Tracking freshwater fishes of conservation concern in Manitoba using environmental DNA

by

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Abstract

The loss and degradation of freshwater habitats in Canada threaten the rich array of plant and animal species supported within these ecosystems, threatening the biodiversity that these ecosystems sustain. Assessing the distribution of the species in these threatened ecosystems is necessary for the effective monitoring of the well-being of their populations and the ecosystem, but it can be difficult, as some species can evade traditional survey methods due to their elusiveness, size, range, or rarity. Environmental DNA (eDNA), which describes the pool of DNA present in an environment that can be collected in and isolated from environmental samples, offers an alternative to traditional surveying that can be more sensitive and less invasive. Therefore, I developed and validated TaqMan™ MGB probe-based eDNA assays for six freshwater fishes of conservation concern in Manitoba: Bigmouth Buffalo *Ictiobus cyprinellus*, Chestnut Lamprey *Ichthyomyzon castaneus*, Golden Redhorse *Moxostoma erythrurum*, Hornyhead Chub *Nocomis biguttatus*, Northern Brook Lamprey *Ichthyomyzon fossor*, and Silver Lamprey *Ichthyomyzon unicuspis*. The designed assays were validated *in situ* using paired eDNA and capture-based sampling and used to map the distribution of the above species from water samples collected and filtered from 129 sites across Manitoba in 2021–2023. I detected the eDNA of four of the target species outside of their historic range: Bigmouth Buffalo in the Roseau, Morris, and Assiniboine rivers; Golden Redhorse in Hazel Creek; Hornyhead Chub in waterbodies of the Winnipeg River watershed where it has not been reported previously; and Northern Brook Lamprey in Boggy River. Apart from Northern Brook Lamprey, the eDNA of all target species was not detected in at least one site within their historic range. Further research is needed to confirm if the lack of eDNA detections of the target species within their historic range is a result of range restriction, or if a refinement of the methods used is needed.

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Dedication

This thesis is dedicated to my two guardian angels: my beloved grandmother, Ruby, and my forever puppy and study buddy, Kodi. Your spirits live on in all of those who had the privilege of knowing you, loving you, and being loved by you.

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CHAPTER 1 GENERAL INTRODUCTION

Canada is renowned for its vast and diverse freshwater ecosystems, which support a rich array of plant and animal species. However, the loss and degradation of freshwater habitats have become pressing concerns in recent years, threatening the biodiversity that these ecosystems sustain (Desforges et al. 2022). Human activities, such as urbanization, agriculture, industrial development, and resource extraction, have significantly altered the natural landscape and water quality of Canada's lakes, rivers, and wetlands (Vörösmarty et al. 2010; Arthington et al. 2016; Desforges et al. 2022). These changes have led to habitat destruction, pollution, and fragmentation, which in turn have had detrimental effects on the health and resilience of freshwater ecosystems (*see* Strayer and Dudgeon 2010; Fuller et al. 2015). Understanding the factors driving the loss and degradation of freshwater habitats and biodiversity in Canada is essential for developing effective conservation strategies and safeguarding the ecological integrity of these vital ecosystems (Ahmed et al. 2022). Reductions in the abundance of or the extirpation of a species can cause a domino effect, leading to decreases in the abundance of the populations of other species through shifts in predator-prey interactions, and reducing the productivity and stability of an ecosystem, as a whole (Ricklefs 2008; Hui 2012). First assessing the distribution and abundance of the species in these threatened ecosystems is necessary for the effective monitoring of the well-being of their populations and the ecosystem as a whole (Gangloff et al. 2016; Arthington et al. 2016); however, the detection of certain freshwater fishes can be challenging due to their elusiveness, size, remote or widespread range, and/or rarity, which has resulted in the status of some fishes remaining largely unchecked (Olden et al. 2007; Ahmed et al. 2022).

Elusive, rare, and small-bodied fishes are difficult to assess as they can evade methods traditionally used to survey fishes (e.g., electrofishing, seines, and gill nets) (Castañeda et al. 2021). While some of these fishes may lack the "charisma" needed to make them a flagship species (e.g., minnows, suckers), or may have a poor reputation with the general public (e.g., suckers, lampreys), these fishes have ecological significance in the habitats where they are found in the form of ecosystem engineers (Vives 1990; Boeker and Geist 2016; Booth et al. 2020), modifying their environment to suit their needs and the needs of other species, and providing key links in the food web of their ecosystems (Scott and Crossman 1973; Boeker and Geist 2016).

The overall objectives of this thesis were to develop and validate environmental DNA (eDNA) assays for six freshwater fishes native to Manitoba: Bigmouth Buffalo *Ictiobus cyprinellus*, Chestnut Lamprey *Ichthyomyzon castaneus*, Golden Redhorse *Moxostoma erythrurum*, Hornyhead Chub *Nocomis biguttatus*, Northern Brook Lamprey *Ichthyomyzon fossor*, and Silver Lamprey *Ichthyomyzon unicuspis*; and to investigate the capacity of using eDNA methods to detect these ecologically important freshwater fishes of conservation concern in Manitoba.

This chapter (i.e., Chapter 1) provides a broad introduction to the study species and methods used throughout the research described throughout this thesis. The first theme of this research encompasses the development and validation of species-specific eDNA assays for each of the above six target fishes, detailed in Chapter 2. The second theme highlights the use of the developed eDNA assays to begin to map the current distribution and range of the six target fishes, outlined in Chapter 3. As outlined in their more recent assessment by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC), continued non-invasive monitoring of Northern Brook and Silver lampreys is necessary to track the well-being of the populations in

Manitoba (COSEWIC 2020). Additionally, understanding the distribution of Chestnut Lamprey, Bigmouth Buffalo, Golden Redhorse, and Hornyhead Chub in Manitoba is essential for the re-assessment of these fishes by COSEWIC, as these fishes have been identified as priorities for re-assessment (COSEWIC 2024). The third theme, and Chapter 4, uses conventional capture-based sampling (i.e., electrofishing) to further validate the designed eDNA assays and highlight any discrepancies between the two methods.

Together, these themes and results begin to address the gaps in knowledge regarding the current range and distribution of some freshwater fishes of conservation concern in Manitoba and provide tools that can be used to non-invasively detect these fishes of interest using their eDNA.

1.1 Suckers (Bigmouth Buffalo and Golden Redhorse)

Fishes in the family Catostomidae, commonly referred to in North America as "suckers," are medium to large-bodied fishes, within the same order (Cypriniformes) as minnows (Scott and Crossman 1973). Most suckers are benthic consumers and serve as important links in the food chain where they are found (Stewart and Watkinson 2004; Booth et al. 2020). Native sucker species resemble invasive carp in their size, feeding habits and ideal habitat, and are suspected to be negatively affected by the introduction and invasion of alien carp species (Stewart and Watkinson 2004; COSEWIC 2009; Fisheries and Oceans Canada 2021). At this time, the status of two (Bigmouth Buffalo *Ictiobus cyprinellus* and Golden Redhorse *Moxostoma erythrurum*) of the seven species of suckers native to Manitoba have been assessed by COSEWIC. However, as briefly reviewed below, much remains unknown regarding their current status. The remaining five species have been reported as widespread throughout Manitoba and are considered secure (Stewart and Watkinson 2004; Mandrak et al. 2023).

1.1.1 Conservation Status and Protection

Once believed to be a rapidly growing fish with a lifespan of a maximum of 10–20 years, Bigmouth Buffalo are now renowned as the longest-living freshwater teleost, with reports of individuals living 125 years and longer (Lackmann et al. 2023). In Canada, the Bigmouth Buffalo occurs as two disjunct populations—in the Great Lakes basin and the Saskatchewan-Nelson River watershed—and each are recognized as discrete and evolutionarily significant Designatable Units (COSEWIC 2009). A suspected range expansion but without increases in abundance (potentially due to the elimination and/or degradation of habitat necessary for reproduction) and "dramatic declines" contributed to the Saskatchewan-Nelson River population being assessed as "Special Concern" in 2009 (COSEWIC 2009). In 2011, the Saskatchewan-Nelson River population was listed as "Special Concern" under the federal Species at Risk Act (SARA). In 2021, a management plan was finalized to protect the habitat of watersheds in which Bigmouth Buffalo are found, to attempt to maintain the existing population levels and distribution of the Saskatchewan-Nelson River population (Fisheries and Oceans Canada 2021). Surveys to determine the distribution, movement and population characteristics of Bigmouth Buffalo have occurred and are ongoing (Enders et al. 2019; Fisheries and Oceans Canada 2021), but more research is still needed (Lackmann et al. 2023).

The status of Golden Redhorse in Canada has not been updated by COSEWIC since their assessment in 1989 when the species received the status of "Not at Risk" in Canada (Goodchild 1990). At the time of status assessment, the abundance of the populations of Golden Redhorse in Canada, which can be found in Ontario and Manitoba, had not been estimated (Goodchild 1990). Goodchild (1990) reported Golden Redhorse had been consistently collected in small numbers in Ontario but suggested this may be due, in part, to the difficulty in distinguishing among co-occurring redhorse sucker species (*Moxostoma* spp.), which often led to redhorses being

identified only to the level of genus during surveys. Furthermore, the assessment of Golden Redhorse occurred shortly after they were first discovered in Manitoba in 1984, making population assessment and trend predictions not possible; however, from the time of their discovery to the present day, Golden Redhorse have been considered rare in Manitoba and likely arrived in Manitoba after the drainage of the postglacial lakes (Franzin et al. 1986; Goodchild 1990; Stewart and Watkinson 2004; Mandrak et al. 2023).

1.1.2 Habitat

Bigmouth Buffalo inhabit a variety of habitats in large rivers and lakes, including oxbows, flood plains, swamps, pools and bayous, and shallow lakes (Scott and Crossman 1973). They can tolerate high levels of turbidity (Scott and Crossman 1973; Becker 1983), high (30–34°C) water temperatures (Minckley et al. 1970 in COSEWIC 2009) and low oxygen levels (Gould and Irvin 1962 in COSEWIC 2009), but they prefer slow moving water (Stewart and Watkinson 2004). Bigmouth Buffalo school in groups of roughly 25 individuals and are benthic and pelagic consumers that are often found near the bottom or the mid-level of a body of water, but they can sometimes be found on the surface of lakes on days when the surface water is warm and still (Scott and Crossman 1973). Bigmouth Buffalo spawn typically in May in small tributary streams, shallow ditches, marshes, backwaters, and shallow bays (Johnson 1963). The flooding of rivers and lakes in spring is a requirement for spawning and recruitment to occur, and it is suspected that Bigmouth Buffalo are unable to spawn in years of drought (Scott and Crossman 1973; Lee et al. 1980; COSEWIC 2009; Lackmann et al. 2023). The preferred substrates for the species vary from mud, clay, silt, and sand, to gravel and rubble (Becker 1983). Bigmouth Buffalo in Manitoba have been reported to have large individual home ranges, travelling 4.2–621.9 km within a year, predominantly between April and October; however, some of their movement is limited and restricted by dams (Enders et al. 2019).

Golden Redhorse are suspected to be tolerant of a wide spectrum of habitat conditions (Goodchild 1990). Preferences for river habitats and small to large streams with permanent pools and varying substrates have been reported for Golden Redhorse in adulthood, but they can also be found in lakes (Scott and Crossman 1973; Lee et al. 1980; Goodchild 1990). Spawning of Golden Redhorse in Manitoba has not been recorded; however, access to smaller streams for spawning with slow-moving waters has been reported as necessary for spawning and nursery habitat, as the young of Golden Redhorse typically inhabit riffles with substrates composed of sand, gravel, bedrock and boulders, and pools without silt or significant aquatic vegetation (Scott and Crossman 1973; Kwak and Skelly 1992). Spawning has been recorded in mid-May in riffles of main streams in Iowa, USA (Scott and Crossman 1973) and early to mid-May in Illinois (Kwak and Skelly 1992). Golden Redhorse are not suspected to migrate large distances (Scott and Crossman 1973) but do migrate to spawning grounds (Kwak and Skelly 1992).

Currently, Golden Redhorse is considered a mid-priority candidate for assessment by COSEWIC (2024); however, much is still unknown about their distribution in Manitoba. Dams and habitat fragmentation are threats faced by other Canadian populations of Golden Redhorse, which negatively affect their distribution (Reid et al. 2008). Thus, understanding the dispersion of Golden Redhorse in Manitoba is an essential step in determining what threats they may be facing.

1.2 Hornyhead Chub

Family Leuciscidae (formerly classified in family Cyprinidae), known colloquially in North America as “minnows,” is one of the most diverse and widely distributed monophyletic groups of fishes in North America (Betancur-R et al. 2017; Schönhuth et al. 2018; Tan and Armbruster 2018; Mandrak et al. 2023). When considering biomass and species diversity,

minnows dominate nearly all watersheds in the temperate zones of North America (Stewart and Watkinson 2004; Mandrak et al. 2023). Minnows have significant ecological importance as they are a key prey item for most commercial and game fishes in Manitoba (Stewart and Watkinson 2004). Habitat type and quality can also be indicated, in part, by the biodiversity and abundance of minnows present (Stewart and Watkinson 2004). Interspecific interaction of minnows occurs, and the presence of some species of minnows may be important for the success of others (Lachner 1952; Scott and Crossman 1973; Vives 1990). *Nocomis* spp. are an example of keystone species as they create necessary habitats for themselves and other leuciscid species by building nests needed for successful reproduction (Vives 1990; Wisenden et al. 2009; Quinn 2020).

Manitoba is home to 26 leuciscid species (Stewart and Watkinson 2004; Mandrak et al. 2023), including the Carmine Shiner *Notropis percobromus*, which was designated with the status of "Endangered" due to threats of habitat loss and pollution (COSEWIC 2018). At this time, only five of the 26 leuciscid species have been assessed by COSEWIC.

Hornyhead Chub *Nocomis biguttatus* is the only *Nocomis* species in Manitoba (Stewart and Watkinson 2004). The species has a restricted distribution in Manitoba, found only in the Whitemouth River and Brokenhead River watersheds (Stewart and Watkinson 2004). Hornyhead Chub are benthic consumers and mainly feed on aquatic invertebrates (e.g., snails, crayfish, insect larvae) and filamentous algae (Scott and Crossman 1973; Becker 1983).

1.2.1 Conservation Status and Protection

The last assessment of the status of Hornyhead Chub in Canada by COSEWIC was in 1988 when it received the designation of "Not at Risk" as they were considered common where found in their restricted range (Dalton 1989). As such, Hornyhead Chub receives no protection in

Canada. However, Hornyhead Chub has also been identified as a mid-priority candidate for assessment by COSEWIC (2024). Water pollution, habitat loss, fragmentation, increased siltation and non-native species have all been reported as negatively impacting the persistence of Hornyhead Chub in Canada and the United States (Mammoliti 2002; Hickerson et al. 2019; Whitney et al. 2020). Understanding their current distribution in Manitoba is fundamental in assessing their status in Canada.

1.2.2 Habitat

Typical habitats for Hornyhead Chub include small to medium-sized streams (often tributaries of large rivers) with clear, slow-moving water, but enough current to keep the substrate clear of mud, silt, or sand, and minimal plant growth (Lachner 1952; Scott and Crossman 1973). The required habitat substrate for Hornyhead Chub ranges from gravel to boulders and is needed for nest building and habitat for the invertebrates they feed upon (Scott and Crossman 1973; Dalton 1989). Male Hornyhead Chub construct large "dome-shaped" nests out of pebbles, where a female will deposit her eggs during spawning (Lachner 1952). The nests built at spawning sites by Hornyhead Chub are used by other minnow species, such as Common Shiner *Luxilis cornutus* and Carmine Shiner, both of which are native to Manitoba (Vives 1990; Mandrak et al. 2023). Interspecific interactions of Carmine Shiner, Common Shiner, and Hornyhead Chub have been observed during sampling for this project in 2021. As Carmine Shiner use the nests of Hornyhead Chub to spawn, the presence and abundance of Hornyhead Chub may be critical for the survival of Carmine Shiner in Manitoba. Furthermore, as the range for Hornyhead Chub and Carmine Shiner largely overlap, Hornyhead Chub populations may also be at risk of habitat loss and pollution.

