the thermomixer for 5 minutes at 56°C with a speed of 350 rpm. QIAshredder spin columns (product number: 79656) were used to isolate the liquid DNA from the filter paper of the NC and replicates (1, 2, 3). The four tubes were centrifuged for two minutes at 11,000 rpm. In new 1.5mL plastic tubes, 400 µL of Buffer AL (product number: 19075) was added to ensure binding of DNA to the DNeasy Mini Spin Columns and 400 µL of 100% ethanol to ensure proper precipitation of the DNA were added to each new tube. The solution (a mix of liquid and precipitate) from the QIAshredders was added to the pre-aliquoted tubes full of Buffer AL and 100% ethanol, and then vortexed for 15 seconds. Mixtures (650 µL) are then pipetted into the DNeasy Mini Spin Columns (product number: 69506) and centrifuged at 8,000 rpm for 1 minute. This step is then repeated following the Qiagen DNeasy Blood and Tissue Extraction Kit protocol. Next steps are followed from the protocol for the wash Buffers

AW1 (product number: 19081) and AW2 (product number: 19072). Following alterations from the protocol include pipetting 105 µL hot Buffer AE (product number: 19077) (approximately 70°C) directly onto the DNeasy Mini Spin Column filters to elute the DNA. The plastic tubes were left incubated at room temperature (15°C to 25°C) for 5 minutes and then centrifuged for 1 minute at 8000 rpm. At this point, the elution of the DNA was complete. For a final step, it was necessary to use the Zymo OneStep PCR Inhibitor Removal Kit (product number: ZD6030; Zymo Research, Irvine, CA, USA) to remove potential contaminants from agricultural runoff. This was completed following the manufacturer's recommendations.

2.5 qPCR Amplification

qPCR was completed using the published probe-based eDNA assay by Currier et al. (2018) targeting a 99-bp fragment of the *COI* gene from the Mapleleaf Mussel's mitochondrial DNA. Three biological replicates were taken per field site, creating a total of nine qPCR replicates. The MMF sites (7/56) were processed by Lauren Teller (summer of 2025). The 49/56 sites were processed by me in November and December (2025). All qPCR analysis was performed in a designated UV-treated hood (15-minute intervals), and ELIMINase was used to surface sterilize the area and equipment.

Every qPCR assay was performed on the StepOnePlus™ Real-time PCR system (product number: 4376592; Thermo Fisher Scientific, Waltham, MA, USA) to amplify the DNA and detect probe fluorescence of the reporter dye VIC. The master mix (MM) was comprised of TaqMan products: 1.75 µL Environmental Master Mix (EMM) 2.0 (product number: 4396838), 7.5 µL of molecular grade water (MGW), 0.3 µL (10 µM) Forward primer (F), 0.3 µL (10 µM)

Reverse primer (R), and 0.15 µL (10 µM) of the minor groove binder (MGB) TaqMan Probe (P). Amounts were made to have enough MM for the 98 well plate. A total of 15 µL was pipetted into each well: 10 µL of the master mix and 5 µL of the extracted template DNA. Wells were allotted to the negative control (NC), the triplicates, and the lab negative control (LNC), resulting in a total of 15 wells per sample site. The positive control used was tissue-derived DNA extracted from the Mapleleaf Mussel. The NTC used MGW instead of DNA to account for pipetting errors or contamination events. The program consisted of an incubation period for 2 minutes at 50°C and then raised to 95°C for 10 minutes for denaturation. The temperature was then lowered down to 60°C for 1 minute during the annealing phase for 40 cycles, each lasting 15 seconds. The cycles lasted for a total of 90 minutes.

3. Results

3.1 qPCR Site Detections

Of the 56 eDNA sampling sites in southern Manitoba sampled and processed through qPCR in the lab, 37/56 sites were non-detections, and 19/56 had different ranges of qPCR detections (i.e., in terms of the proportion of replicates that resulted in detectable amplification of Mapleleaf Mussel DNA: Figure 6). The greatest proportion of qPCR detections occurred at the MMF sample sites: Berens River (site code: BER-A), Pigeon River (site code: PR), Bloodvein River (site code: BVR), Assiniboine River (site code: AR-BR), and the Assiniboine and Souris River confluence (site code: AR-SR). The sampling site on the Red River (site code: RR-LP) had the lowest detection. Many other sites had detections in between (e.g., site codes: BRA-A and QR-A) (see Table S1 for site codes, coordinates, and qPCR detections).