1.3 Lampreys (Chestnut, Northern Brook, and Silver Lampreys)

Lampreys are one of two groups of extant jawless fishes and have existed for at least 360 million years (Gess et al. 2006). Much of the lamprey life cycle before metamorphosis is the same among species; however, at metamorphosis, the divergence into one of two life history types (parasitic and non-parasitic) occurs, at which point, species vary greatly in the rate of sexual maturity, the extent of migration, and whether they feed following metamorphosis (Dawson et al. 2015; Docker and Potter 2019). Three species of lampreys are native to Manitoba: Chestnut Lamprey *Ichthyomyzon castaneus*, Silver Lamprey *Ichthyomyzon unicuspis*, and Northern Brook Lamprey *Ichthyomyzon fossor*. Silver and Northern Brook lampreys are considered "paired" species as they are very similar or indistinguishable until metamorphosis, at which point Silver Lamprey delay sexual maturation and begin a parasitic feeding stage, while the non-parasitic Northern Brook Lamprey forgoes the parasitic feeding stage, becoming sexually mature within 6–10 months of metamorphosis without ever feeding again (Docker 2009). The "paired" species concept has been debated, and genetic analysis of Northern Brook and Silver lampreys suggests both species could be ecotypes of a single species; however, conventional taxonomy still recognizes them as distinct species (Neave et al. 2007; Docker 2009; Docker et al. 2012). The Chestnut Lamprey, like the Silver Lamprey, is parasitic. The three lamprey species can be distinguished externally post-metamorphosis using differences in body size, dentition, and pigmentation; however, some misidentifications of adult Chestnut and Silver lampreys have been reported (Stewart and Watkinson 2004; COSEWIC 2010, 2020). Larger larval Chestnut Lamprey can be distinguished from larval Northern Brook and Silver lampreys using morphological traits; however, it is not yet possible to identify Northern Brook and Silver lampreys to the level of species pre-metamorphosis using morphological traits and conclusively identifying Chestnut Lamprey below lengths of approximately 80–100 mm is also challenging

(Neave et al. 2007). The most reliable method of identification of larval native lampreys at this time is through genetic analysis, which can differentiate Chestnut Lamprey from Silver and Northern Brook lampreys (Docker et al. 2012). Pre-metamorphic differentiation of Northern and Silver lampreys is not yet possible due to a lack of fixed genetic differences between the species (Docker et al. 2012).

As larvae, lampreys are primary consumers and filter feed on organic detritus, diatoms, microscopic algae and protozoans (Sutton and Bowen 1994; Yap and Bowen 2003; Docker and Potter 2019; Polyakova et al. 2019). During metamorphosis, lampreys undergo dramatic transformations, including extensive changes to their digestive system, which force a period of non-feeding for both non-parasitic and parasitic lampreys (Youson 1980; Docker 2009; Manzon et al. 2015). After metamorphosis, the digestive systems of parasitic lampreys become functional once again and they begin to feed on the blood and/or tissues of their hosts, often non-lethally, by using their toothed oral disc to attach, then their toothed tongue to rasp shallow holes in the side of their hosts (Hall 1963; Docker 2009; Renaud and Cochran 2019). The hosts/prey of lampreys can vary by species and distribution; however, parasitism of a wide range of sizes and species of fishes (e.g., salmonids, suckers, sturgeons, bass, pikes, minnows) and cetaceans (e.g., whales, dolphins) by lampreys have been documented (*see* Renaud and Cochran 2019; Quintella et al. 2021). Non-parasitic lampreys typically do not feed after metamorphosis, although rare occurrences of facultative parasitism have been reported in some species (Renaud and Cochran 2019). Larval lampreys serve as prey items for many of the fishes that they prey upon later in their life and seasonally, can make up the bulk of the diet for some of these fishes (e.g., Burbot *Lota lota*) (Scott and Crossman 1973).

1.3.1 Conservation Status and Protection

Silver and Northern Brook lampreys were assessed by COSEWIC in 2020; however, designations of the statuses of "Special Concern" (Saskatchewan – Nelson River populations) and "Data Deficient" (Hudson Bay – James Bay populations) for Silver Lamprey, and "Endangered" (Saskatchewan – Nelson River populations) for Northern Brook Lamprey by COSEWIC warrant continued monitoring of these species in Manitoba (COSEWIC 2020). Protection under SARA has not yet been afforded to these species in Manitoba, although the Great Lakes – Upper St. Lawrence populations of both species are listed on SARA as "Special Concern," given evidence that they have been negatively impacted by sea lamprey control measures (Maitland et al. 2015; COSEWIC 2020; Neave et al. 2021), and no known conservation activities are underway (Government of Canada 2022).

Chestnut Lamprey were last assessed by COSEWIC in 2010 and received the designation "Data Deficient" due to a lack of information regarding their distribution, abundance, and threats to the species (COSEWIC 2010). Two Designatable Units (DUs) of Chestnut Lamprey are currently recognized in Canada (Great Lakes – Upper St. Lawrence, Saskatchewan – Nelson River), where DUs recognize irreplaceable (discrete and significant) diversity below the level of a taxonomic species. However, the existence of the Great Lakes – Upper St. Lawrence populations of Chestnut Lamprey is uncertain as detections of the species in the Canadian Great Lakes have not occurred since a few individuals were reported following a re-examination of museum specimens (Renaud et al. 1996). If Chestnut Lamprey are still present in the Great Lakes – Upper St. Lawrence region, they are likely very rare, and at great risk of extirpation (Neave et al. 2021). The eDNA assays developed and validated for Chestnut Lamprey in Manitoba would be an essential tool to help address this critical uncertainty in the Great Lakes

region, as both DUs of Chestnut Lamprey are candidates for re-assessment by COSEWIC (2024).

1.3.2 Habitat

Suitable habitat for lampreys requires optimal substrate size, depth and composition, water velocity (≤ 0.05 m/s), and water temperature (< 28°C, on average) (Dawson et al. 2015). As lampreys spend most of their lives as larvae buried in the muddy, silty or sandy substrate of small rivers, streams and creeks, their distribution can be limited based on suitable habitat availability (Dawson et al. 2015). After metamorphosis from the larval life stage, Northern Brook Lamprey migrate short distances upstream to shallow areas with coarse gravel substrate and moderate water current to spawn (Scott and Crossman 1973; Collerone 2014). Chestnut and Silver lampreys migrate downstream post-metamorphosis into larger waterbodies (i.e., rivers or lakes) to feed on larger-bodied fishes for a period before they reach sexual maturity (Scott and Crossman 1973; Renaud and Cochran 2019; COSEWIC 2020). As they reach sexual maturity, the parasitic lampreys migrate upstream to spawn in similar habitats as Northern Brook Lamprey, or in rivers (Renaud and Cochran 2019; COSEWIC 2020). All lampreys are semelparous and die after spawning, with death occurring within a few days or weeks, to up to a couple months after spawning (Johnson et al. 2015; Moser et al. 2019).

1.4 Environmental DNA (eDNA)

In the last decade, a promising method of species detection, collectively known as environmental DNA (eDNA) surveying, has increased in popularity. In general, eDNA describes the pool of DNA present in an environment that can be collected in and isolated from environmental samples, then used to assess the presence of a target species in that environment at the time of sampling (Taberlet et al. 2012; Pawlowski et al. 2020). Environmental DNA is different from genomic DNA as it is not extracted directly from specimens but rather from their

environment (Pawlowski et al. 2020). The source of eDNA can be any DNA an organism can shed or excrete in its environment that contains intra- or extracellular DNA (e.g., tissue, gametes, mucus, feces) (Thomsen et al. 2012a; Pawlowski et al. 2020). The experimental protocol for obtaining, extracting, and analyzing eDNA from an environment varies between studies, environments, and target organisms. However, the typical workflow for eDNA sampling involves collecting and isolating the eDNA by filtering or precipitating DNA collected in an environmental sample (e.g., soil, water, sediment, air, biofilm), extracting the eDNA from the filter or precipitate, and amplifying targets of interest from the eluted DNA using polymerase chain reaction (PCR), then assessing the amplified DNA for the presence of the target DNA.

The first reports of eDNA for species detection in aquatic ecosystems occurred in the late 1980s by microbiologists to detect microorganisms in aquatic sediment (Ogram et al. 1987). The application of eDNA techniques to detect aquatic vertebrates was first reported in the early 2000s (Ficetola et al. 2008; Goldberg et al. 2011; Jerde et al. 2011; Thomsen et al. 2012a). In the decade since those early applications, research using eDNA-based monitoring has developed rapidly, with research occurring worldwide (Lodge et al. 2012; Thomsen and Willerslev 2015; Jerde et al. 2019; Postaire et al. 2020), applying eDNA techniques to detect aquatic organisms, including marine mammals (Foote et al. 2012; Stoeckle et al. 2018), amphibians (Goldberg et al. 2011; Pope et al. 2020), invasive species (Amberg et al. 2015; Gingera et al. 2016, 2017) and rare species (Wilcox et al. 2013; COSEWIC 2020; Budd et al. 2021). As eDNA surveying and analysis can detect species presence, and in some cases, relative abundance, without the need to capture, handle or observe individuals, eDNA is considered a non-invasive method of species detection. When compared to traditional methods of surveying that rely on the capture of specimens, eDNA surveying can be more effective at detecting fish or other organisms at low

densities (Robinson et al. 2019; Piggott et al. 2021; Bradley et al. 2022). While eDNA-based surveying can be an effective method of species detection, monitoring and/or management, it is critical to understand the many factors that can affect eDNA detections so eDNA results can be accurately interpreted.

1.4.1 Environmental DNA Protocol

The methods and equipment used to collect, filter, extract and amplify eDNA from water samples can significantly affect overall DNA yield and thus the likelihood of detection, especially when eDNA concentrations are low (Deiner et al. 2015; Hinlo et al. 2017b; Kumar et al. 2020; Takahara et al. 2020). Many in-house formulations and commercial kits for eDNA extraction exist, and the method for eDNA extraction chosen should consider the objective of the research and the source of the eDNA (Kumar et al. 2020). Once the extracted eDNA is eluted, it can be analyzed for the presence of target DNA using various methods using PCR (e.g., quantitative PCR, droplet digital PCR, end-point PCR) or high-throughput sequencing (e.g., DNA barcoding, metabarcoding; Table 1). The method of analysis used is dependent on the objective of the study; studies looking to detect a single or few species typically use species-specific primers and PCR-based analyses to amplify the DNA from only the target species (Gustavson et al. 2015; Gingera et al. 2016, p. 201; Kusanke et al. 2020), whereas broadscale biodiversity analyses are possible using metabarcoding and "universal" or general primers, then sequencing the amplified PCR product using high-throughput sequencing to determine which species' DNA is present in a sample (Hänfling et al. 2016; Andruszkiewicz et al. 2017b; Stat et al. 2019). While more taxa can be detected simultaneously with metabarcoding, PCR-based analyses can detect DNA at lower concentrations than metabarcoding (Harper et al. 2018) and are often used for eDNA analyses, especially for rare species. Of the PCR-based analyses, quantitative PCR (qPCR) is most commonly used (*see* Section 1.4.1.2) as it is more specific than

end-point PCR (i.e., conventional PCR where amplification is determined visually on an agarose gel) (Williams et al. 2017; Schloesser et al. 2018), and more cost-effective and faster than droplet digital PCR (ddPCR), although ddPCR can offer more reliable quantification of eDNA concentrations in water than qPCR (Doi et al. 2015).

The amplification of eDNA can be affected by the presence of PCR inhibitors in an eDNA sample and lead to false negative results or the inaccurate quantification of eDNA when samples are inhibited. The inhibition of an eDNA sample can occur when high concentrations of common PCR inhibitors (e.g., humic acid, fulvic acid, tannins, phytic acid) are present in the environment where the eDNA sample was collected or by the presence of high concentrations of non-target DNA in the sample (Wilson 1997; Lance and Guan 2020). Inhibitor removal treatments and/or the use of PCR master mixes that are resistant to common inhibitors can remove or negate the effects of PCR inhibitors on amplification, and they can increase the likelihood of species detection when inhibitors are present (Goldberg et al. 2016; Williams et al. 2017). Furthermore, internal positive controls of exogenous DNA or synthetic DNA can be included in PCR reactions to identify the presence of PCR inhibition (Goldberg et al. 2016; Klymus et al. 2020).

Due to the sensitive nature of PCR, clean procedures must be in place to prevent contamination, and field and laboratory controls and replicates must be integrated throughout the eDNA workflow to assess for contamination (negative controls), the precision of the analysis (biological and technical replicates) and the sensitivity of the assay (positive controls) (Goldberg et al. 2016). Contamination should be limited by thoroughly decontaminating all surfaces and equipment using available decontamination solutions (e.g., ELIMINase®) or sodium hypochlorite (bleach) solutions, followed by ultra-violet (UV) irradiation, when possible

Table 1.1. Comparison of methods used to analyze environmental DNA samples relative to sensitivity, specificity, cost, throughput, mechanism, and best use. Polymerase chain reaction (PCR)-based methods include conventional/end-point PCR, quantitative PCR (qPCR) and droplet digital PCR (ddPCR) compared to high-throughput sequencing analysis, including DNA sequencing and metabarcoding.

	PCR	qPCR	ddPCR	Sequencing & Metabarcoding
Sensitivity	Limited sensitivity	Highly sensitive; allows for quantification	Highly sensitive; more reliable quantification	Broader approach; lower sensitivity
Specificity	Less specific than probe-based PCR	Probe increases specificity	Probe increases specificity	High specificity
Cost	More cost-effective	More expensive than PCR	More expensive than qPCR	Most expensive
Throughput & Processing	Lower throughput and slower processing than qPCR/ddPCR	High throughput and quick analysis for one or few species than sequencing	High throughput and quicker analysis for one or few species than sequencing	Highest simultaneous throughput of multiple species; slowest processing time
Mechanism	Designed primer pair	Designed primer pair and probe	Designed primer pair and probe	Sequenced PCR product generated using “universal” or general primers
Best Use	Single/few species	Single/few species	Single/few species requiring reliable quantification	Many species/broadscale biodiversity

(Goldberg et al. 2016), and limiting contact with exogenous sources of the species' DNA. When possible, dedicated eDNA facilities and laboratories should be established and used to prevent the contact of the species' DNA with laboratory equipment and personnel (*see* Abbott et al. 2021).