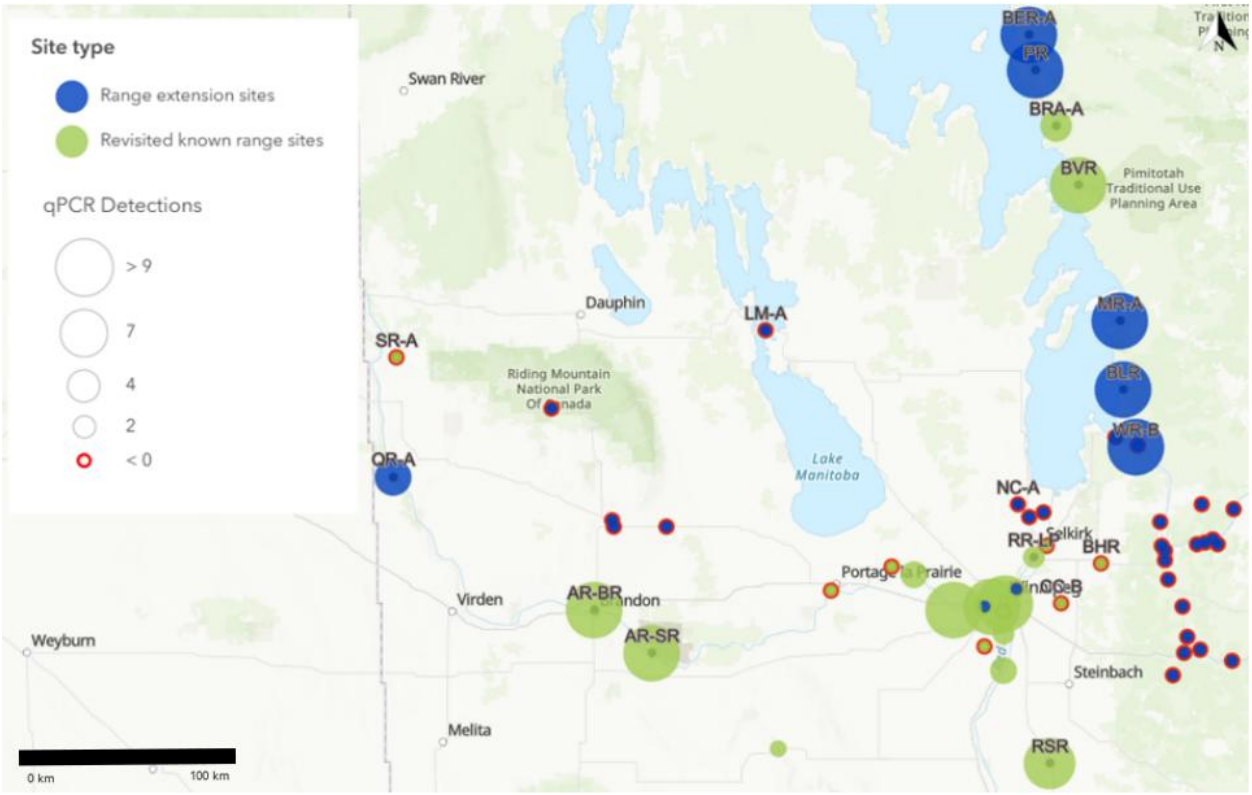


Figure 6. Analysis of the qPCR detections of the 56 sampled sites in southern Manitoba (summer 2025) from eDNA of the Mapleleaf Mussel. Circle size indicates the proportion of qPCR detections in the nine replicates (with nine being the largest and zero being the smallest, indicating a non-detection). Green points indicate the 21 revisited known (historical) range sites, and blue shows the 35 new (potential range extension) sites. Sites outlined in red were non-detections. Significant sites are labelled with their site code (see Table S1 for the remaining sites' information). This map was created in ArcGIS Online.

3.2 Detection Frequency of Known Range Sites vs Range Extension Sites

Sampling known range sites and the potential range extension sites depicts varying detection frequencies (Figure 7). A total of 19/56 sites showed a detection of at least 1 qPCR replicate. Higher detection frequencies occurred in the revisited (known) range sites (13/21 = 0.62) than in the newly sampled range extension sites (6/35 = 0.17) (see Table S5 for calculations).

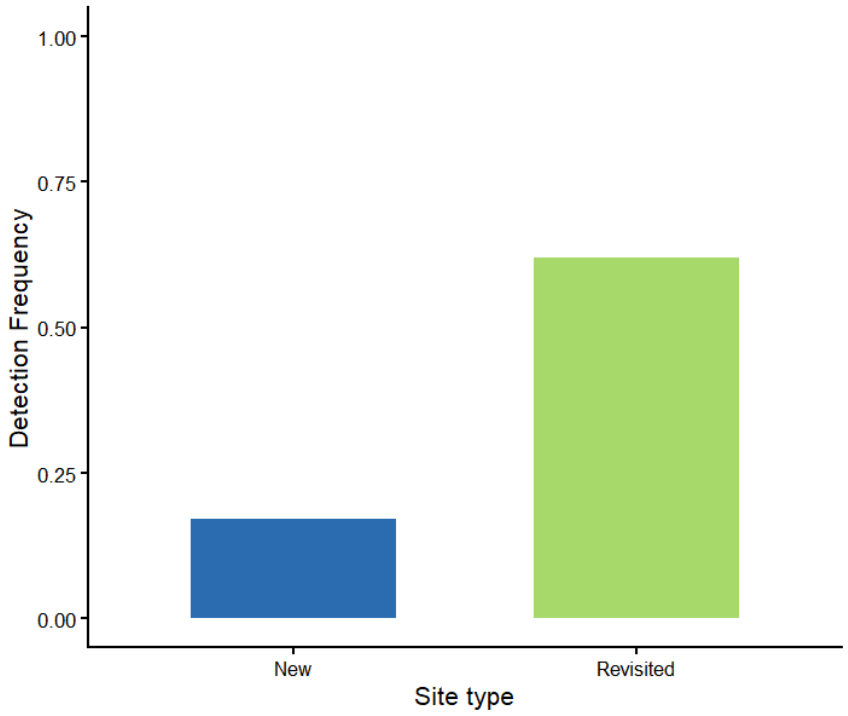


Figure 7. Detection frequency of the Mapleleaf Mussel at revisited (known/historical range) and potential range extension (new) sites based on qPCR analysis of the 56 sites from eDNA sampling (2025). Detection frequency represents the proportion of sites with at least one positive detection (qPCR replicate $1 \geq 9$) (see Table S1 for qPCR replicate values). Bars represent the proportions of detections from 0 to 1 (0 = non-detection, 1 = detection) within each site type category.

4. Discussion

Summer 2025 eDNA sampling detected the Mapleleaf Mussel at 19/56 sites in southern Manitoba (Figure 6). From the analyzed results, there were positive detections in six new range extension sites at Qu'Appelle River (site code: QR-A), Black River (site code: BLR), Berens River (site code: BER-A), Manigotagan River (site code: MR-A), Winnipeg River (site code: WR-B), and Pigeon River (site code: PR), denoting that the Mapleleaf Mussel may occupy a larger range and distribution in southern Manitoba than previously known. In addition, 21 of the sites were revisited based on locations reported from past physical sampling, of these sites 13/21 had detections (e.g., Bradbury River (site code: BRA-A), Bloodvein River (site code: BVR), and Assiniboine River sites), suggesting that the environment still supports this species (Figure 6, Table S1, Table S3). In total, at 37/56 of the sites, we did not detect eDNA of the Mapleleaf Mussel. Four of these sites (Shell River, Cooks Creek, and Brokenhead River (site codes: SR-A, CC-A, CC-B, and BHR)) had unexpected results. When compared to past historical sampling, the mussels were detected in these waterbodies (Table S1) (COSEWIC 2016). All new potential range extension sampling sites were identified to have the preferred habitat to support the mussel, but 30/35 did not have positive detections (e.g., Nutimik Lake (site codes: Nutimik-A, Nutimik-B), Eleanor Lake (site code: Eleanor-A), Whitemud River (site code: WMDR-A), and Whitemouth River (site codes: WM-J, WM-H, etc.)) (Table S1, Table S4, Table S5). Throughout the laboratory analysis, there was no evidence of contamination as the negative controls from the field, lab, and qPCR (NTC) never showed amplification or any other signs of contamination.