1.4.1.2 Environmental DNA Assays

Before the detection of a species' DNA can occur, PCR assays must be developed and/or validated for the procedures, protocols and methods used. Earlier eDNA studies used end-point PCR (e.g., Ficetola et al. 2008; Jerde et al. 2011); however, real-time qPCR, which gives real-time amplification information as the reaction proceeds, is more commonly used because it is more sensitive than end-point PCR (Piggott 2016; Xia et al. 2018) and does not require post-PCR sample handling like end-point PCR, reducing the risk of contamination of and with PCR product (Heid et al. 1996). Detection of target DNA using qPCR relies on the detection of a fluorescent reporter by the qPCR machine; SYBR® Green and TaqMan probe-based chemistries are commonly used for qPCR analyses in eDNA surveys and differ in their specificity for target DNA and fluorophore binding methods. The detection of the target DNA using SYBR® Green qPCR assays is achieved when the SYBR® Green intercalating dye binds to double-stranded DNA non-specifically and emits green light that can be detected by the qPCR machine. As the SYBR® Green intercalating dye binds to any double-stranded DNA present (e.g., target or non-target DNA, primer-dimers), SYBR® Green qPCR assays can be less specific and can lead to false positive detections or inaccurate quantification (Smith and Osborn 2009). Probe-based qPCR assays require the binding of the two primers and a probe to the target DNA for the successful amplification of the target DNA and fluorescence to occur, increasing the specificity of probe-based assays in comparison to SYBR® Green assays (So et al. 2020; Kronenberger et al. 2022). TaqMan® probe-based assays are commonly used in eDNA analysis using qPCR and

depend on exonuclease activity by the *Taq* polymerase enzyme to cleave the fluorophore present on the probe during extension on the new strand of DNA, resulting in the production of fluorescence. Two types of TaqMan® probes are typically used in eDNA analyses: the dual-labelled probes, which consist of a reporter dye at the 5' end of the probe and a quencher dye at the 3' end of the probe (Heid et al. 1996); and TaqMan® minor groove-binding (MGB) probes (Kutyavin et al. 2000). TaqMan® MGB probes have a reporter dye at the 5' end of the probe and a non-fluorescent quencher and MGB at the 3' end of the binding region of the probe; the MGB molecule increases the melting temperature of the probe, which stabilizes the probe-target DNA complex by folding the complex into the minor groove of the double-stranded DNA (Kutyavin et al. 2000). The greater stability allows for TaqMan® MGB probes to be significantly shorter than traditional probes (~15-20 nucleotides), and to have greater specificity than dual-reporter probes; however, TaqMan® MGB probe-based assays can produce amplification with as few as two to three base pair mismatches between the probe and binding sequence (Kutyavin et al. 2000; Yao et al. 2006; Wilcox et al. 2013), making cross-amplification with closely related non-target species possible. Thus, thoroughly validating an assay against non-target DNA before use is necessary for accurate results.

1.4.2 Factors Affecting eDNA Detection

When detection rates are compared, eDNA frequently outperforms other methods of fish surveying (e.g., netting, electrofishing), particularly when surveying populations at low densities (Pilliod et al. 2013; Hinlo et al. 2017a; Nevers et al. 2018; Piggott et al. 2021). However, the detectability of eDNA can be affected by environmental and biological factors, leading to false-negative results (i.e., the target species is present, but their DNA is not detected) (Hunter et al. 2019) and false-positive results (i.e., the DNA of a target species is detected, but the species is not present) (Kamoroff and Goldberg 2018). The detection of a target species can be affected by

the production of eDNA, the state of eDNA, the transport of eDNA and the degradation of eDNA (Jo et al. 2019).

1.4.2.1 Production of eDNA

The production of eDNA occurs when an organism sheds or excretes anything containing intra- or extracellular DNA into its environment (Pawlowski et al. 2020). In fish, the production of eDNA can be variable, resulting in variations in eDNA detections throughout the year. Most notably, a significant increase in eDNA detections and concentrations is often reported during spawning, likely due to the shedding of gametes, aggregation of individuals, and tissue damage from interspecific interaction (Gingera et al. 2016; Tillotson et al. 2018; Hayami et al. 2020; Hayer et al. 2020; Ruan et al. 2020). Many studies have reported increased shedding rates with increasing water temperatures, likely due to higher metabolic and physiological activity (e.g., Dejean et al. 2011; Strickler et al. 2015; Jo et al. 2019; Andruszkiewicz Allan et al. 2021). Higher rates of eDNA shedding have also been reported in juvenile fish (Maruyama et al. 2014), likely due to increased metabolic activity and growth. Non-living sources of eDNA can also produce positive eDNA detections, such as carcasses of the target species, or feces from predators that have consumed the target species (Merkes et al. 2014).

1.4.2.2 State of eDNA

In an environment, eDNA can be found in a mixture of different states (e.g., intramembranous, extramembranous, particulate, free/dissolved) (Barnes and Turner 2016; Jo et al. 2021b; Mauvisseau et al. 2022). The state (or states) that eDNA is collected in may affect the likelihood of detection, as some states of eDNA cannot be completely recovered and detected through typical eDNA workflows, leading to false negatives and/or inaccurate quantification of eDNA recovered (Jo et al. 2021b; Mauvisseau et al. 2022). No known methods exist to identify

the state of eDNA readily in a sample to ensure the eDNA is properly extracted (Mauvisseau et al. 2022).

1.4.2.3 Transport of eDNA

The transportation of eDNA within a waterbody can occur through dilution, vertical movement (e.g., from the surface to sediment), horizontal movement (e.g., downstream), or resuspension (e.g., from sediment into the water column). The transport of eDNA away from the source is often considered one of the main causes of decreased eDNA detection in flowing waters even when the species is present. Conversely, the resuspension of eDNA-containing sediment or the mixing of water in stratified waterbodies (Jeunen et al. 2020) can lead to the detection of a target species that is no longer present (i.e., via "ancient" DNA, DNA that was not shed recently). The flow or discharge of a waterbody can affect how far eDNA is transported from the source of eDNA (Shogren et al. 2018) and can lead to the dilution of eDNA near the source of eDNA. How far eDNA can be detected from a target organism appears to be a combination of many factors, including the type of waterbody (e.g., lake, ocean, stream, river), the flow rate of the water body and the species of interest. Therefore, the distance at which eDNA has been reported from a target organism can vary and has been reported (e.g., using caged individuals of a species not found in that waterbody) in freshwater streams at a distance of 239.5 m (Jane et al. 2015) and up to 5000 m from the target organism in freshwater rivers (Laporte et al. 2020). As in freshwater environments, the distance from which eDNA can travel from its origin in marine environments and remain detectable varies depending on abiotic factors (e.g., temperature, flow rate, biomass of origin), and reports vary from 5 to 600 km before degradation or dilution beyond detectability occurs (Thomsen et al. 2012b; Jeunen et al. 2019; Andruszkiewicz et al. 2019). However, some argue that flow rate only affects the detection rate

of eDNA when eDNA production rates are not constant, or when the source of eDNA is no longer present (Pilliod et al. 2014).

1.4.2.4 Degradation of eDNA

Immediately after being shed, eDNA begins to decay exponentially, but the rate of decay is highly variable and dependent on the state of the eDNA and the environment it is in (Thomsen et al. 2012a; Strickler et al. 2015; Andruszkiewicz Allan et al. 2021; Jo et al. 2021b; Mauvisseau et al. 2022). The degradation of eDNA is biphasic, consisting of an initial phase of rapid degradation that occurs within 24 hours and is responsible for 80–90% of the degradation, followed by a slower rate of degradation (Maruyama et al. 2014; Shogren et al. 2018). In general, the rate at which eDNA decays in an environment increases with water temperatures of 20°C or greater and decreases in environments with basic (pH >5.0) or alkaline (pH >9.0) water (Mauvisseau et al. 2022). While temperature can be directly involved in the degradation of eDNA at very high temperatures (>50°C) through the denaturation of DNA, studies suggest that most temperature-related eDNA degradation is likely indirect, and higher rates of degradation presumably occur through higher levels of microbial metabolism as temperature increases (Zhu 2006; Poté et al. 2009; Fu et al. 2012; Barnes et al. 2014; Strickler et al. 2015; Jo et al. 2019). By measuring the amount of biofilm present in an aquatic system, studies have demonstrated a strong, negative effect on the persistence of eDNA in an environment with the presence of biofilm, suggesting conditions that favour the presence and proliferation of the microbes involved in the degradation of eDNA can explain the variability in rates of degradation and thus persistence of eDNA in an environment (Strickler et al. 2015; Salter 2018; Shogren et al. 2018). While higher fish biomass and greater fish abundance can contribute to higher productions of eDNA, both may also contribute to an increase in the abundance of microbes in an environment, ultimately leading to increased eDNA degradation (Jo et al. 2019). Cooler water temperatures

can reduce overall eDNA degradation, potentially by reducing microbial growth and activity, supported by reports of eDNA detections up to 58 days post-exposure in cool water (e.g., <5°C) (Strickler et al. 2015).

Because the degradation of eDNA continues after samples are collected, filtering on-site followed by the immediate preservation of the filters is recommended (Laramie et al. 2015; Spens et al. 2017; Takahara et al. 2020). When filtration on-site is not possible, samples can be preserved by chilling on ice (Deiner et al. 2015; Yamanaka et al. 2017; Sales et al. 2019), with the addition of DNA preservatives (e.g., benzalkonium chloride, sodium acetate, ethanol, Longmire's lysis buffer) (Williams et al. 2016; Hinlo et al. 2017b; Hunter et al. 2019; Takahara et al. 2020; Jo et al. 2021a), or by freezing (Ladell et al. 2019; Takahara et al. 2020). Filters and extracted DNA can be stored in freezers held at -20 (shorter- to medium-term) to -80°C (long-term, >1 year) to preserve the captured and extracted DNA (Williams et al. 2016; Hinlo et al. 2017b; Hundermark and Takahashi 2020; Cunningham et al. 2024), although freeze-thaw cycles should be limited as they may decrease concentrations of detectable DNA (Takahara et al. 2015; Hinlo et al. 2017b). Room-temperature storage of preserved filters and water samples with insignificant DNA degradation has been reported (Renshaw et al. 2015; Williams et al. 2016; Spens et al. 2017; Yamanaka et al. 2017), with one study reporting "no discernible loss" in total eDNA concentration after 150 days (Wegleitner et al. 2015).

1.4.3 Applications of eDNA Detections

As more has been discovered about the behaviour of eDNA in an environment, more research has investigated how eDNA results can be extrapolated to answer questions further than presence and absence. Recent studies have investigated the relationship between eDNA signal and abundance, many via occupancy modelling (e.g., Dorazio and Erickson 2018; Hunter et al.

2018, 2019; Strickland and Roberts 2019; Pope et al. 2020; Buxton et al. 2021; Piggott et al. 2021). While many studies have reported a positive relationship between total eDNA concentration and target organism abundance (Doi et al. 2015; Tillotson et al. 2018; Bradley et al. 2022), some studies have suggested that the relationship between eDNA concentrations and abundance is not always predictable (Lacoursière-Roussel et al. 2016; Andruszkiewicz et al. 2017a; Hinlo et al. 2017a).

While eDNA surveying can be used to address some challenges in biological monitoring due to its increased sensitivity compared to traditional methods, eDNA surveys have their pitfalls in their inability to provide detailed information on the precise location from which an eDNA detection originates, any detailed or demographic information about the target organism (e.g., health, age, size, sex, maturation status, living/dead), and in their ability to produce errors (e.g., false negatives/positives) due to human error and inhibition of samples or environmental variables (Jo et al. 2021b). Therefore, using results from eDNA surveys to perform targeted surveys using traditional methods may provide a combination that addresses the shortcomings of both methods, resulting in a more refined and efficient tool for monitoring stocks, species of conservation concern and/or invasive species.

1.5 Ethical Statement and Permits

There was no animal care or independent use of live animals for experimental purposes in the study. All tissue extractions from the specimens collected during paired sampling (e.g., for assay validation using tissue-derived DNA) complied with the Abbreviated Protocol for Minimal Animal Involvement approved by the Fort Garry Campus Animal Care Committee at the University of Manitoba and Scientific Collection (General) Permits (Permit No. 44342926; 60261548) issued by the Government of Manitoba. All paired sampling was completed alongside

Fisheries and Oceans biologists (Fisheries and Oceans Canada, Freshwater Institute, Winnipeg, Manitoba). All water samples were collected from public grounds in compliance with Aquatic Invasive Species (AIS) Permits (Permit No. 23-2022; Permit No. 24-2024), as AIS were present at some of the sites sampled.

Literature Cited

Abbott C, Coulson M, Gagné N, et al (2021) Guidance on the Use of Targeted Environmental DNA (eDNA) Analysis for the Management of Aquatic Invasive Species and Species at Risk. DFO Canadian Science Advisory Secretariat Research Document 2021/019. https://waves-vagues.dfo-mpo.gc.ca/library-bibliotheque/40960791.pdf

Ahmed SF, Kumar PS, Kabir M, et al (2022) Threats, challenges and sustainable conservation strategies for freshwater biodiversity. Environmental Research 214:113808. https://doi.org/10.1016/j.envres.2022.113808

Amberg JJ, Grace McCalla S, Monroe E, et al (2015) Improving efficiency and reliability of environmental DNA analysis for Silver Carp. Journal of Great Lakes Research 41:367–373. https://doi.org/10.1016/j.jglr.2015.02.009

Andruszkiewicz Allan E, Zhang WG, Lavery A, Govindarajan A (2021) Environmental DNA shedding and decay rates from diverse animal forms and thermal regimes. Environmental DNA 3:492–514. https://doi.org/10.1002/edn3.141

Andruszkiewicz EA, Koseff JR, Fringer OB, et al (2019) Modeling environmental DNA transport in the coastal ocean using Lagrangian particle tracking. Front Mar Sci 6:477. https://doi.org/10.3389/fmars.2019.00477

Andruszkiewicz EA, Sassoubre LM, Boehm AB (2017a) Persistence of marine fish environmental DNA and the influence of sunlight. PLoS ONE 12:e0185043. https://doi.org/10.1371/journal.pone.0185043

Andruszkiewicz EA, Starks HA, Chavez FP, et al (2017b) Biomonitoring of marine vertebrates in Monterey Bay using eDNA metabarcoding. PLoS ONE 12:e0176343. https://doi.org/10.1371/journal.pone.0176343

Arthington AH, Dulvy NK, Gladstone W, Winfield IJ (2016) Fish conservation in freshwater and marine realms: status, threats and management. Aquatic Conservation 26:838–857. https://doi.org/10.1002/aqc.2712

Barnes MA, Turner CR (2016) The ecology of environmental DNA and implications for conservation genetics. Conservation Genetics 17:1–17. https://doi.org/10.1007/s10592-015-0775-4

Barnes MA, Turner CR, Jerde CL, et al (2014) Environmental conditions influence eDNA persistence in aquatic systems. Environ Sci Technol 48:1819–1827. https://doi.org/10.1021/es404734p

Becker GC (1983) Fishes of Wisconsin. The University of Wisconsin Press, Madison

Betancur-R R, Wiley EO, Arratia G, et al (2017) Phylogenetic classification of bony fishes. Evolutionary Biology 17:162. https://doi.org/10.1186/s12862-017-0958-3

Boeker C, Geist J (2016) Lampreys as ecosystem engineers: burrows of *Eudontomyzon* sp. and their impact on physical, chemical, and microbial properties in freshwater substrates. Hydrobiologia 777:171–181. https://doi.org/10.1007/s10750-016-2774-y

Booth MT, Hairston NG, Flecker AS (2020) Consumer movement dynamics as hidden drivers of stream habitat structure: suckers as ecosystem engineers on the night shift. Oikos 129:194–208. https://doi.org/10.1111/oik.06396

Bradley DL, Morey KC, Bourque DA, et al (2022) Environmental DNA detection and abundance estimates comparable to conventional methods for three freshwater larval species at a power plant discharge. Environmental DNA 4:700–714. https://doi.org/10.1002/edn3.286

Budd AM, Cooper MK, Port AL, et al (2021) First detection of critically endangered scalloped hammerhead sharks (*Sphyrna lewini*) in Guam, Micronesia, in five decades using environmental DNA. Ecological Indicators 127:107649. https://doi.org/10.1016/j.ecolind.2021.107649

Buxton A, Matechou E, Griffin J, et al (2021) Optimising sampling and analysis protocols in environmental DNA studies. Scientific Reports 11:11637. https://doi.org/10.1038/s41598-021-91166-7

Castañeda RA, Ackerman JD, Chapman LJ, et al (2021) Approaches and research needs for advancing the protection and recovery of imperilled freshwater fishes and mussels in Canada. Canadian Journal of Fisheries and Aquatic Sciences 78:1356–1370. https://doi.org/10.1139/cjfas-2020-0374

Collerone S (2014) Distribution and habitat of larval and spawning Silver (*Ichthyomyzon unicuspis*), Chestnut (*I. castaneus*), and Northern Brook (*I. fossor*) lampreys in southeastern Manitoba. Honour's thesis, University of Manitoba, Winnipeg

COSEWIC (2009) COSEWIC assessment and status report on the Bigmouth Buffalo *Ictiobus cyprinellus* (Great Lakes - Upper St. Lawrence populations and Saskatchewan - Nelson River populations) in Canada. Committee on the Status of Endangered Wildlife in Canada, Ottawa

COSEWIC (2018) COSEWIC assessment and status report on the Carmine Shiner *Notropis percobromus* in Canada. Committee on the Status of Endangered Wildlife in Canada, Ottawa

COSEWIC (2010) COSEWIC assessment and status report Chestnut Lamprey *Ichthyomyzon castaneus* (Great Lakes - Upper St. Lawrence populations and Saskatchewan-Nelson River populations) in Canada. Committee on the Status of Endangered Wildlife in Canada, Ottawa

COSEWIC (2020) COSEWIC assessment and status report on the Northern Brook Lamprey *Ichthyomyzon fossor* (Great Lakes - Upper St. Lawrence populations and Saskatchewan - Nelson River populations) and the Silver Lamprey *Ichthyomyzon unicuspis* (Great Lakes

- Upper St. Lawrence populations) in Canada. Committee on the Status of Endangered Wildlife in Canada, Ottawa

COSEWIC (2024) COSEWIC candidate wildlife species. https://www.cosewic.ca/index.php/en/reports/candidate-wildlife-species#fwf

Cunningham SW, Tessler M, Johnson-Rosemond J, et al (2024) Environmental DNA Isolation, Validation, and Preservation Methods. In: DeSalle R (ed) DNA Barcoding. Springer US, New York, pp 171–180

Dalton KW (1989) Status of the Hornyhead Chub, *Nocomis biguttatus*, in Canada. Canadian Field-Naturalist 103:180–185

Dawson HA, Quintella BR, Almeida PR, et al (2015) The Ecology of Larval and Metamorphosing Lampreys. In: Docker MF (ed) Lampreys: Biology, Conservation and Control, Springer, Dordrecht, pp 75–137

Deiner K, Walser JC, Mächler E, Altermatt F (2015) Choice of capture and extraction methods affect detection of freshwater biodiversity from environmental DNA. Biological Conservation 183:53–63. https://doi.org/10.1016/j.biocon.2014.11.018

Desforges JE, Clarke J, Harmsen EJ, et al (2022) The alarming state of freshwater biodiversity in Canada. Canadian Journal of Fisheries and Aquatic Sciences 79:352–365. https://doi.org/10.1139/cjfas-2021-0073

Docker MF (2009) A review of the evolution of non-parasitism in lampreys and update of the paired species concept. In: Brown LR, Chase SD, Mesa MG, et al. (eds) Biology, Management, and Conservation of Lampreys in North America. American Fisheries Society Symposium 72, Bethesda, pp 71–114

Docker MF, Mandrak NE, Heath DD (2012) Contemporary gene flow between "paired" Silver (*Ichthyomyzon unicuspis*) and Northern Brook (*I. fossor*) lampreys: implications for conservation. Conservation Genetics 13:823–835. https://doi.org/10.1007/s10592-012-0332-3

Docker MF, Potter IC (2019) Life History Evolution in Lampreys: Alternative Migratory and Feeding Types. In: Docker MF (ed) Lampreys: Biology, Conservation and Control. Springer, Dordrecht, pp 287–409

Doi H, Uchii K, Takahara T, et al (2015) Use of droplet digital PCR for estimation of fish abundance and biomass in environmental DNA surveys. PLoS ONE 10:e0122763. https://doi.org/10.1371/journal.pone.0122763

Dorazio RM, Erickson RA (2018) ednaoccupancy: An r package for multiscale occupancy modelling of environmental DNA data. Molecular Ecology Resources 18:368–380. https://doi.org/10.1111/1755-0998.12735

Enders EC, Charles C, Watkinson DA, et al (2019) Analysing habitat connectivity and home ranges of Bigmouth Buffalo and Channel Catfish using a large-scale acoustic receiver network. Sustainability 11:3051. https://doi.org/10.3390/su11113051

Ficetola GF, Miaud C, Pompanon F, Taberlet P (2008) Species detection using environmental DNA from water samples. Biology Letters 4:423–425. https://doi.org/10.1098/rsbl.2008.0118

Fisheries and Oceans Canada (2021) Management plan for the Bigmouth Buffalo (*Ictiobus cyprinellus*) in Canada (Saskatchewan-Nelson River populations). Fisheries and Oceans Canada, Ottawa

Foote AD, Thomsen PF, Sveegaard S, et al (2012) Investigating the potential use of environmental DNA (eDNA) for genetic monitoring of marine mammals. PLoS ONE 7:e41781. https://doi.org/10.1371/journal.pone.0041781

Franzin WG, Parker BR, Harbicht SM (1986) First record of the Golden Redhorse, *Moxostoma erythrurum*, from the Red River in Manitoba. The Canadian Field-Naturalist 100:270–271

Fu XH, Wang L, Le YQ, Hu JJ (2012) Persistence and renaturation efficiency of thermally treated waste recombinant DNA in defined aquatic microcosms. Journal of Environmental Science and Health 47:1975–1983. https://doi.org/10.1080/10934529.2012.695260

Fuller MR, Doyle MW, Strayer DL (2015) Causes and consequences of habitat fragmentation in river networks. Annals of the New York Academy of Sciences 1355:31–51. https://doi.org/10.1111/nyas.12853

Gangloff MM, Edgar GJ, Wilson B (2016) Imperilled species in aquatic ecosystems: emerging threats, management and future prognoses. Aquatic Conservation: Marine and Freshwater Ecosystems 26:858–871. https://doi.org/10.1002/aqc.2707

Gess RW, Coates MI, Rubidge BS (2006) A lamprey from the Devonian period of South Africa. Nature 443:981–984. https://doi.org/10.1038/nature05150

Gingera TD, Bajno R, Docker MF, Reist JD (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 8:287–300. https://doi.org/10.3391/mbi.2017.8.3.03

Gingera TD, Steeves TB, Boguski DA, et al (2016) Detection and identification of lampreys in Great Lakes streams using environmental DNA. Journal of Great Lakes Research 42:649–659. https://doi.org/10.1016/j.jglr.2016.02.017

Goldberg CS, Pilliod DS, Arkle RS, Waits LP (2011) Molecular detection of vertebrates in stream water: a demonstration using Rocky Mountain tailed frogs and Idaho giant salamanders. PLoS ONE 6:e22746. https://doi.org/10.1371/journal.pone.0022746

Goldberg CS, Turner CR, Deiner K, et al (2016) Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods in Ecology and Evolution 7:1299–1307. https://doi.org/10.1111/2041-210X.12595

Goodchild CD (1990) Status of the Golden Redhorse, *Moxostoma erythrurum*, in Canada. Canadian Field-Naturalist 104:103–111

Government of Canada (2022) Northern Brook Lamprey, Saskatchewan - Nelson population (2022) - Response Statement. https://species-registry.canada.ca/index-en.html#/documents/attachment/14274-14275. Accessed 23 Jul 2022

Gustavson MS, Collins PC, Finarelli JA, et al (2015) An eDNA assay for Irish *Petromyzon marinus* and *Salmo trutta* and field validation in running water. Journal of Fish Biology 87:1254–1262. https://doi.org/10.1111/jfb.12781

Hall JD (1963) An ecological study of the Chestnut Lamprey, *Ichthyomyzon castaneus* Girard, in the Manistee River, Michigan. PhD thesis, University of Michigan, Ann Arbor

Hänfling B, Handley LL, Read DS, et al (2016) Environmental DNA metabarcoding of lake fish communities reflects long-term data from established survey methods. Molecular Ecology 25:3101–3119. https://doi.org/10.1111/mec.13660

Harper LR, Lawson Handley L, Hahn C, et al (2018) Needle in a haystack? A comparison of eDNA metabarcoding and targeted qPCR for detection of the Great Crested Newt (*Triturus cristatus*). Ecology and Evolution 8:6330–6341. https://doi.org/10.1002/ece3.4013

Hayami K, Sakata MK, Inagawa T, et al (2020) Effects of sampling seasons and locations on fish environmental DNA metabarcoding in dam reservoirs. Ecology and Evolution 10:5354–5367. https://doi.org/10.1002/ece3.6279

Hayer CA, Bayless MF, George A, et al (2020) Use of environmental DNA to detect Grass Carp spawning events. Fishes 5:27. https://doi.org/10.3390/fishes5030027

Heid CA, Stevens J, Livak KJ, Mickey WP (1996) Real time quantitative PCR. Genome Research 6:986–994. https://doi.org/10.1101/gr.6.10.986

Hickerson BT, Maitland BM, Walters AW (2019) Effects of multiple nonnative fish on an imperiled cyprinid, Hornyhead Chub. Trans Am Fish Soc 148:1132–1145. https://doi.org/10.1002/tafs.10203

Hinlo R, Furlan E, Suitor L, Gleeson D (2017a) Environmental DNA monitoring and management of invasive fish: comparison of eDNA and fyke netting. MBI 8:89–100. https://doi.org/10.3391/mbi.2017.8.1.09

Hinlo R, Gleeson D, Lintermans M, Furlan E (2017b) Methods to maximise recovery of environmental DNA from water samples. PLoS ONE 12:e0179251. https://doi.org/10.1371/journal.pone.0179251

Hui D (2012) Food Web: Concepts and Applications. Nature Education Knowledge 3:6

Hundermark EL, Takahashi MK (2020) Improving the yield of environmental DNA from filtered aquatic samples. Conservation Genetics Resources 12:49–51. https://doi.org/10.1007/s12686-018-1067-3

Hunter ME, Ferrante JA, Meigs-Friend G, Ulmer A (2019) Improving eDNA yield and inhibitor reduction through increased water volumes and multi-filter isolation techniques. Scientific Reports 9:5259. https://doi.org/10.1038/s41598-019-40977-w

Hunter ME, Meigs-Friend G, Ferrante JA, et al (2018) Surveys of environmental DNA (eDNA): a new approach to estimate occurrence in Vulnerable manatee populations. Endangered Species Research 35:101–111. https://doi.org/10.3354/esr00880

Jane SF, Wilcox TM, Mckelvey KS, et al (2015) Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. Molecular Ecology Resources 15:216–227. https://doi.org/10.1111/1755-0998.12285

Jerde CL, Mahon AR, Chadderton WL, Lodge DM (2011) "Sight-unseen" detection of rare aquatic species using environmental DNA. Conservation Letters 4:150–157. https://doi.org/10.1111/j.1755-263X.2010.00158.x

Jerde CL, Wilson EA, Dressler TL (2019) Measuring global fish species richness with eDNA metabarcoding. Molecular Ecology Resources 19:19–22. https://doi.org/10.1111/1755-0998.12929

Jeunen G, Lamare MD, Knapp M, et al (2020) Water stratification in the marine biome restricts vertical environmental DNA (eDNA) signal dispersal. Environmental DNA 2:99–111. https://doi.org/10.1002/edn3.49

Jeunen GJ, Knapp M, Spencer HG, et al (2019) Environmental DNA (eDNA) metabarcoding reveals strong discrimination among diverse marine habitats connected by water movement. Molecular Ecology Resources 19:426–438. https://doi.org/10.1111/1755-0998.12982

Jo T, Murakami H, Yamamoto S, et al (2019) Effect of water temperature and fish biomass on environmental DNA shedding, degradation, and size distribution. Ecology and Evolution 9:1135–1146. https://doi.org/10.1002/ece3.4802

Jo T, Sakata MK, Murakami H, et al (2021a) Universal performance of benzalkonium chloride for the preservation of environmental DNA in seawater samples. Limnology and Oceanography: Methods 19:758–768. https://doi.org/10.1002/lom3.10459

Jo T, Takao K, Minamoto T (2021b) Linking the state of environmental DNA to its application for biomonitoring and stock assessment: targeting mitochondrial/nuclear genes, and different DNA fragment lengths and particle sizes. Environmental DNA 271–283. https://doi.org/10.1002/edn3.253

Johnson NS, Buchinger TJ, Li W (2015) Reproductive Ecology of Lampreys. In: Docker MF (ed) Lampreys: Biology, Conservation and Control. Springer, Dordrecht, pp 265–303

Johnson RP (1963) Studies on the life history and ecology of the Bigmouth Buffalo, *Ictiobus cyprinellus* (Valenciennes). Journal of the Fisheries Research Board of Canada 20:1397–1429. https://doi.org/10.1139/f63-095

Kamoroff C, Goldberg CS (2018) Environmental DNA quantification in a spatial and temporal context: a case study examining the removal of Brook Trout from a high alpine basin. Limnology 19:335–342. https://doi.org/10.1007/s10201-018-0551-5

Klymus KE, Ruiz Ramos DV, Thompson NL, Richter CA (2020) Development and testing of species-specific quantitative PCR assays for environmental DNA applications. Journal of Visualized Experiments e61825. https://doi.org/10.3791/61825

Kronenberger JA, Wilcox TM, Mason DH, et al (2022) eDNAssay: a machine learning tool that accurately predicts amplification. Molecular Ecology Resources 00:2994–3005. https://doi.org/10.1111/1755-0998.13681

Kumar G, Eble JE, Gaither MR (2020) A practical guide to sample preservation and pre-PCR processing of aquatic environmental DNA. Molecular Ecology Resources 20:29–39. https://doi.org/10.1111/1755-0998.13107

Kusanke LM, Panteleit J, Stoll S, et al (2020) Detection of the endangered European Weather Loach (*Misgurnus fossilis*) via water and sediment samples: testing multiple eDNA workflows. Ecology and Evolution 10:8331–8344. https://doi.org/10.1002/ece3.6540

Kutyavin IV, Afonina IA, Mills A, et al (2000) 3'-Minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Research 28:655–661. https://doi.org/10.1093/nar/28.2.655

Kwak TJ, Skelly TM (1992) Spawning habitat, behavior, and morphology as isolating mechanisms of the Golden Redhorse, *Moxostoma erythrurum*, and the Black Redhorse, *M. duquesnei*, two syntopic fishes. Environmental Biology of Fishes 34:127–137. https://doi.org/10.1007/BF00002388

Lachner EA (1952) Studies of the biology of the Cyprinid fishes of the chub genus *Nocomis* of Northeastern United States. The American Midland Naturalist 48:433–466. https://doi-org.uml.idm.oclc.org/10.2307/2422260

Lackmann AR, Sereda J, Pollock M, et al (2023) Bet-hedging Bigmouth Buffalo (*Ictiobus cyprinellus*) recruit episodically over a 127-year timeframe in Saskatchewan. Canadian Journal of Fisheries and Aquatic Sciences 80:313–329. https://doi.org/10.1139/cjfas-2022-0122

Lacoursière-Roussel A, Rosabal M, Bernatchez L (2016) Estimating fish abundance and biomass from eDNA concentrations: variability among capture methods and environmental

conditions. Molecular Ecology Resources 16:1401–1414. https://doi.org/10.1111/1755-0998.12522

Ladell BA, Walleser LR, McCalla SG, et al (2019) Ethanol and sodium acetate as a preservation method to delay degradation of environmental DNA. Conservation Genetics Resources 11:83–88. https://doi.org/10.1007/s12686-017-0955-2

Lance RF, Guan X (2020) Variation in inhibitor effects on qPCR assays and implications for eDNA surveys. Canadian Journal of Fisheries and Aquatic Sciences 77:23–33. https://doi.org/10.1139/cjfas-2018-0263

Laporte M, Bougas B, Côté G, et al (2020) Caged fish experiment and hydrodynamic bidimensional modeling highlight the importance to consider 2D dispersion in fluvial environmental DNA studies. Environmental DNA 2:362–372. https://doi.org/10.1002/edn3.88

Laramie MB, Pilliod DS, Goldberg CS, Strickler KM (2015) Environmental DNA sampling protocol - filtering water to capture DNA from aquatic organisms. Techniques and Methods. https://doi.org/10.3133/tm2a13