The likelihood of having 19/56 of the sites detect the Mapleleaf Mussel throughout the summer 2025 sampling season may be attributed to sampling during the mussel's spawning season in early spring to early summer (Figure 6). This was a significant sampling time that may

have had larger amounts of the mussels' DNA from gametes or glochidia aggregates in the water (COSEWIC 2016). Many of the sites with higher detection frequencies were sites that have previously detected the Mapleleaf Mussel from past physical sampling efforts (Figure 7). From the increased sampling efforts of new range extension sites, there were six newly detected waterbodies that may contain the Mapleleaf Mussel across southern Manitoba. New detection sites, such as Berens River and Pigeon River, had proximity to previously known range sites at Bloodvein River and Bradbury River. The cause of detection is likely that the Mapleleaf Mussel was already present in Berens and Pigeon River, but past physical sampling efforts had not occurred for those waterbodies listed in the COSEWIC (2016) status report and therefore would not possibly have had historical detections. New detections in the Manigotagan River (site code: MR-A), Black River (site code: BLR), and Winnipeg River (site code: WR-B) are near each other but did not have any near known range sites indicting the mussel would have been present from previous sampling. Detections of the mussel in that area do align with the host fish dispersal patterns as the Channel Catfish reaches up to the Bloodvein and Pigeon Rivers, passing through Lake Winnipeg, hence the positive detections at those sites (Stewart and Watkinson 2014). The new detection at the Qu'Appelle River site has no previous detection of sampling sites in its proximity; the closest connection to historical sampling attempts was in Shell River, which did not detect the eDNA of the Mapleleaf Mussel from the sample site this summer, but the mussel has been known to be present there. There is a connection through the Assiniboine River to the Qu'Appelle and Shell Rivers, so this may be the reason for detection at Qu'Appelle, as eDNA can travel distances across lotic systems (Deiner and Altermatt 2014). New detections imply that the range of the Mapleleaf Mussel in Manitoba is larger than previously known, but it is not direct evidence of occurrence. Results from my study denote that when the mussel is next

up for COSEWIC reassessment, it is necessary that further re-sampling of the new positively detected sites this past summer (2025) will need to be revisited and analyzed to fully determine the validity of the Mapleleaf Mussel's detection in these locations of southern Manitoba. Additionally, further range extension sites must be sampled to truly understand the full range distribution the Mapleleaf Mussel occupies in Manitoba.

4.1 Reasons for Non-detections

Overall, there were 37/56 sites that were not determined to detect the Mapleleaf Mussel (Figure 6). This may be due to abiotic factors, or likely the DNA was not detectable because the mussel was not within sampling range or may have been carried downstream (in river systems) (Deiner et al. 2016; Thomsen et al. 2012). The current in the water will not carry the DNA in an even distribution; there is spatial heterogeneity in transport, thus, it is possible to miss the sampling target, or wave turbulence may also have caused destruction to the cells (Deiner et al. 2016; Wood et al. 2021). DNA degradation within the water body could have occurred prior to the time of sampling, as the water may have been too warm for sites sampled in July, which may have caused the DNA cells to lyse (Saito and Doi 2021; Strickler et al. 2015). This may have been a reason why the site in Shell River (site code: SR-A) was not detectable. Additionally, for the unexpected results, samples for the four historically known range sites, because they were non-detections the sites were re-run in the lab to validate if there were any errors throughout the qPCR analysis. However, the results ultimately still had no detections. Extirpation potentially may be a possibility in these locations, as the mussels were last sampled in Shell River, Cooks

Creek, and Brokenhead River prior to 2016; and pollution may have impacted their survivability (COSEWIC 2016).

Alternate reasoning for non-detections may be due to qPCR inhibition during laboratory processing; however, inhibition was not indicated as sites were amplified, and the Zymo OneStep PCR Inhibitor Removal Kit was used suggesting that the non-detections would not have been caused by amplification failure. In general, there could be potential for false negatives as the Mapleleaf Mussel's DNA concentration throughout the water body is quite low and would not be detected in the 40 cycles the qPCR ran for. If there were more cycles and the qPCR was running for a longer period, there may have been detections. The mussel could very well be in a sampled water body that did not have a detection. If sampling occurred at a different time or location along the river system or water body of interest, this could result in a positive detection of the mussel (see Table S1 for sampling months and coordinates). To determine the validity of a detection, resampling all sites must occur to solidify results. As seen in Table S1, some of the amplified values from the qPCR detections had a high cycle threshold (Ct) value, indicating that it took more cycles to amplify the eDNA than a lower Ct value. The Ct value is a relative measure of the initial concentration of the target DNA amplified in the qPCR reaction; lower Ct values indicate higher starting concentrations of the eDNA (Sigsgaard et al. 2015). Likely, if there were lower levels of Mapleleaf Mussel eDNA in the water, there would be a high Ct value because it took longer to amplify the DNA. For example, the site RR-LP had a very high Ct value of 39.241, making it clear that there was such a small amount of DNA that was amplified, and because there were only 40 cycles, the DNA nearly missed the window for amplification. The degraded template DNA requires many qPCR cycles to be able to amplify the target species, hence the potential of nearly having a false negative (Ficetola et al. 2015). These very low

concentrations and Ct values from sampling in water make it difficult to interpret how close the mussel is to the sample within that water body when only sampling a small part of the lake, river, or creek.

Anthropogenic threats that may cause non-detections of the native Manitoba Mussel could be from overlapping distribution with the human-introduced invasive zebra mussel (Manitoba Government 2023). As they may have induced mortality at the Red River sites (e.g., site codes: RR-A, RR-NC), the new range extension site on Lake Manitoba (site code: LM-A), and any sites that have a close connection to Lake Winnipeg (e.g., site codes: NC-A) (see Figure 1 and Figure 6 for overlapping map distribution comparisons and Table S1 for site coordinate information). Additionally, urban and industrial pollution are concerning reasons for non-detections close to the city from wastewater effluent and heavy metals, which may cause mortality to the mussel (Francis et al. 2025; Pip 1995). In general, in rural areas, agricultural run-off may cause eutrophication, altering the Mapleleaf Mussels' food quality, reducing feeding efficiency, and expelling more energy, altering their survivability (Ćmiel et al. 2020; Kelly et al. 2018). As well, within water systems of Manitoba, there are naturally occurring compounds derived from organic matter (e.g., polyphenolics, humic/fulvic acids, tannins, melanin) or sediment particles that can induce qPCR inhibition, which is why the Zymo OneStep PCR Inhibitor Removal Kit was used to help purify the DNA by removing these inhibitors before qPCR analysis.