Lee DS, Gilbert CR, Hocutt CH, et al (1980) Atlas of North American Freshwater Fishes. North Carolina State Museum of Natural History, Raleigh

Lodge DM, Turner CR, Jerde CL, et al (2012) Conservation in a cup of water: estimating biodiversity and population abundance from environmental DNA. Molecular Ecology 21:2555–2558. https://doi.org/10.1111/j.1365-294X.2012.05600.x

Maitland PS, Renaud CB, Quintella BR, et al (2015) Conservation of Native Lamprey. In: Docker MF (ed) Lampreys: Biology, Conservation and Control. Springer, Dordrecht, pp 375–428

Mammoliti CS (2002) The effects of small watershed impoundments on native stream fishes: A focus on the Topeka Shiner and Hornyhead Chub. Transactions of the Kansas Academy of Science 105:219–231. https://doi.org/10.1660/0022-8443(2002)105[0219:TEOSWI]2.0.CO;2

Mandrak NE, Curry RA, Dumont P, et al (2023) Zoogeography of the Freshwater Fishes of Canada. In: Freshwater Fisheries in Canada: Historical and Contemporary Perspectives on the Resources and Their Management. American Fisheries Society, Bethesda

Manzon RG, Youson JH, Holmes JA (2015) Lamprey Metamorphosis. In: Docker MF (ed) Docker MF (ed) Lampreys: Biology, Conservation and Control. Springer, Dordrecht, pp 139–214

Maruyama A, Nakamura K, Yamanaka H, et al (2014) The release rate of environmental DNA from juvenile and adult fish. PLoS ONE 9:e114639. https://doi.org/10.1371/journal.pone.0114639

Mauvisseau Q, Harper LR, Sander M, et al (2022) The multiple states of environmental DNA and what is known about their persistence in aquatic environments. Environmental Science and Technology 56:5322–5333. https://doi.org/10.1021/acs.est.1c07638

Moser ML, Hume JB, Aronsuu KK, et al (2019) Lamprey Reproduction and Early Life History: Insights from Artificial Propagation. In: Docker MF (ed) Lampreys: Biology, Conservation and Control. Springer, Dordrecht, pp 187–245

Neave FB, Booth RMW, Philipps RR, et al (2021) Changes in native lamprey populations in the Great Lakes since the onset of Sea Lamprey (*Petromyzon marinus*) control. Journal of Great Lakes Research 47:S378–S387. https://doi.org/10.1016/j.jglr.2020.10.005

Neave FB, Mandrak NE, Docker MF, Noakes DL (2007) An attempt to differentiate sympatric *Ichthyomyzon* ammocoetes using meristic, morphological, pigmentation, and gonad analyses. Canadian Journal of Zoology 85:549–560. https://doi.org/10.1139/Z07-032

Nevers MB, Byappanahalli MN, Morris CC, et al (2018) Environmental DNA (eDNA): a tool for quantifying the abundant but elusive Round Goby (*Neogobius melanostomus*). PLoS ONE 13:e0191720. https://doi.org/10.1371/journal.pone.0191720

Ogram A, Sayler GS, Barkay T (1987) The extraction and purification of microbial DNA from sediments. Journal of Microbiological Methods 7:57–66. https://doi.org/10.1016/0167-7012(87)90025-X

Olden JD, Hogan ZS, Zanden MJV (2007) Small fish, big fish, red fish, blue fish: size-biased extinction risk of the world's freshwater and marine fishes. Global Ecology and Biogeography 16:694–701. https://doi.org/10.1111/j.1466-8238.2007.00337.x

Pawlowski J, Apothéloz-Perret-Gentil L, Altermatt F (2020) Environmental DNA: What's behind the term? Clarifying the terminology and recommendations for its future use in biomonitoring. Molecular Ecology 29:4258–4264. https://doi.org/10.1111/mec.15643 1

Piggott MP (2016) Evaluating the effects of laboratory protocols on eDNA detection probability for an endangered freshwater fish. Ecology and Evolution 6:2739–2750. https://doi.org/10.1002/ece3.2083

Piggott MP, Banks SC, Broadhurst BT, et al (2021) Comparison of traditional and environmental DNA survey methods for detecting rare and abundant freshwater fish. Aquatic Conservation: Marine and Freshwater Ecosystems 31:173–184. https://doi.org/10.1002/aqc.3474

Pilliod DS, Goldberg CS, Arkle RS, Waits LP (2013) Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. Canadian Journal of Fisheries and Aquatic Sciences 70:1123–1130. https://doi.org/10.1139/cjfas-2013-0047

Pilliod DS, Goldberg CS, Arkle RS, Waits LP (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

Polyakova NV, Kucheryavyy AV, Pavlov DS, Tsimbalov IA (2019) Feeding habits of larval European River Lamprey *Lampetra fluviatilis* from the Chernaya River (Baltic Sea basin). Journal of Ichthyology 59:216–224. https://doi.org/10.1134/S0032945219020164

Pope KL, Goldberg CS, Nelson NL, et al (2020) Designing environmental DNA surveys in complex aquatic systems: Backpack sampling for rare amphibians in Sierra Nevada meadows. Aquatic Conservation: Marine and Freshwater Ecosystems 30:1975–1987. https://doi.org/10.1002/aqc.3444

Postaire BD, Bakker J, Gardiner J, et al (2020) Environmental DNA detection tracks established seasonal occurrence of Blacktip Sharks (*Carcharhinus limbatus*) in a semi-enclosed subtropical bay. Scientific Reports 10:11847. https://doi.org/10.1038/s41598-020-68843-0

Poté J, Ackermann R, Wildi W (2009) Plant leaf mass loss and DNA release in freshwater sediments. Ecotoxicology and Environmental Safety 72:1378–1383. https://doi.org/10.1016/j.ecoenv.2009.04.010

Quinn NWS (2020) Batch spawning in five species of minnows (Cyprinidae) from Ontario, Canada. Canadian Field-Naturalist 133:325–328. https://doi.org/10.22621/cfn.v133i4.1914

Quintella BR, Clemens BJ, Sutton TM, et al (2021) At-sea feeding ecology of parasitic lampreys. Journal of Great Lakes Research 47:S72–S89. https://doi.org/10.1016/j.jglr.2021.07.008

Reid SM, Mandrak NE, Carl LM, Wilson CC (2008) Influence of dams and habitat condition on the distribution of redhorse (*Moxostoma*) species in the Grand River watershed, Ontario. Environmental Biology of Fishes 81:111–125. https://doi.org/10.1007/s10641-006-9179-0

Renaud CB, Cochran PA (2019) Post-metamorphic feeding in lampreys. In: Docker MF (ed) Lampreys: Biology, Conservation and Control. Springer, Dordrecht, pp 247–285

Renaud CB, Ribey SC, Chapleau F (1996) Four records of the Chestnut Lamprey, *Ichthyomyzon castaneus*, new to Ontario. Canadian Field-Naturalist 110:450–453

Renshaw MA, Olds BP, Jerde CL, et al (2015) The room temperature preservation of filtered environmental DNA samples and assimilation into a phenol-chloroform-isoamyl alcohol DNA extraction. Molecular Ecology Resources 15:168–176. https://doi.org/10.1111/1755-0998.12281

Ricklefs RE (2008) Disintegration of the ecological community. American Naturalist 172:741–750. https://doi.org/10.1086/593002

Robinson AT, Paroz YM, Clement MJ, et al (2019) Environmental DNA sampling of small-bodied minnows: performance relative to location, species, and traditional sampling. North American Journal of Fisheries Management 39:1073–1085. https://doi.org/10.1002/nafm.10344

Ruan R, Wang D, Yue H, et al (2020) Using environmental DNA to detect *Hypophthalmichthys molitrix* during the spawning period in the Yangtze River. Conservation Genetics Resources 12:37–39. https://doi.org/10.1007/s12686-018-1062-8

Sales NG, Wangensteen OS, Carvalho DC, Mariani S (2019) Influence of preservation methods, sample medium and sampling time on eDNA recovery in a neotropical river. Environmental DNA 1:119–130. https://doi.org/10.1002/edn3.14

Salter I (2018) Seasonal variability in the persistence of dissolved environmental DNA (eDNA) in a marine system: the role of microbial nutrient limitation. PLoS ONE 13:e0192409. https://doi.org/10.1371/journal.pone.0192409

Schloesser NA, Merkes CM, Rees CB, et al (2018) Correlating Sea Lamprey density with environmental DNA detections in the lab. Management of Biological Invasions 9:483–495. https://doi.org/10.3391/mbi.2018.9.4.11

Schönhuth S, Vukić J, Šanda R, et al (2018) Phylogenetic relationships and classification of the Holarctic family Leuciscidae (Cypriniformes: Cyprinoidei). Molecular Phylogenetics and Evolution 127:781–799. https://doi.org/10.1016/j.ympev.2018.06.026

Scott W, Crossman E (1973) Freshwater Fishes of Canada. Fisheries Research Board of Canada, Ottawa

Shogren AJ, Tank JL, Egan SP, et al (2018) Water flow and biofilm cover influence environmental DNA detection in recirculating streams. Environmental Science and Technology 52:8530–8537. https://doi.org/10.1021/acs.est.8b01822

Smith CJ, Osborn AM (2009) Advantages and limitations of quantitative PCR (qPCR)-based approaches in microbial ecology. FEMS Microbiology Ecology 67:6–20. https://doi.org/10.1111/j.1574-6941.2008.00629.x

So KYK, Fong JJ, Lam IPY, Dudgeon D (2020) Pitfalls during in silico prediction of primer specificity for eDNA surveillance. Ecosphere 11:e03193. https://doi.org/10.1002/ecs2.3193

Spens J, Evans AR, Halfmaerten D, et al (2017) Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. Methods in Ecology and Evolution 8:635–645. https://doi.org/10.1111/2041-210X.12683

Stat M, John J, DiBattista JD, et al (2019) Combined use of eDNA metabarcoding and video surveillance for the assessment of fish biodiversity. Conservation Biology 33:196–205. https://doi.org/10.1111/cobi.13183

Stewart KW, Watkinson DA (2004) The Freshwater Fishes of Manitoba. University of Manitoba Press, Winnipeg

Stoeckle MY, Mishu MD, Charlop-Powers Z (2018) GoFish: a streamlined environmental DNA presence/absence assay for marine vertebrates. bioRxiv e0198717. https://doi.org/10.1101/331322

Strayer DL, Dudgeon D (2010) Freshwater biodiversity conservation: recent progress and future challenges. Journal of the North American Benthological Society 29:344–358. https://doi.org/10.1899/08-171.1

Strickland GJ, Roberts JH (2019) Utility of eDNA and occupancy models for monitoring an endangered fish across diverse riverine habitats. Hydrobiologia 826:129–144. https://doi.org/10.1007/s10750-018-3723-8

Strickler KM, Fremier AK, Goldberg CS (2015) Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. Biological Conservation 183:85–92. https://doi.org/10.1016/j.biocon.2014.11.038

Sutton TM, Bowen SH (1994) Significance of organic detritus. Canadian Journal of Fisheries and Aquatic Sciences 51:2380–2387

Taberlet P, Coissac E, Hajibabaei M, Rieseberg LH (2012) Environmental DNA. Molecular Ecology 21:1789–1793. https://doi.org/10.1111/j.1365-294X.2012.05542.x

Takahara T, Minamoto T, Doi H (2015) Effects of sample processing on the detection rate of environmental DNA from the Common Carp (*Cyprinus carpio*). Biological Conservation 183:64–69. https://doi.org/10.1016/j.biocon.2014.11.014

Takahara T, Taguchi J, Yamagishi S, et al (2020) Suppression of environmental DNA degradation in water samples associated with different storage temperature and period using benzalkonium chloride. Limnology and Oceanography: Methods 18:437–445. https://doi.org/10.1002/lom3.10374

Tan M, Armbruster JW (2018) Phylogenetic classification of extant genera of fishes of the order Cypriniformes (Teleostei: Ostariophysi). Zootaxa 4476:6–39. https://doi.org/10.11646/zootaxa.4476.1.4

Thomsen PF, Kielgast J, Iversen LL, et al (2012a) Monitoring endangered freshwater biodiversity using environmental DNA. Molecular Ecology 21:2565–2573. https://doi.org/10.1111/j.1365-294X.2011.05418.x

Thomsen PF, Kielgast J, Iversen LL, et al (2012b) Detection of a diverse marine fish fauna using environmental DNA from seawater samples. PLoS ONE 7:e41732. https://doi.org/10.1371/journal.pone.0041732

Thomsen PF, Willerslev E (2015) Environmental DNA - an emerging tool in conservation for monitoring past and present biodiversity. Biological Conservation 183:4–18. https://doi.org/10.1016/j.biocon.2014.11.019

Tillotson MD, Kelly RP, Duda JJ, et al (2018) Concentrations of environmental DNA (eDNA) reflect spawning salmon abundance at fine spatial and temporal scales. Biological Conservation 220:1–11. https://doi.org/10.1016/j.biocon.2018.01.030

Vives SP (1990) Nesting ecology and behavior of Hornyhead Chub *Nocomis biguttatus*, a Keystone Species in Allequash Creek, Wisconsin. The American Midland Naturalist 124:46–56. https://doi.org/10.2307/2426078

Vörösmarty CJ, McIntyre PB, Gessner MO, et al (2010) Global threats to human water security and river biodiversity. Nature 467:555–561. https://doi.org/10.1038/nature09440

Wegleitner BJ, Jerde CL, Tucker A, et al (2015) Long duration, room temperature preservation of filtered eDNA samples. Conservation Genetics Resources 7:789–791. https://doi.org/10.1007/s12686-015-0483-x

Whitney JE, Waters R, Holloway JA (2020) Muddying the waters: investigating the generality of silt-resistance in mound-building *Nocomis* spp. using hornyhead chub (*Nocomis biguttatus*) and redspot chub (*Nocomis asper*). Environmental Biology of Fishes 103:815–831. https://doi.org/10.1007/s10641-020-00984-7

Wilcox TM, McKelvey KS, Young MK, et al (2013) Robust detection of rare species using environmental DNA: the importance of primer specificity. PLoS ONE 8:e59520. https://doi.org/10.1371/journal.pone.0059520

Williams KE, Huyvaert KP, Piaggio AJ (2017) Clearing muddied waters: capture of environmental DNA from turbid waters. PLoS ONE 12:e0179282. https://doi.org/10.1371/journal.pone.0179282

Williams KE, Huyvaert KP, Piaggio AJ (2016) No filters, no fridges: a method for preservation of water samples for eDNA analysis. BMC Research Notes 9:298. https://doi.org/10.1186/s13104-016-2104-5

Wilson IG (1997) Inhibition and facilitation of nucleic acid amplification. Applied and Environmental Microbiology 63:3741–3751. https://doi.org/10.1128/aem.63.10.3741-3751.1997

Wisenden BD, Unruh A, Morantes A, et al (2009) Functional constraints on nest characteristics of pebble mounds of breeding male Hornyhead Chub *Nocomis biguttatus*. Journal of Fish Biology 75:1577–1585. https://doi.org/10.1111/j.1095-8649.2009.02384.x

Xia Z, Johansson ML, Gao Y, et al (2018) Conventional versus real-time quantitative PCR for rare species detection. Ecology and Evolution 8:11799–11807. https://doi.org/10.1002/ece3.4636

Yamanaka H, Minamoto T, Matsuura J, et al (2017) A simple method for preserving environmental DNA in water samples at ambient temperature by addition of cationic surfactant. Limnology 18:233–241. https://doi.org/10.1007/s10201-016-0508-5

Yao Y, Nellåker C, Karlsson H (2006) Evaluation of minor groove binding probe and TaqMan probe PCR assays: influence of mismatches and template complexity on quantification. Molecular and Cellular Probes 20:311–316. https://doi.org/10.1016/j.mcp.2006.03.003

Yap MR, Bowen SH (2003) Feeding by Northern Brook Lamprey (*Ichthyomyzon fossor*) on sestonic biofilm fragments: Habitat selection results in ingestion of a higher quality diet. Journal of Great Lakes Research 29:15–25. https://doi.org/10.1016/S0380-1330(03)70475-4

Youson JH (1980) Morphology and physiology of lamprey metamorphosis. Canadian Journal of Fisheries and Aquatic Sciences 37:1687–1710. https://doi.org/10.1139/f80-216

Zhu B (2006) Degradation of plasmid and plant DNA in water microcosms monitored by natural transformation and real-time polymerase chain reaction (PCR). Water Research 40:3231–3238. https://doi.org/10.1016/j.watres.2006.06.040

CHAPTER 2 DEVELOPING AND VALIDATING ENVIRONMENTAL DNA QUANTITATIVE PCR ASSAYS

2.1 Introduction

To detect a species with eDNA, one must design an eDNA assay. A brief comparison of the different types of eDNA assays and analyses can be found in Chapter 1 (*see* Section 1.4 Environmental DNA); however, this chapter will overview the design and validation of probe-based quantitative polymerase chain reaction (qPCR) assays using TaqMan™ minor-groove-binder (MGB) probes. These assays consist of a forward primer, a reverse primer, and a probe. TaqMan™ MGB probes consist of reporter dye at the 5' end of the probe and a non-fluorescence quencher and MGB at the 3' end of the binding region of the probe. Through the activity of the *Taq* polymerase enzyme during the extension of a new strand of DNA, the fluorescent reporter dye is cleaved, which results in the production of fluorescence that can be detected and quantified by the qPCR machine. The MGB molecule at the 3' end of the probe increases the melting temperature of the probe and allows for significantly shorter probes with greater specificity than traditional dual-reporter probes (Kutyavin et al. 2000; Yao et al. 2006; Wilcox et al. 2013). However, regardless of the assay type and method of analysis used, throughout validation of the designed assay and method is necessary for accurate results.