4.2 Comparison between Historical Physical Sampling vs eDNA Techniques

There are pros and cons to eDNA sampling and traditional physical sampling. With physical surveying methods, human identification error is possible, sampling is difficult in turbid waters, and there is a possibility of causing damage from handling, as the species is at risk. The search effort was done by feel and could not be far from shore or exceed waist-deep water (~1 m), for safety measures, and because it was not feasible to visualize the mussels any deeper within the water body due to turbidity or murkiness (COSEWIC 2016). VanTassel et al. (2020) denoted that in their physical sampling efforts, the Mapleleaf Mussel could not be collected in upstream sampling locations, likely because sampling was too difficult or the species may not have been present at the time. Sampling for mussels in Ontario, Reid (2016), declared a similar sentiment, as sampling was tactile; any deep areas would be too dangerous and not effectively sampled, so they were avoided. In comparison, with the user-friendly nature of eDNA techniques, experts are no longer required to enter the field to collect data samples (Biggs et al. 2015). Individuals can avoid the risk that physical sampling has while also sampling remote areas across distances in lotic systems by using eDNA (as eDNA can travel almost 10 km while still being detected) (Deiner and Altermatt 2014). Therefore, the work of eDNA is optimal because it is sensitive and can yield higher detection rates of the target species, without the obligation to spend a large amount of time and effort capturing them to collect their data. Sampling of deep, turbid, hard-to-access waterbodies is possible and easier using eDNA techniques than traditional physical sampling, and much safer to use if the water body is hard to physically survey. There has been low search effort in sampling the Red River as it is more dangerous to physically sample due to its strong current and turbid waters (COSEWIC 2016). Additionally, Currier et al. (2018) validated that there is no difference between surface and

subsurface sampling using eDNA techniques to target benthic invertebrates (i.e., Mapleleaf Mussel) eDNA, so water sampling for this study occurred on the surface of the water body to detect the mussel.

As the status of the Mapleleaf Mussel was assessed as Threatened and listed on SARA in Manitoba, COSEWIC (2016), it is also a rare and cryptic species, indicating that eDNA sampling is the most influential non-invasive approach to detect the mussel's distributional range patterns and reduces the resources allotted to physical sampling. Jerde et al. (2011) reiterated that it is sometimes impossible to detect a rare species by physical sampling attempts, because the animal is so cryptic that it becomes a waste of effort and resources. Therefore, the work of eDNA is optimal because it is sensitive and can yield higher detection rates of the target species, without the obligation to spend a large amount of time and effort capturing them to collect their data (Sigsgaard et al. 2015). From my study, using eDNA techniques was a valuable tool to locate the presence of the Mapleleaf Mussel in the southern Manitoba region and rivals physical sampling to avoid harm to the species through handling, and detection of the mussel's range extension sites is possible. Although it is important to give credit to traditional surveys as they can be used as a valuable preliminary assessment and knowledge, because without them, we would not have known the range of the Mapleleaf Mussel in Manitoba. The surveys summarized in COSEWIC (2016) were the fundamental basis of information on the distribution of the Mapleleaf Mussel in Manitoba between the 1970s and 2015. eDNA analysis is essential to advance conservation research, as monitoring rare and endangered individuals provides a deeper understanding of the ecosystem dynamics, although ultimately, traditional methods are needed to initially create a research study (Sakata et al. 2021). Comparing revisited sampling site detections using eDNA from summer 2025 analysis exhibits similar positive detections found in all the same

waterbodies except for the concerning non-detections in the four sites: Cooks Creek (site codes: CC-A and CC-B), Brokenhead River (site code: BHR), and Shell River (site code: SR-A), which should have had positive detections.

Some studies demonstrate that eDNA is a better tool to detect rare species, and others believe both eDNA and physical sampling are important. Generally, species of conservation concern are likely located through the analysis of the eDNA sample more effectively than in traditional sampling (Schmelzle and Kinziger 2016). Quadrat sampling to estimate species abundance or capture-based entrapment, such as netting, are plausible examples of the difficulties of quantifying or estimating the presence of the species. On occasion it may be too difficult or dangerous to capture the organism. These traditional methods are helpful, but it is difficult to determine without expertise to predict the right time and area where that organism will appear (Jerde et al. 2011). Even research experts have difficulty finding target species, as most organisms prefer to evade humans. eDNA may partially replace traditional approaches from a genetic standpoint because it is an efficient process for data analysis (Currier et al. 2018). A study on the rare great crested newt (*Triturus cristatus*) in the United Kingdom highlights the limitations of traditional sampling methods. As the species is elusive and occurs at low population densities, detecting individuals through physical capture or observation is time consuming and dependant on extensive knowledge from experts to monitor the rarity of the newt species (Biggs et al. 2015). Therefore, the scientists from this study realized that eDNA offers an acute technique for detecting the genomics of elusive species like the great crested newt and producing reliable data when capturing techniques are not as efficient (Biggs et al. 2015). In contrast, the study by Currier et al. (2018) consisted of comparing eDNA sampling to past quadrat sampling of unionid mussels in the Ontario region and found that the mussels were

detectable in every location using eDNA techniques. It is important in some contexts to use past historical sampling to validate the eDNA techniques to make sure the species is there, especially if the distribution is unclear. Physical sampling may be necessary to ensure the species is present in the sampling area before eDNA techniques follow.

5. Conclusion

As future work continues, further directions for this study include resampling the positive detections sites, sampling of new range sites, and resampling the non-detection sites where the mussel was not detected where it previously has been. This study was a preliminary analysis to understand the distributional range of the Mapleleaf Mussel in Manitoba to aid in a distributional assessment for when the mussel is next up for status reassessment through COSEWIC. Alternate directions to comprehensively understand the threats to the Mapleleaf Mussel would be to study the invasive zebra mussels or anthropogenic factors (pollution into water systems). To truly understand the range of the Mapleleaf Mussel, it would be interesting to increase the sampling effort in more remote areas to see if it is present in more rural or northern regions of Manitoba. The dispersal of the Channel Catfish (fish host) must be considered for future sampling upon reassessment for the Mapleleaf Mussel in Manitoba, as their range should overlap. This distributional information identifies the area of critical habitat that the mussel resides in and must be protected through management plans to conserve the species.