2.1.1 Assay Design

The first step in designing an assay is to determine a candidate region(s) to target. Typically, mitochondrial DNA (mtDNA) is targeted for eDNA assay development as mtDNA gene sequences have been characterized for many fish species (Hubert et al. 2008), there is significant divergence in mtDNA gene sequences across species yet intraspecific diversity of mtDNA sequences is low, and multiple (hundreds or thousands) of mt genomes in each cell (compared to a single nuclear genome) increases sensitivity (Goldberg et al. 2016; Tsuji et al.

2019). However, nuclear DNA has also been targeted for eDNA assay development (Minamoto et al. 2017). Many mt genes, such as cytochrome oxidase c subunit I (CO1), cytochrome oxidase *b* (CytB), NADH dehydrogenase subunit 1, NADH dehydrogenase subunit 4, and 16S ribosomal RNA, have been the target for eDNA assay development (Tsuji et al. 2019). The CO1 gene is often targeted as it is considered the "Barcode of Life" and sequences are widely available on public databases (Ratnasingham and Hebert 2007), including for almost all freshwater fishes of Canada (Hubert et al. 2008).

2.1.2 Assay Validation

Before detections of eDNA using a developed assay can be reliable, the assay must undergo validation so future results can be interpreted accurately and with confidence. To ensure species- or genus-specificity, developed assays undergo a three-step process: *in silico* validation, *in vitro* validation, and *in situ* validation (Goldberg et al. 2016; Abbott et al. 2021).

2.1.2.1 In Silico Validation

The objective of *in silico* validation is to predict the level of specificity of an assay by comparing the primer and probe sequences to all available sequence data. In this stage, researchers confirm that the primer and probe sequences match and will bind to the targeted gene region of the target species and, ideally, show sufficient mismatches to sequences of the same region of non-target species. At a minimum, the designed assay should not be predicted to bind and amplify regions of DNA of non-target species that are found within the geographical region in which the assay will be used; ideally, it should also not amplify DNA from non-target species that the samples might encounter (e.g., DNA of a species researched in the same laboratory).

2.1.2.2 In Vitro Validation

Testing the developed assays using known concentrations of tissue-derived DNA is a key step in the validation of an assay, commonly referred to as *in vitro* validation. During this step,

assays are tested against either tissue-derived DNA from multiple specimens of the target species and tissue-derived DNA from species identified as risks for non-target amplification, or synthetic oligo fragments of the target region of the target species and non-target species (e.g., gBlock®, Integrated DNA Technologies). While determining specificity, the optimal primer and probe concentrations are also determined for each assay using target DNA and different concentrations of the primers and the probe. If multiple concentrations of primers and probe yield equivalent amplification, the lowest concentration is selected to minimize the cost per reaction and risk of non-specific amplification.

2.1.2.3 Determining Limit of Detection, Limit of Quantification, and eDNA Concentration

Once assays are validated *in silico* and *in vitro*, the limit of detection (LOD), the limit of quantification (LOQ), and the assay efficiency for each assay can be determined. The method by which the LOD and LOQ of a designed assay for its target are determined can vary depending on the specific eDNA assay, the experimental conditions, and the study design; thus, definitions for both can vary in the literature (Forootan et al. 2017; Hunter et al. 2018; Klymus et al. 2020a; LeBlanc et al. 2020). However, methods to determine the LOD and LOQ are either statistical, which calculates the point at which the amplified signal is distinguishable from background fluorescence, or empirical, which tests serial dilutions of known concentrations of a target sample, including (*see* Burd 2010):

- **Probit analysis:** A statistical method used to determine the LOD by analyzing the relationship between the concentration of the target DNA and the probability of detection.
- **End-point detection**: Determines the lowest concentration of target DNA that can be reliably detected in a sample.

- **Standard curve:** Generates a standard curve using known concentrations of target DNA and determines the LOD and LOQ as the lowest concentration and lowest quantifiable concentration that falls within the linear range of the curve.
- **Precision-based:** Calculates the LOD and LOQ based on the precision of the assay, considering factors such as repeatability and reproducibility of measurements.
- **Standard addition**: Adds known amounts of target DNA to samples of different concentrations and defines the LOQ as the lowest concentration that can be accurately quantified.

To assess the LOD, LOQ, and assay efficiency for the methods and primer and probe combination used, these methods compare the threshold cycle (C_t) values (i.e., the number of cycles it takes for the fluorescence generated by the reaction to surpass the background fluorescence) and known concentrations of target DNA, which can be quantified tissue-derived DNA from the target species or synthetic DNA oligonucleotides of the target species (e.g., gBlocks®). The C_t value indicates how many cycles it takes for a detectable amount of product to be generated and, all else being equal, varies inversely with the concentration of target DNA in a sample (i.e., a low C_t value is indicative of a higher DNA concentration). Plate-to-plate variations of LOD estimates are common; thus, many researchers suggest repeating LOD analyses and estimating it multiple times, when possible (Hunter et al. 2018; Klymus et al. 2020a).

2.1.2.4 In Situ Validation

Before broad-scale use of a developed assay, *in situ* validation of designed assays should occur to ensure the assay works *in situ* (i.e., on-site), as environmental factors (*see* Section 1.4 Environmental DNA) and biological factors (e.g., competing DNA) can affect the amplification of the target DNA (Wilcox et al. 2013). Various routes can be taken to validate assays *in situ*,

such as paired sampling, where eDNA samples are taken before capture-based sampling, or spiking eDNA samples with a known concentration of target DNA to simulate environmental inhibition. When possible, samples should be collected from sites where the target species is expected to occur, as well as sites where it is not expected to occur to assess for the possibility of false positives, where amplification occurs without the presence of target DNA.

2.2 Methods

2.2.1 Assay Design

Assays were designed using Geneious Prime software (https://www.geneious.com) and Primer Express v3.0.1 (Applied Biosystems). Alignments of target gene sequences (i.e., CO1, CytB) were generated using Geneious for each species, including multiple sequences of all species within the family of each target species (i.e., Leuciscidae for Hornyhead Chub (*Nocomis biguttatus)*, Catostomidae for Bigmouth Buffalo (*Ictiobus cyprinellus*) and Golden Redhorse (*Moxostoma erythrurum*), and Petromyzontidae for Chestnut (*Ichthyomyzon castaneus*), Northern Brook (*I. fossor*), and Silver (*I. unicuspis*) lampreys) that occurred in the Saskatchewan-Nelson River biogeographical zone, as defined by COSEWIC (COSEWIC 2020). Additional alignments of all species within the family of each target species that occur in Canada were generated to assess the level of specificity of the assay once designed. The sequences used for the alignments were pulled from a custom database of sequences generated by Genomic Network for Fish Identification, Stress and Health (GEN-FISH, unpublished). Any regions where there were differences in the nucleotides of the target species and non-target species were noted.

Unique regions of the target species sequences were inputted into Primer Express to develop candidate primers and probe combinations for each assay. Melting temperatures and difference in melting temperatures between the primers and probe, the percentage of guanine and cytosine (GC) content of the sequence, the likelihood of hairpin and dimer structures, and

amplicon, primers and probe lengths were all considered following the guidelines outlined by Primer Express (Thermo Fisher Scientific 2022). The primer pairs and probe with the highest scores were considered for further testing.

To eliminate any candidate primers and probes that were not species-specific, the primers and probes were tested against a CO1 sequence alignment of 2,381 sequences, 600 bp in length, including sequences from all freshwater fishes in Canada and some invasive freshwater fishes, also generated by GEN-FISH (unpublished), to see if the assay matches to the complementary sequences of any non-target species. Any primers or probes that were predicted to bind 100% to the non-target species *in vitro* were not considered. Of the remaining candidate primers and probe sets, the sets with the highest score on Primer Express and showed the lowest likelihood of non-specific binding were chosen to undergo assay validation. For the species-specific assays, FAM was chosen as the probe dye when possible; however, when it was not possible to use FAM (i.e., the 5' end of the probe begins with guanine), VIC was used (Table 2.1).

The protocol described above was also followed to design the assays targeting regions of the CytB gene. An alignment of 5,601 sequences of 626 bp in length, containing sequences of most freshwater fishes in Canada and many invasive freshwater fishes, generated by GEN-FISH (unpublished) was used to determine if non-target binding is occurring with any of the developed CytB assays. The CytB assays were designed using Geneious Prime and Primer Express. The sequences that were used to design the primers and probes were pulled from a custom database of CytB sequences generated by GEN-FISH (unpublished) and from NCBI Gen-Bank. Accession numbers and the date accessed were recorded for all sequences. When possible, multiple sequences for each species from different biogeographical zones were used.

2.2.1.2 In Silico Validation

Primer-BLAST (Basic Local Alignment Search Tool; GenBank www.ncbi.nlm.nih.gov/blast) was used to compare the primer and probe sequences to the available sequence data and identify species where the assay binds to sequences of DNA of non-target species either partially or entirely. Any species that are closely related to the target species and/or co-occur with the target species that did not bind to the assay during the initial Primer-BLAST search were manually searched to identify the likelihood of non-target amplification. The specificity of the primer pairs and probe for each assay were further investigated using a CO1 sequence alignment of 5,466 sequences generated by GEN-FISH (unpublished) containing sequences from all congeneric species of Canadian freshwater fishes. Any primer pairs that bound at least 80% (e.g., 5 mismatches out of 25 nucleotides) to the forward and reverse primer were considered risks for non-target amplification and were recorded. The percentage of the probe bound to the non-target sequences was assessed and recorded for all species identified as risks for non-target amplification during the assessment of the primers, and for all species where the probe bound to non-target regions at least 80%. All species that were considered risks for non-target amplification were categorized by their distribution in Canada and only species that occur in Manitoba (including species that are native or invasive/at risk of invasion) were considered for *in vitro* validation. If assays had a high likelihood of non-target amplification (e.g., 100% of primers or probe bound to closely related non-target species found in Manitoba), the assay was re-designed until an assay with enough mismatches was designed.

2.2.1.3 In Vitro Validation

The tissue-derived DNA was extracted from fin clippings or other tissue using the DNeasy Blood and Tissue Kit following the purification of total DNA from animal tissues

Table 2.1 Summary of the TaqMan™ probe-based CO1 assays used in this project. Each assay consists of two primers and one probe which targets a sequence on the cytochrome *c* oxidase subunit 1 (CO1) gene for the species of interest. The optimal annealing temperature (T_a), amplicon size (base pair, bp), and probe reporter dye for each assay are given.

Species	Name	Marker	Sequence (5'-3')	T_a (°C)	Size (bp)	Dye	Validation
Bigmouth Buffalo	BMBF CO1	Forward	CCCCACTTGCGGGTAATCT	60	85	VIC	Complete
		Reverse	ATTGATGAAACTCCTGCTAGGTGAA				
		Probe	AGCTTCAGTAGACTTGACTA				
Chestnut Lamprey	CHLP CO1	Forward	GGATGAACTGTATACCCCCCTTT	58	85	FAM	Complete
		Reverse	CGAGGTGTAGGGAGAAGATTGTTAA				
		Probe	TCACACAGGAGCCTCTA				
Golden Redhorse	GDRH CO1	Forward	AGCATTCCCCCGGATGA	58	80	FAM	Complete
		Reverse	CAACTCCGGAAGAAGCCAATAA				
		Probe	CAATATAAGCTTCTGACTCCT				
Hornyhead Chub	HHCH CO1	Forward	ATCAGACACCCCTCTTTGTTTGA	57	80	FAM	Complete
		Reverse	TCCAGCAGCTAGGACAGGTAATG				
		Probe	TAACTGCTGTTCTTCTACTACTA				

protocol (spin-column protocol), with modifications to increase genomic DNA yield and purity. Lysis time was increased to 18–24 hours to increase the digestion of tissue during incubation and yield of DNA. To ensure genomic DNA was RNA-free, 3 µl of RNase A was added to samples after incubation and mixed by vortexing, then incubated for 5 minutes at 56°C. To increase the final DNA concentration, 60 µl of Buffer AE heated at 70°C for a minimum of 15 minutes was used to elute the final eluate. Once extracted and eluted, the tissue-derived DNA was quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific). All tissue extractions occurred in a separate laboratory from any qPCR or eDNA extractions, in a UV3 HEPA PCR workstation (Ultra-Violet Products Ltd.) equipped with high-efficiency particulate air (HEPA) filtration. The surfaces in the PCR workstation, all equipment used, and the countertops surrounding the workstation were wiped down with ELIMINase® decontaminant, then 70% ethanol. All equipment within the PCR workstation was exposed to ultra-violet (UV) light for a minimum of 15 minutes between tissue extractions.

As non-target amplification during *in vitro* validation can be a result of contamination (Goldberg et al. 2016; Klymus et al. 2020b), any reactions where non-target amplification occurred was re-run with serial dilutions of the non-target DNA and target DNA from 10 ng/µl to 1 x 10^{-4} ng/µl. In cases where non-target DNA continued to be amplified at low dilutions, DNA was extracted from the tissue of a different non-target specimen to confirm the non-target amplification was not due to contamination of non-target tissue with target DNA. If the assay continued to amplify the non-target DNA, the primers and probe were designated as non-specific and re-designed.