References

Ardura, A., Zaiko, A., Martinez, J.L., Samulioviene, A., Semenova, A., and Garcia-Vazquez, E. 2015. eDNA and specific primers for early detection of invasive species – A case study on the bivalve *Rangia cuneata*, currently spreading in Europe. Marine Environmental Research **112**(1): 48–55. doi:10.1016/j.marenvres.2015.09.013.

Barnes, M.A., and Turner, C.R. 2016. The ecology of environmental DNA and implications for conservation genetics. Conservation Genetics **17**(1): 1–17. doi:10.1007/s10592-015-0775-4.

Beng, K.C., and Corlett, R.T. 2020. Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. Biodiversity and Conservation **29**(7): 2089–2121. doi:10.1007/s10531-020-01980-0.

Bernos, T.A., Yates, M.C., Docker, M.F., Fitzgerald, A., Hanner, R., Heath, D., Imrit, A., Livernois, J., Myler, E., Patel, K., Sharma, S., Young, R., and Mandrak, N.E. 2023. Environmental DNA (eDNA) applications in freshwater fisheries management and conservation in Canada: overview of current challenges and opportunities. Canadian Journal of Fisheries and Aquatic Sciences **80**(7): 1170–1186. doi:10.1139/cjfas-2022-0162.

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Dejean, T., Griffiths, R.A., Foster, J., Wilkinson, J.W., Arnell, A., Brotherton, P., Williams, P., and Dunn, F. 2015. Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (*Triturus cristatus*). Biological Conservation **183**: 19–28. doi:10.1016/j.biocon.2014.11.029.

Borcherding, J. 1991. The annual reproductive cycle of the freshwater mussel *Dreissena polymorpha* Pallas in lakes. Oecologia **87**(2): 208–218. doi:10.1007/BF00325258.

Butchart, S.H.M., Walpole, M., Collen, B., van Strien, A., Scharlemann, J.P.W., Almond, R.E.A., Baillie, J.E.M., Bomhard, B., Brown, C., Bruno, J., Carpenter, K.E., Carr, G.M., Chanson, J., Chenery, A.M., Csirke, J., Davidson, N.C., Dentener, F., Foster, M., Galli, A., Galloway, J.N., Genovesi, P., Gregory, R.D., Hockings, M., Kapos, V., Lamarque, J.-F., Leverington, F., Loh, J., McGeoch, M.A., McRae, L., Minasyan, A., Morcillo, M.H., Oldfield, T.E.E., Pauly, D., Quader, S., Revenga, C., Sauer, J.R., Skolnik, B., Spear, D., Stanwell-Smith, D., Stuart, S.N., Symes, A., Tierney, M., Tyrrell, T.D., Vié, J.-C., and

Watson, R. 2010. Global biodiversity: indicators of recent declines. Science **328**(5982): 1164–1168. doi:10.1126/science.1187512.

Butler, S.E., and Wahl, D.H. 2011. Distribution, movements and habitat use of channel catfish in a river with multiple low-head dams. River Research and Applications **27**(10): 1182–1191. doi:10.1002/rra.1416.

Clarke, A.H. 1981. The freshwater molluscs of Canada. National Museum of Natural Sciences, National Museums of Canada, Ottawa. https://doi.org/10.5962/bhl.title.132376

Ćmiel, A.M., Strużyński, A., Wyrębek, M., Lipińska, A.M., Zając, K., and Zając, T. 2020. Response of freshwater mussel recruitment to hydrological changes in a eutrophic floodplain lake. The Science of the Total Environment **703**: 135467. doi:10.1016/j.scitotenv.2019.135467.

Committee on the Status of Endangered Wildlife in Canada (COSEWIC). 2016. COSEWIC assessment and status report on the Mapleleaf *Quadrula quadrula*, Great Lakes - Upper St. Lawrence population and Saskatchewan - Nelson Rivers population, in Canada. Committee on the Status of Endangered Wildlife in Canada. Ottawa, Ontario.

Committee on the Status of Species at Risk in Ontario (COSSARO). 2017. Ontario species at risk evaluation report for Mapleleaf (*Quadrula quadrula*). Committee on the Status of Species at Risk in Ontario. Peterborough, Ontario.

Currier, C.A., Morris, T.J., Wilson, C.C., and Freeland, J.R. 2018. Validation of environmental DNA (eDNA) as a detection tool for at-risk freshwater pearly mussel species (Bivalvia: Unionidae). Aquatic Conservation **28**(3): 545–558. doi:10.1002/aqc.2869.

Davison, P.I., Copp, G.H., Créach, V., Vilizzi, L., and Britton, J.R. 2017. Application of environmental DNA analysis to inform invasive fish eradication operations. Natural Science **104**(3–4): 35–37. doi:10.1007/s00114-017-1453-9.

Deiner, K., and Altermatt, F. 2014. Transport distance of invertebrate environmental DNA in a natural river. PLoS ONE **9**(2): e88786. doi:10.1371/journal.pone.0088786.

Deiner, K., Bik, H.M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., Creer, S., Bista, I., Lodge, D.M., De Vere, N., Pfrender, M.E., and Bernatchez, L. 2017.

Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. Molecular Ecology **26**(21): 5872–5895. doi:10.1111/mec.14350.

Deiner, K., Fronhofer, E.A., Mächler, E., Walser, J.-C., and Altermatt, F. 2016. Environmental DNA reveals that rivers are conveyer belts of biodiversity information. Nature Communication 7(1): 12544. doi:10.1038/ncomms12544.

Dolson, R. M. L., McNichols-O'Rourke, K. A., and Morris, T. J. 2023. A literature review of freshwater mussel survey methods and techniques used in deep, turbid environments. Can. Tech. Rep. Fish. Aquat. Sci. 3505: v + 70 p.

Ficetola, G.F., Pansu, J., Bonin, A., Coissac, E., Giguet-Covex, C., De Barba, M., Gielly, L., Lopes, C.M., Boyer, F., Pompanon, F., Rayé, G., and Taberlet, P. 2015. Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. Molecular Ecology Resources **15**(3): 543–556. doi:10.1111/1755-0998.12338.

Fordham, D.A. 2024. Identifying species traits that predict vulnerability to climate change. Cambridge Prisms: Extinction **2**: e21. doi:10.1017/ext.2024.24.

Francis, J.D., Yanaç, K., Uyaguari-Diaz, M.I., and Biddle, J.F. 2025. Seasonal patterns of viromes in urban aquatic environments of Manitoba. Applied Environmental Microbiology **91**(10): e0040824. doi:10.1128/aem.00408-24.

Gingera, T., Bajno, R., Docker, M., and Reist, J. 2017. Environmental DNA as a detection tool for zebra mussels *Dreissena polymorpha* (Pallas, 1771) at the forefront of an invasion event in Lake Winnipeg, Manitoba, Canada. Management of Biological Invasions **8**(3): 287–300. doi:10.3391/mbi.2017.8.3.03.

Green, D.M. 2005. Essays: Designatable units for status assessment of endangered species. Conservation Biology **19**(6): 1813–1820. doi:10.1111/j.1523-1739.2005.00284.x.

Hoffman, J.R., Morris, T.J., and Zanatta, D.T. 2018. Genetic evidence for canal-mediated dispersal of Mapleleaf, *Quadrula quadrula* (Bivalvia:Unionidae) on the Niagara Peninsula, Canada. Freshwater Science **37**(1): 82–95. doi:10.1086/696127.

Hoffman, J.R., Willoughby, J.R., Swanson, B.J., Pangle, K.L., and Zanatta, D.T. 2017. Detection of barriers to dispersal is masked by long lifespans and large population sizes. Ecology and Evolution **7**(22): 9613–9623. doi:10.1002/ece3.3470.

Jerde, C.L., Mahon, A.R., Chadderton, W.L., and Lodge, D.M. 2011. "Sight-unseen" detection of rare aquatic species using environmental DNA: eDNA surveillance of rare aquatic species. Conservation Letters **4**(2): 150–157. doi:10.1111/j.1755-263X.2010.00158.x.

Jo, T., and Minamoto, T. 2021. Complex interactions between environmental DNA (eDNA) state and water chemistries on eDNA persistence suggested by meta-analyses. Molecular Ecology Resources **21**(5): 1490–1503. doi:10.1111/1755-0998.13354.

Kelly, K.E., Belcher, K., and Khakbazan, M. 2018. Economic targeting of agricultural beneficial management practices to address phosphorus runoff in Manitoba. Canadian Journal of Agricultural Economics **66**(1): 143–166. doi:10.1111/cjag.12143.

Livingstone, I. 1995-06-30. bivalve / bivalve. BIODIDAC accessed March 13, 2026, http://omeka.uottawa.ca/biodidac/items/show/5695.

Manitoba Government. 2023. Zebra mussels (*Dreissena polymorpha*). Aquatic Invasive Species accessed March 14, 2026, Province of Manitoba | Aquatic Invasive Species

Mathias, P.T., Hoffman, J.R., Wilson, C.C., and Zanatta, D.T. 2018. Signature of postglacial colonization on contemporary genetic structure and diversity of *Quadrula quadrula* (Bivalvia: Unionidae). Hydrobiologia **810**(1): 207–225. doi:10.1007/s10750-016-3076-0.

Mauvisseau, Q., Harper, L.R., Sander, M., Hanner, R.H., Kleyer, H., and Deiner, K. 2022. The multiple states of environmental DNA and what is known about their persistence in aquatic environments. Environmental Science & Technology **56**(9): 5322–5333. doi:10.1021/acs.est.1c07638.

Morris, T.J., Sheldon, M.N., and McNichols-O'Rourke, K.A. 2020. First records of two freshwater mussel Species at Risk, Mapleleaf (*Quadrula quadrula*) and Lilliput (*Toxolasma parvum*), in the Canard River, Ontario, with implications for freshwater mussel recovery in the Detroit River. Canadian Field-Naturalist **134**(2): 179–188. doi:10.22621/cfn.v134i2.2549.

Pilliod D.S., Goldberg C.S., Arkle R.S., and Waits L.P. 2014. Factors influencing detection of eDNA from a stream-dwelling amphibian. Molecular Ecology Resources **14**: 109–116, https://doi.org/10.1111/1755-0998.12159

Pip, E. 1995. Cadmium, lead and copper in freshwater mussels from the Assiniboine River, Manitoba, Canada. Journal of Molluscan Studies **61**(3): 295–302. doi:10.1093/mollus/61.3.295.

Pip, E. 2000. The decline of freshwater molluscs in southern Manitoba. Canadian Field-naturalist **114**(4): 555–560. doi:10.5962/p.364025.

Reid, S.M. 2016. Search effort and imperfect detection: Influence on timed-search mussel (Bivalvia: Unionidae) surveys in Canadian rivers. Knowledge and Management of Aquatic Ecosystems (417): 17. doi:10.1051/kmae/2016004.

Reid, S.M., Brumpton, A., Hogg, S., and Morris, T. 2014. A comparison of two-timed search methods for collecting freshwater mussels in Great Lakes Coastal Wetlands. Freshwater mollusk biology and conservation **17**(1): 16–23. Freshwater Mollusk Conservation Society. doi:10.31931/fmbc.v17i1.2014.16-23.

Sakata, M.K., Watanabe, T., Maki, N., Ikeda, K., Kosuge, T., Okada, H., Yamanaka, H., Sado, T., Miya, M., and Minamoto, T. 2021. Determining an effective sampling method for eDNA metabarcoding: a case study for fish biodiversity monitoring in a small, natural river. Limnology **22**(2): 221–235. doi:10.1007/s10201-020-00645-9.

Schlaepfer, D. R., Braschler, B., Rusterholz, H., and Baur, B. 2018. Genetic effects of anthropogenic habitat fragmentation on remnant animal and plant populations: a meta-analysis. Ecosphere **9**(10): e02488. https://doi.org/10.1002/ecs2.2488

Schmelzle, M.C., and Kinziger, A.P. 2016. Using occupancy modelling to compare environmental DNA to traditional field methods for regional-scale monitoring of an endangered aquatic species. Molecular Ecology Resources **16**(4): 895–908. doi:10.1111/1755-0998.12501.