All designed and ordered CO1 assays were tested using qPCR and the entire assay (i.e., primers and probe). Primer pairs of designed CytB assays deemed likely to be specific were ordered and

tested first using end-point PCR to assess the efficiency of target amplification and the specificity of the assay and to test for the presence of primer dimers. A standard protocol was used for all end-point PCR reactions, consisting of 3 µl molecular grade water (MGW), 5 µl DreamTaq Green PCR Mastermix, 1 µl of both forward and reverse primers (10 µM), and 5 µl of template DNA; for a total reaction volume of 15 µl. The cycling conditions were set to 95ºC for 5 minutes for initial denaturation, followed by 35 cycles of 95ºC for 30 seconds for denaturation, the assay annealing temperature (Table 2.1, Table 2.2, Table 2.3, Table 2.4) for 30 seconds for primer annealing, and 72ºC for 30 seconds for primer extension, followed by 72ºC for 5 minutes for a final extension step. Once the cycle was completed, the thermocycler was set to hold indefinitely at 4ºC. Each run included one negative control which consisted of MGW in place of DNA, and one replicate of each DNA sample. The products from the end-point PCR were visualized using 1.5% agarose gel with 300 mL of 1% ethidium bromide. Into each well, 6 µl of PCR product was loaded, and to the leftmost and rightmost lane of the gel, 3 µl of 1 kilobase pair ladder (Invitrogen™ 1 Kb Plus DNA Ladder, Thermo Fisher Scientific) was added. The DNA was visualized on the agarose gel after electrophoresis at a voltage of 100V, and an amperage of 200 mAmp for 20–30 minutes, and subsequently visualized using a UV light imager (Bio-Rad Laboratories Inc. ChemiDoc™ XRS+ Gel Imaging System, Hercules, CA, USA) for visualization of DNA fragments.

2.2.1.4 Determining Limit of Detection and Limit of Quantification

This project followed the definitions for LOD and LOQ, and the protocol outlined by Klymus et al. (2020), in which the LOD reflects the confidence in the ability of the assay to detect eDNA in a sample, whereas the LOQ reflects the precision of the quantification of eDNA in a sample. To determine the LOD and LOQ of each of the designed CO1 assays, a standard

Table 2.2 Summary of the TaqMan™ probe-based assays designed to target a region of the CO1 gene of Silver and Northern Brook lampreys. Each assay consists of two primers and one probe which targets a sequence on the cytochrome *c* oxidase subunit 1 (CO1) gene for the species of interest. The optimal annealing temperature (T_a), amplicon size (base pair, bp), and probe reporter dye for each assay are given. All assays tested *in vitro* were determined to be not species-specific as they amplified Chestnut Lamprey *Ichthyomyzon castaneus* DNA.

Name	Oligo Name	Marker	Sequence (5'-3')	T_a (°C)	Size (bp)	Dye	Validation
NBLPCO12022	NBLP_COI_M116	Forward	CAATCATAATTGGAGGCTTTGGT	57	80	VIC	Completed qPCR; not species-specific*
	NBLP_COI_M245	Reverse	GTTTATACGTGGGAAGGCCATATC				
	NBLP_COI_M199	Probe	TGCCACTAATACTAAGCG				
NBLP_M116	NBLP_COI_M116	Forward	CAATCATAATTGGAGGCTTTGGT	56	119	FAM	Completed qPCR; not species-specific*
	NBLP_COI_M284	Reverse	TAGAAGTAATGATGGTGGAAGTAATCAAAA				
	NBLP_COI_M231	Probe	TTCCCACGTATAAACAACATAA				
NBLP_131	NBLP_COI_M131	Forward	CCATGCTTTCGTCATAATCTTTTTC	56	115	FAM	Completed PCR; likely not species-specific*
	NBLP_COI_M245	Reverse	GTTTATACGTGGGAAGGCCATATC				
	NBLP_COI_M167	Probe	AATCATAATTGGAGGCTTTGG				
NBLP_399	NBLP_COI_M399	Forward	TTCACCTCGCTGGAATTTCAT	53	150	FAM	Completed PCR; likely not species-specific*
	NBLP_COI_M548	Reverse	GTAGAGATAATAGGAGAAGGACTGCAGTAA				
	NBLP_COI_M495	Probe	CTTTATTTGTTTGATCCGTCCTA				

*Assay binds to and amplifies Chestnut Lamprey DNA.

Table 2.3 Summary of the TaqMan™ probe-based CytB assays used in this project. Each of the assays consists of two primers and one probe which targets a sequence on the cytochrome *b* (CytB) gene for the species of interest. The optimal annealing temperature (T_a), amplicon size (base pair, bp), and probe reporter dye for each assay are given.

Species	Name	Marker	Sequence (5'-3')	T_a (°C)	Size	Dye	Validation
Chestnut Lamprey	CHLP CytB	Forward	TGGAGGAGTTCTAGCCTTAGTAATAGC	55	90	FAM	Tested primers using end-point PCR
		Reverse	CGAAATTGTATGCTTCGTTGTTTAG				
		Probe	TCTCCTAATTATCCCTCTTAC				
Northern Brook/Silver lampreys	NBLP CytB	Forward	TTGGAGGAGTTTTAGCTTTAGTCATG	57	90	FAM	Complete
		Reverse	GAAATTGTATGCCTCGCTGCTT				
		Probe	TATCCTAATCCTCCTAATCAT				
Bigmouth Buffalo	BMBF CytB	Forward	CGCCCCATTACCCAATTTCT	58	90	FAM	Tested primers using end-point PCR
		Reverse	GAATGGGTGCTCTACTGGCATT				
		Probe	CCCTTGTTGCCGATATA				
Golden Redhorse	GDRH CytB	Forward	TCGGTGTTGTCCTTCTTCTGCTA	58	90	FAM	Tested primers using end-point PCR
		Reverse	CCGTGGCACCTCAAAACG				
		Probe	ATACGTGCTTCCATGAGGA				
Hornyhead Chub	HHCH CytB	Forward	GCCGGTGCCACAGTTCTC	57	90	FAM	Tested primers using end-point PCR
		Reverse	GGAAATTTTATCTGCGTCGGAAT				
		Probe	TCTACTATTCTTACATGAGACAGG				

Table 2.4 Summary of the TaqMan™ probe-based assays validated in situ for each target species. Each of the assays consists of two primers and one probe which targets a sequence on the cytochrome *b* (CytB) gene for the species of interest. The optimal annealing temperature (T_a), amplicon size (base pair, bp), and probe reporter dye for each assay are given.

Target Species	Name	Marker	Sequence (5'-3')	T_a (°C)	Size (bp)	Dye
Bigmouth Buffalo	BMBF CO1	Forward	CCCCACTTGCGGGTAATCT	60	85	VIC
		Reverse	ATTGATGAAACTCCTGCTAGGTGAA			
		Probe	AGCTTCAGTAGACTTGACTA			
Golden Redhorse	GDRH CO1	Forward	AGCATTCCCCCGGATGA	58	80	FAM
		Reverse	CAACTCCGGAAGAAGCCAATAA			
		Probe	CAATATAAGCTTCTGACTCCT			
Hornyhead Chub	HHCH CO1	Forward	ATCAGACACCCCTCTTTGTTTGA	57	80	FAM
		Reverse	TCCAGCAGCTAGGACAGGTAATG			
		Probe	TAACTGCTGTTCTTCTACTACTA			
Northern Brook/Silver lampreys	NBLP CytB	Forward	TTGGAGGAGTTTTAGCTTTAGTCATG	57	90	FAM
		Reverse	GAAATTGTATGCCTCGCTGCTT			
		Probe	TATCCTAATCCTCCTAATCAT			
Chestnut Lamprey	CHLP CO1	Forward	GGATGAACTGTATACCCCCCTTT	58	85	FAM
		Reverse	CGAGGTGTAGGGAGAAGATTGTTAA			
		Probe	TCACACAGGAGCCTCTA			

curve was generated using the data from a 6-point, 4-fold serial dilution with 8 replicates per dilution using gene fragments of the barcode region of the CO1 gene for the target species (gBlock®, Integrated DNA Technologies), with a starting concentration between 71.0 and 77.6 copies/µl (Table 2.5), following the protocol outlined in Klymus et al. (2020a). For CytB assays, tissue-extracted DNA standardized to 10 ng/µl was used in place of gBlock®. For this project, the CV for the LOQ was set to a standard value of 35. The data generated from each standard curve for each species was run through the LOD/LOQ calculator R script (Merkes et al. 2019; Klymus et al. 2020a) using R (version 4.1.1).

2.2.1.5 In Situ Validation

One designed assay per species was validated *in situ* by pairing eDNA sampling with capture-based electrofishing to link positive eDNA detections with physical collections of the species of interest (*see* Chapter 4). To test for false positive detections using the designed assays, extracted eDNA from water samples collected at sites outside of the known and possible range of the target species were analyzed using the designed target assays to ensure that reactions using the assay did not result in amplification in the absence of the target DNA (*see* Chapter 3).

2.3 Results

2.3.1 Assay Design

Species-specific TaqMan™ MGB probe-based assays were designed, validated, and used for each target species except for paired species, Northern Brook and Silver lampreys (*see* Chapter 1), where one assay that detects both species was designed, as regions with fixed genetic differences that could distinguish the two species reliably using eDNA are not yet known (Table 2.1, Table 2.2, Table 2.3, Table 2.4). Two assays were designed per species (or species pair); one targeting the CO1 gene, and one targeting the CytB gene (Table 2.1, Table 2.3). Candidate species-specific TaqMan™ MGB probe-based assays that target regions of the CO1 gene of

Table 2.5 Non-target species tested for non-target amplification in-vitro using designed species-specific CO1 and CytB assays. Bolded rows are the target species for the assay tested. "N" indicates no amplification detected and "Y" indicates detectable amplification within the 45 cycles run using qPCR.

a)

Common Name	Scientific Name	Amplification	Assay
Bigmouth Buffalo	***Ictiobus cyprinellus***	**Y**	**BMBF CO1**
Blackchin Shiner	*Miniellus heterodon*	N	BMBF CO1
Blacknose Shiner	*Notropis heterolepis*	N	BMBF CO1
Brassy Minnow	*Hybognathus hankinsoni*	N	BMBF CO1
Common Carp	*Cyprinus carpio*	N	BMBF CO1
Common Shiner	*Luxilus cornutus*	N	BMBF CO1
Fathead Minnow	*Pimephales promelas*	N	BMBF CO1
Flathead Chub	*Platygobio gracilis*	N	BMBF CO1
Lake Chub	*Couesius plumbeus*	N	BMBF CO1
Longnose Sucker	*Catostomus catostomus*	N	BMBF CO1
Mimic Shiner	*Paranotropis volucellus*	N	BMBF CO1
Northern Pearl Dace	*Margariscus nachtriebi*	N	BMBF CO1
Quillback	*Carpiodes cyprinus*	N	BMBF CO1
Sand Shiner	*Miniellus stramineus*	N	BMBF CO1
Silver Chub	*Macrhybopsis storeriana*	N	BMBF CO1
Spottail Shiner	*Hudsonius hudsonius*	N	BMBF CO1
Weed Shiner	*Alburnops texanus*	N	BMBF CO1
White Sucker	*Catostomus commersonii*	N	BMBF CO1

b)

Common Name	Scientific Name	Amplification	Assay
Bigmouth Shiner	*Ericymba dorsalis*	N	HHCH CO1
Blackchin Shiner	*Miniellus heterodon*	N	HHCH CO1
Bluntnose Minnow	*Pimephales notatus*	N	HHCH CO1
Brassy Minnow	*Hybognathus hankinsoni*	N	HHCH CO1
Carmine Shiner	*Notropis percobromus*	N	HHCH CO1
Common Carp	*Cyprinus carpio*	N	HHCH CO1
Common Shiner	*Luxilus cornutus*	N	HHCH CO1
Emerald Shiner	*Notropis atherinoides*	N	HHCH CO1
Fathead Minnow	*Pimephales promelas*	N	HHCH CO1
Flathead Chub	*Platygobio gracilis*	N	HHCH CO1
Hornyhead Chub	***Nocomis biguttatus***	**Y**	**HHCH CO1**
Mimic Shiner	*Paranotropis volucellus*	N	HHCH CO1
Northern Pearl Dace	*Margariscus nachtriebi*	N	HHCH CO1
Sand Shiner	*Miniellus stramineus*	N	HHCH CO1
Silver Chub	*Macrhybopsis storeriana*	N	HHCH CO1
Spottail Shiner	*Hudsonius hudsonius*	N	HHCH CO1
Weed Shiner	*Alburnops texanus*	N	HHCH CO1
Western Blacknose Dace	*Rhinichthys obtusus*	N	HHCH CO1

c)

Common Name	Scientific Name	Amplification	Assay
Golden Redhorse	***Moxostoma erythrurum***	**Y**	**GDRH CO1**
Silver Chub	*Macrhybopsis storeriana*	N	GDRH CO1
White Sucker	*Catostomus commersonii*	N	GDRH CO1

d)

Common Name	Scientific Name	Amplification	Assay
American Brook Lamprey	*Lethenteron appendix*	N	CHLP CO1
Chestnut Lamprey	***Ichthyomyzon castaneus***	**Y**	CHLP CO1
Northern Brook Lamprey	*Ichthyomyzon fossor*	N	CHLP CO1
Sea Lamprey	*Petromyzon marinus*	N	CHLP CO1
Silver Lamprey	*Ichthyomyzon unicuspis*	N	CHLP CO1

e)

Common Name	Scientific Name	Amplification	Assay
American Brook Lamprey	*Lethenteron appendix*	N	NBLP CytB
Chestnut Lamprey	*Ichthyomyzon castaneus*	N	NBLP CytB
Northern Brook Lamprey	***Ichthyomyzon fossor***	**Y**	NBLP CytB
Sea Lamprey	*Petromyzon marinus*	N	NBLP CytB
Silver Lamprey	***Ichthyomyzon unicuspis***	**Y**	NBLP CytB

Bigmouth Buffalo and Golden Redhorse were developed by A. Khan (University of Manitoba), and validated *in silico, in vitro* and *in situ* (Table 2.1). All other assays were designed for and validated during this project.

2.3.1.2 In Silico Validation

The forward and reverse primer, and probe sequences of each candidate assay were tested against an alignment of all freshwater fishes in Canada, and the NCBI GenBank genetic database to determine the likelihood of non-target amplification determined by the number and location of mismatches between the target and non-target sequence. An emphasis was placed on mismatches between co-occurring non-target species (i.e., other species that are found in Manitoba) rather than species that are known to not occur in Manitoba. A minimum of 5 mismatches were necessary between the designed assay sequences and the target gene region of co-occurring sequences. Assays were designed until at least one candidate assay worthy of ordering was determined through *in silico* validation per species, per target gene. A minimum of one potential assay per target gene, per species, was ordered and tested further *in vitro.*

Bigmouth Buffalo CO1

The designed primers were run through PrimerBLAST to compare the genetic sequence of Bigmouth Buffalo at the binding region of the primers to non-target species. A total of 16 species found in Manitoba were identified as potential risks of non-target amplification due to a low number of mismatches between the binding region of the primers and their genetic sequence at the target region of the CO1 gene. All species were noted, and the likelihood of the probe binding to the respective non-target region of those species was then assessed manually using nBLAST. A minimum of 5 mismatches were noted between the designed assay and all identified Manitoban non-target species; thus, the assay was ordered and continued to *in vitro* validation.

Chestnut Lamprey CO1

In total, 18 assay options were designed for Chestnut Lamprey targeting the CO1 gene. Amplicon lengths ranged from 80 to 141 base pairs. An emphasis was placed on mismatches between co-occurring Silver and Northern Brook lampreys; however, each candidate assay was assessed for the number of mismatches between it and the target binding region of Southern Brook Lamprey *Ichthyomyzon gagei*, Ohio Lamprey *Ichthyomyzon bdellium*, Mountain Brook Lamprey *Ichthyomyzon greeleyi*, Sea Lamprey *Petromyzon marinus*, and American Brook Lamprey *Lethenteron appendix*. *In silico* validation of the designed assay suggested the designed Chestnut Lamprey CO1 assay will amplify its paired species *Ichthyomyzon gagei*, as well as *I. bdellium* and *I. greeleyi*; however, none occur in Manitoba.