Sigsgaard, E.E., Carl, H., Møller, P.R., and Thomsen, P.F. 2015. Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. Biological Conservation **183**: 46–52. doi:10.1016/j.biocon.2014.11.023.

Stewart, K. W., and Watkinson, D. 2014. *Ictalurus punctatus*, channel catfish. The Freshwater Fishes of Manitoba. University of Manitoba Press. Winnipeg, Manitoba. pp. 137-140.

Taberlet, P., Coissac, E., Hajibabaei, M., and Rieseberg, L. H. 2012. Environmental DNA. Molecular Ecology **21**(8), 1789-1793. https://doi.org/10.1111/j.1365-294x.2012.05542.x

Thomsen, P.F., and Willerslev, E. 2015. Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. Biological Conservation **183**(1): 4–18. doi:10.1016/j.biocon.2014.11.019.

Thomsen, P.F., Kielgast, J., Iversen, L.L., Wiuf, C., Rasmussen, M., Gilbert, M.T.P., Orlando, L., and Willerslev, E. 2012. Monitoring endangered freshwater biodiversity using environmental DNA. Molecular Ecology **21**(11): 2565–2573. doi:10.1111/j.1365-294X.2011.05418.x.

VanTassel, N.M., Beaver, C.E., Watkinson, D.A., Morris, T.J., and Zanatta, D.T. 2020. Absence of genetic structure reflects post-glacial history and present-day host use in Mapleleaf (*Quadrula quadrula*) mussel from Manitoba, Canada. Canadian Journal of Zoology **98**(8): 551–556. doi:10.1139/cjz-2019-0227.

Vaughn, C.C. 2018. Ecosystem services provided by freshwater mussels. Hydrobiologia **810**(1): 15–27. doi:10.1007/s10750-017-3139-x.

Wang, P., Yan, Z., Yang, S., Wang, S., Zheng, X., Fan, J., and Zhang, T. 2019. Environmental DNA: an emerging tool in ecological assessment. Bulletin of Environmental Contamination and Toxicology **103**(5): 651–656. doi:10.1007/s00128-019-02720-z.

Wilcox, T.M., McKelvey, K.S., Young, M.K., Jane, S.F., Lowe, W.H., Whiteley, A.R., Schwartz, M.K., and Willson, R.C. 2013. Robust detection of rare species using environmental DNA: the importance of primer specificity. PLoS ONE **8**(3): e59520. doi:10.1371/journal.pone.0059520.

Wood, Z.T., Lacoursière-Roussel, A., LeBlanc, F., Trudel, M., Kinnison, M.T., Garry McBrine, C., Pavey, S.A., and Gagné, N. 2021. Spatial heterogeneity of eDNA transport improves

stream assessment of threatened salmon presence, abundance, and location. Frontiers in Ecology and Evolution **9**. doi:10.3389/fevo.2021.650717.

Appendices

Table S1. Key features of the 56 sampling locations of the field sites in southern Manitoba of Mapleleaf Mussel are highlighted below based on water body, site code, location (latitude/longitude), replicate detections of the water samples, qPCR detections, and the Ct average. The Ct value indicates the number of cycles that surpassed during qPCR to amplify the DNA (/40). Of the sampled sites, 49 were sampled and collected by the Docker Lab; the seven remaining sites were collected by MMF (site codes: BER-A, PR, BRA-A, BVR, AR-BR, AR-SR, and PR-A). All lab work was processed in the Docker Lab.

Water body	Site Code	Sampling Date (2025)	Latitude	Longitude	Water replicate detection (/3)	qPCR detection (/9)	Ct average
Red River	RR-A	May 12	49.8548	-97.142	0/3	0/9	Not detected
Assiniboine River	AR-A	May 12	49.886	-97.13	3/3	9/9	34.671
Assiniboine River	AR-B	May 12	49.8729	-97.22	3/3	9/9	34.495
Sturgeon Creek	SC-A	May 12	49.8728	-97.268	0/3	0/9	Not detected
Assiniboine River	AR-F	May 13	49.9476	-98.3288	0/3	0/9	Not detected
Assiniboine River	AR-E	May 13	50.0526	-97.9084	0/3	0/9	Not detected
Assiniboine River	AR-C	May 13	50.0117	-97.7597	1/3	3/9	35.702
Assiniboine River	AR-D	May 13	49.8591	-97.477	3/3	9/9	35.640
La Salle River	RR-LR	May 20	49.7516	-97.1372	1/3	2/9	35.587
Rat River	RR-RT	May 20	49.5881	-97.138	1/3	3/9	36.757
Roseau River	RSR	May 20	49.1774	-96.8246	2/3	8/9	35.3F
La Salle River	LR-A	May 20	49.6983	-97.2688	0/3	0/9	Not detected
Bunn's Creek	BC	May 21	49.9498	-97.0583	0/3	0/9	Not detected
Red River	RR-LP	May 21	50.091	-96.937	2/3	2/9	39.241
Cooks Creek	CC-A	May 21	50.1404	-96.8423	0/3	0/9	Not detected
Netley Creek	RR-NC	May 21	50.2923	-96.8674	0/3	0/9	Not detected
Wavey Creek	WC	May 21	50.2707	-96.9653	0/3	0/9	Not detected

Netley Creek	NC-A	May 21	50.3265	-97.0443	0/3	0/9	Not detected
Whitemouth River	WHTR-ER	May 22	49.8761	-95.9063	0/3	0/9	Not detected
Whitemouth River	WHTR-WF	May 22	50.1193	-96.0346	0/3	0/9	Not detected
Winnipeg River	WR-A	May 22	50.1418	-96.0495	0/3	0/9	Not detected
Brokenhead River	BHR	May 22	50.0618	-96.4658	0/3	0/9	Not detected
Cooks Creek	CC-B	May 22	49.8854	-96.7403	0/3	0/9	Not detected
Assiniboine River	AR-BR	May 23	49.8605	-99.9574	3/3	9/9	34.332
Assiniboine and Souris River	AR-SR	May 23	49.6659	-99.5631	3/3	9/9	33.442
Pembina River	PR-A	May 23	49.2382	-98.6876	1/3	1/9	38.173
Berens River	BER-A	May 27	52.3410	-96.9686	3/3	9/9	34.991
Pigeon River	PR	May 27	52.1955	-96.9204	3/3	9/9	30.84683
Bradbury River	BRA-A	May 27	51.9581	-96.7780	2/3	4/9	36.63857
Bloodvein River	BVR	May 27	51.7075	-96.6275	3/3	9/9	26.88652
Rice Creek	RC	May 28	50.3242	-95.7825	0/3	0/9	Not detected
Manigotagan River	MR-A	May 28	51.1276	-96.3389	3/3	9/9	33.354
Sandy River	Sandy-A	May 28	50.5832	-96.2202	0/3	0/9	Not detected
Black River	BLR	May 28	50.8281	-96.3218	3/3	9/9	36.418
Winnipeg River	WR-B	May 28	50.574	-96.233	3/3	9/9	35.609
Catfish Creek	CFC	May 28	50.6181	-96.3754	0/3	0/9	Not detected
Winnipeg River	WR-G	June 25	50.2441	-96.0591	0/3	0/9	Not detected
Winnipeg River	WR-B1	June 25	50.3035	-95.5592	0/3	0/9	Not detected
Whitemouth River	WM-C	June 26	49.7384	-95.879	0/3	0/9	Not detected
Whitemouth River	WM-H	June 26	49.9905	-96.0091	0/3	0/9	Not detected
Whitemouth River	WM-J	June 26	50.0761	-96.0325	0/3	0/9	Not detected

Eleanor Lake	Eleanor-A	June 27	50.1459	-95.8064	0/3	0/9	Not detected
Nutimik Lake	Nutimik-B	June 27	50.1729	-95.7006	0/3	0/9	Not detected
Nutimik Lake	Nutimik-A	June 27	50.1492	-95.6663	0/3	0/9	Not detected
Dorothy Lake	Dorothy-B	June 27	50.1523	-95.7557	0/3	0/9	Not detected
Whitemouth River	WM-11	July 7	49.6649	-95.8954	0/3	0/9	Not detected
Birch River	Birch-C	July 7	49.6798	-95.7892	0/3	0/9	Not detected
Whitemouth River	WM-A	July 7	49.5676	-95.977	0/3	0/9	Not detected
Boggy River	Boggy-C	July 7	49.6304	-95.5661	0/3	0/9	Not detected
Little Saskatchewan River	LS-A	July 24	50.74	-100.252	0/3	0/9	Not detected
Shell River	SR-A	July 24	50.9657	-101.315	0/3	0/9	Not detected
Lake Manitoba	LM-A	July 24	51.0823	-98.7797	0/3	0/9	Not detected
Whitemud River	WMDR-A	July 25	50.2294	-99.456	0/3	0/9	Not detected
Qu'Appelle River	QR-A	July 25	50.4417	-101.333	2/3	5/9	35.353
Little Saskatchewan River	LS-B	July 25	50.2527	-99.8288	0/3	0/9	Not detected
Little Saskatchewan River	LS-C	July 25	50.2259	-99.8229	0/3	0/9	Not detected

Table S2. A summary of sampling sites conducted in Fall 2024 by Jessie Ogden and compared to the revisited detections for Mapleleaf Mussel in summer, 2025 to ensure the presence of the Mapleleaf Mussel in this region. The field sampling was conducted by Lauren Teller and me. The same protocols of processing the eDNA were conducted for DNA extractions and qPCR in 2024, as in the summer 2025 sampling.

Water body and site code	Sampling Date 2024	qPCR Detections (/9) 2024	Sampling Date 2025	qPCR Detections (/9) 2025
Assiniboine River (AR-A)	September 11	9/9	May 12	9/9
Assiniboine River (AR-B)	September 11	9/9	May 12	9/9
Red River (RR-A)	September 11	0/9	May 12	0/9
Sturgeon Creek (SC-A)	October 4	2/9	May 12	0/9
Assiniboine River (AR-C)	October 4	0/9	May 13	3/9
Assiniboine River (AR-D)	October 4	8/9	May 13	9/9

Table S3. Listed are waterbodies where previous historical physical sampling sites found the Mapleleaf Mussel based on summarized data from the COSEWIC (2016) report. These waterbodies were used as revisited "known range" sites for the eDNA sampling for the Mapleleaf Mussels in summer 2025. Compared are the detections from the same waterbodies from summer 2025 eDNA sampling techniques.

Waterbodies Mapleleaf Mussel was found present based on past physical sampling methods	Detections from summer 2025 sampling efforts using eDNA techniques
Cooks Creek	Not detected
Brokenhead River	Not detected
Shell River	Not detected
Red River	Detected
Roseau River	Detected
Assiniboine River	Detected
La Salle River	Detected
Rat River	Detected
Bloodvein River	Detected
Bradbury River	Detected
Wanipigow	Not sampled
Seine River	Not sampled
Pembina River	Detected
Morris River	Not sampled
Maskwa River	Not sampled

Table S4. Summary of newly sampled range extension waterbodies for analysis on the range distribution of the Mapleleaf Mussel in southern Manitoba is larger than previously known from summer sampling, 2025. Positive detections indicate possible range extension sites; non-detections indicate that the mussels DNA was not within range of the sample site.

Water body	Detection
Bunn's Creek	Not detected
Winnipeg River	Detected
Nutimik Lake	Not detected
Eleanor Lake	Not detected
Qu'Appelle River	Detected
Little Saskatchewan River	Not detected
Dorothy Lake	Not detected
Souris River	Detected
Whitemud River	Not detected
Whitemouth River	Not detected
Berens River	Detected
Black River	Detected
Pigeon River	Detected
Lake Manitoba	Not detected
Manigotagan River	Detected
Birch River	Not detected
Boggy River	Not detected

Table S5. General summary of the results from eDNA sampling 2025 for the new (range extension) and the revisited (known range sites) detections. Comparisons of positive detections and non-detection amounts per site type are listed.

Site type	Number of sites sampled	Positive detections	Non-detections
New sites (/56)	35/56 = 0.625 (62.5%)	6/35 = 0.171 (17.1%)	29/35 = 0.828 (82.8%)
Revisited sites (/56)	21/56 = 0.375 (37.5%)	13/21 = 0.619 (61.9%)	8/21 = 0.381 (38.1%)