Golden Redhorse CO1

The designed primers were run through PrimerBLAST to compare the genetic sequence at the binding region of the primers of Golden Redhorse to non-target species. Two species that occur in Manitoba were identified as being the greatest risks for non-target amplification during *in silico* validation of the designed Golden Redhorse CO1 assay: White Sucker *Catostomus commersonii* and Silver Chub *Macrhybopsis storeriana*; however, both had sufficient mismatches to the designed primers (5 and 6 mismatches, respectively). The likelihood of the probe binding to White Sucker and Silver Chub was assessed manually using nBLAST and binding of the probe to the non-target DNA was determined to be unlikely as at least four mismatches were noted for each species. The likelihood of non-target amplification of other *Catostomus* spp. and *Moxostoma* spp. found in Manitoba was manually assessed using PrimerBLAST and nBLAST with a specificity stringency of up to 9 mismatches allowed, and non-target binding was not observed.

Hornyhead Chub CO1

In silico validation of the chosen CO1 assay for Hornyhead Chub indicated a high likelihood of non-target amplification of congeners of Hornyhead Chub, Redtail Chub *Nocomis effusus* and Redspot Chub *Nocomis asper*. As both species are not present in Manitoba, and adequate mismatches were noted for all Manitoban species, the assay continued to *in vitro* validation. Three assays were considered *in silico*; however, only the assay with the most mismatches to other *Nocomis* spp. and Manitoban species was chosen.

Northern Brook/Silver Lamprey CO1

Similar to the Chestnut Lamprey CO1 assay, an emphasis was placed on designing an assay that would target a region where sufficient genetic diversity is noted between Northern Brook/Silver lampreys and Chestnut Lamprey; however, each candidate assay was also assessed for the number of mismatches between it and the target binding region of *Petromyzon marinus* and *Lethenteron appendix* as Northern Brook and Silver lampreys also co-occur in the Great Lakes with these lampreys. Two rounds of assay designing occurred, and thus, two rounds of *in silico* validation (Table 2.2).

During the first round, three assays were designed, with amplicon lengths between 80 and 85 bp. The assay chosen (NBLPCO12022) had the greatest number of mismatches with Chestnut Lamprey; 3 in the forward primer, 2 in the reverse primer and 1 in the probe.

Four assays were designed during round 2, with amplicon lengths of 110–150 bp. Forward and reverse primers from three of the four assays (NBLP_116, NBLP_131 and NBLP_399) were ordered and tested further *in vitro*, as they each had 7, 6, and 7 mismatches total with Chestnut Lamprey, respectively.

Bigmouth Buffalo CytB

In total, four candidate assays were assessed for their specificity *in silico* for the CytB gene of Bigmouth Buffalo. Other fishes within the genus *Ictiobus* have the most similar genetic sequence at the target region to Bigmouth Buffalo. As Bigmouth Buffalo is the only *Ictiobus* sp. in Manitoba, non-target amplification of other *Ictiobus* spp. is not a concern; however, to increase the likelihood of use of this assay elsewhere in the species' range, the assay that bound to the area that was most distinct from the other *Ictiobus* spp. was chosen. The validated assay is suspected to amplify Black Buffalo *Ictiobus niger*, Smallmouth Buffalo *Ictiobus bubalus*, but may not amplify Fleshylip Buffalo *Ictiobus labiosus*, or at minimum, will have a lesser affinity for *I. labiosus* due to one mismatch in each primer and two mismatches in the probe.

Chestnut Lamprey CytB

Four candidate assays were designed to target the CytB gene of Chestnut Lamprey and tested *in silico*. The assay with the greatest number of mismatches to Silver and Northern Brook lampreys was ordered. Not surprisingly, *in silico* validation noted the assay is likely to also bind and amplify *Ichthyomyzon gagei* as it is the paired species of Chestnut Lamprey (Docker 2009).

Golden Redhorse CytB

Two candidate assays were designed to target the CytB gene of Golden Redhorse. During *in silico* validation, the probe of one assay was noted to bind to Threespine Stickleback *Gasterosteus aculeatus*, which is native to Manitoba. Thus, the assay was not considered. As the remaining assay showed no likelihood of non-target amplification, it was ordered and continued to *in vitro* validation.

Hornyhead Chub CytB

Nine candidate assays were designed to target the CytB gene of Hornyhead Chub. Regions unique to Hornyhead Chub in comparison to other Manitoban fishes that were suitable in length for primer and probe binding had a high predicted likelihood of dimerization, per the Primer Express software. The assay with the lowest possibility of dimerization, while maintaining specificity, was chosen for *in vitro* validation. This assay, like the assay designed to target the CO1 gene of Hornyhead Chub, was noted as having a high likelihood of non-target amplification of *Nocomis asper* and *Nocomis effusus*, neither of which are found in Canada.

Northern Brook/Silver Lamprey CytB

Two candidate assays were designed to target the CytB gene of paired species Northern Brook and Silver lampreys. The primers of both assays were ordered and tested further *in vitro* as 12 mismatches to the CytB gene of Chestnut Lamprey at the target region of the gene were noted for both assays.

2.3.1.3 In Vitro Validation

Assays were validated *in vitro* either using qPCR or end-point PCR and visualized on a gel, except for the Silver/Northern Brook assays, which were first validated using end-point PCR, and then validated using qPCR (*see* Section 2.2.1.3).

CO1 Assays

Species-specificity was confirmed for all CO1 assays using tissue-extracted DNA (Table 2.5), apart from the Silver/Northern Brook CO1 assay, which amplified tissue-extracted Chestnut Lamprey DNA and gBlock® Chestnut Lamprey CO1 oligonucleotides (Table 2.1; Figure 2.1).

CytB Assays

Assays that were designed to target the CytB gene for Bigmouth Buffalo, Chestnut Lamprey, Golden Redhorse, and Hornyhead Chub were tested *in vitro* for specificity using tissue-extracted DNA of non-target species (Figure 2.2). Non-target amplification for each assay was tested by running PCR reactions of tissue-extracted DNA of target and non-target species. The initial specificity of the designed Hornyhead Chub CytB assay was assessed using the DNA of Emerald Shiner *Notropis atherinoides,* Common Shiner *Luxilus cornutus,* and Carmine Shiner *Notropis percobromus*. The specificity of the Bigmouth Buffalo assay was assessed using the DNA of Quillback *Carpiodes cyprinus*, Common Carp *Cyprinus carpio,* and White Sucker. The specificity of the assay designed to target the CytB gene of Golden Redhorse was assessed using the DNA of Shorthead Redhorse *Moxostoma macrolepidotum,* Silver Redhorse *Moxostoma anisurum*, and White Sucker. Lastly, the specificity of the Chestnut Lamprey CytB assay was determined using samples of Northern Brook Lamprey DNA extracted from two specimens, as extracted Silver Lamprey DNA was not available at the time of testing.

Distinguishable bands indicating successful amplification were visualized in the wells containing DNA of Hornyhead Chub, Golden Redhorse, and Chestnut Lamprey, compared to the wells containing the DNA of the associated non-target species. A faint band at the target amplicon size was visualized in the well containing the DNA of Bigmouth Buffalo. No distinguishable amplification could be visualized in the wells containing the DNA of non-target species (Figure 2).

An assay targeting the CytB gene of Northern Brook and Silver lampreys was designed and validated *in silico,* then, validated *in vitro* using tissue-extracted DNA of American Brook Lamprey, Chestnut Lamprey, and Sea Lamprey. The assay did not amplify any of the non-target

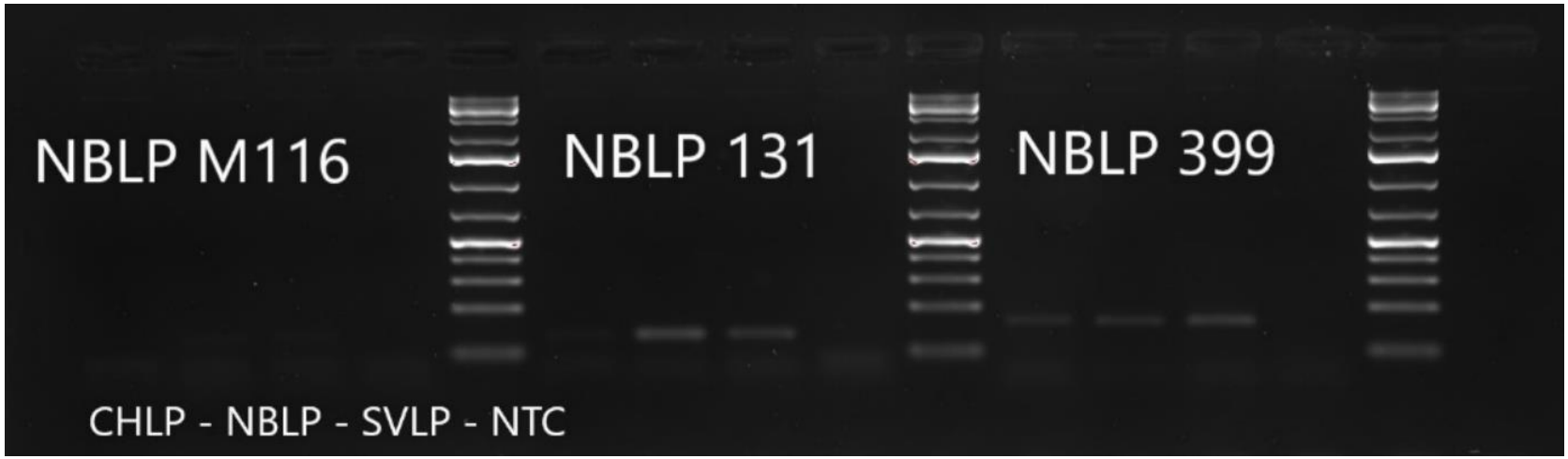


Figure 2.1 Photograph of resulting DNA bands after gel electrophoresis of PCR products from validation of assays targeting the CO1 gene of Northern Brook and Silver lampreys designed. Assays that were designed to target the cytochrome oxidase subunit 1 (CO1) gene of Northern Brook (NBLP) and Silver (SVLP) lampreys. The assays were tested *in vitro* for specificity using tissue-extracted DNA Chestnut Lamprey (CHLP). A no-template control (NTC) of molecular grade water was included in place of DNA to assess for contamination of the reagents used. Included in the rightmost well for each set of reactions per assay was a 1-kilobase pair ladder to aid with visualization. From left to right (per assay): Chestnut Lamprey DNA, Northern Brook Lamprey DNA, Silver Lamprey DNA, No-Template Control, ladder.

Figure 2.2 Photograph of resulting DNA bands after gel electrophoresis of PCR products from validation of CytB assays designed. Assays that were designed to target the cytochrome *b* (CytB) gene for Hornyhead Chub (HHCH), Bigmouth Buffalo (BMBF), Golden Redhorse (GDRH) and Chestnut Lamprey (CHLP) were tested *in vitro* for specificity using tissue-extracted DNA of non-target species. A no-template control (NTC) of molecular grade water was included in place of DNA to assess for contamination of the reagents used. At the outer borders and between different assays, a 1-kilobase pair ladder was included. EMSH = Emerald Shiner, CMSH = Common Shiner, CRSH = Carmine Shiner, QUBK = Quillback, CARP = Common Carp, WHSK = White Sucker, SHRH = Shorthead Redhorse, SVRH = Silver Redhorse, NBLP = Northern Brook Lamprey.

DNA; thus, the assay was considered species-specific for paired-species Northern Brook and Silver lampreys.

2.3.1.4 Determining Limit of Detection and Limit of Quantification

The LOD and LOQ were determined for all species-specific CO1 assays (i.e., Bigmouth Buffalo, Chestnut Lamprey, Golden Redhorse, Hornyhead Chub) using gBlock® oligonucleotides of a genetic sequence that was most like the consensus of the CO1 region determined from aligning available CO1 sequence for each species (Table 2.6, Table 2.7).

The starting concentration (copies/µl) varied per species depending on the amplicon size (bp) of the fragment (Table 2.6). The starting concentration for the gBlock® designed and used for each of the *Ichthyomyzon* spp. lampreys was 71.0 copies/µl. For Bigmouth Buffalo, Golden Redhorse, and Hornyhead Chub, the starting concentration was 77.6 copies/µl.

The LOD and LOQ were not determined for any CytB assays due to the lack of availability of oligonucleotides of the CytB gene for the target species. Instead, a standard curve was generated using a 6-point, 4-fold dilution series with 8 replicates per dilution to assess the efficiency and proportion of variance of the assay (Figure 2.3).

2.4 Discussion & Conclusion

2.4.1 Species-Specificity and Assay Sensitivity

The ability to design universally species-specific assays, where assays can be designed to be used in any waterbody and maintain species-specificity, is likely incredibly rare, if not impossible for many species and genes. Thus, clear communication of the limitations of assays is necessary and should not be considered a pitfall of eDNA when specificity can be maintained within the scope of the designed project. Rather, this highlights the importance of thorough validation of designed assays for each new geographic area or application, regardless of their

previous use, for all studies to ensure species-specificity is maintained and the limitations of the assay are clear to the end user.

A region of the CO1 gene of Silver and Northern Brook lampreys that was suitable for eDNA assay design and contained enough differences from Chestnut Lamprey could not be found during the timeline of this project. Additional sequences of the CO1 gene of Chestnut, Silver, and Northern Brook lampreys, especially longer reads, would be beneficial to highlight differences between the species.

2.4.2 Variations in Limit of Detection and Limit of Quantification

While both values provide data needed to better understand and quantify eDNA qPCR data, the LOD and LOQ of an eDNA assay can vary depending on several factors, including the type of sample used for determining the LOD and LOQ (i.e., the possibility of interference or inhibition during PCR), the extraction and amplification protocols followed, and the sensitivity of the detection technique (e.g., PCR, qPCR) (Thalinger et al. 2021). When reporting LOD and LOQ results, it is important to include information about these factors. In summary, researchers should calculate and re-calculate the LOD and LOQ whenever new assays are used, or any methods or protocols in the workflow are changed, including changes in lab personnel. Ultimately, the LOD and LOQ values for an assay should be used as guidelines to help determine the sensitivity of an assay but must be considered flexible values that can, and do, change (Burd 2010).

A comparatively high value for the LOD was noted for the Bigmouth Buffalo CO1 assay (Table 2.7). Further examination of the assay suggests the initial probe sequence may have been written incorrectly, and an adenosine nucleotide was added at the 3’ end of the probe, decreasing the suitability of the probe (Table 2.1). Future research could investigate if removing the

Table 2.6 Starting concentration for serial dilutions to determine the limit of quantification for the designed species-specific CO1 assays. Gene fragments (gBlock®, Integrated DNA Technologies) of the cytochrome *c* oxidase subunit 1 (CO1) gene for the target species were designed and used for each species. The starting concentration (copies/µl) varied per species depending on the amplicon size (bp) of the fragment.

Common Name	Scientific Name	Gene	Size	Concentration (copies/µl)	Accession Number
Chestnut Lamprey	*Ichthyomyzon castaneus*	CO1	652	71.0	BCF631-07
Northern Brook Lamprey	*Ichthyomyzon fossor*	CO1	652	71.0	BNAFA409-08 (NAFF 3024)
Silver Lamprey	*Ichthyomyzon unicuspis*	CO1	652	71.0	RMAYA067-06 (NAFF 067)
Bigmouth Buffalo	*Ictiobus cyprinellus*	CO1	597	77.6	CFF221-16 KX145263
Hornyhead Chub	*Nocomis biguttatus*	CO1	597	77.6	BCF613-07 EU5244160
Golden Redhorse	*Moxostoma erythrurum*	CO1	597	77.6	BCFB507-06 EU524876

Table 2.7. The limit of detection and quantification of designed species-specific CO1 assays. The limit of detection (LOD) and limit of quantification (LOQ) were determined by generating standard curves from 6-point, 4-fold serial dilution with 8 replicates per dilution using gene fragments (gBlock®) of the barcode region of the CO1 gene for the target species. The resulting data was analyzed using methods described by (Klymus et al. 2020a).

Assay	Target Gene	Target Species	LOD	LOQ
BMBF CO1	CO1	Bigmouth Buffalo *Ictiobus cyprinellus*	4.20	12
CHLP CO1	CO1	Chestnut Lamprey *Ichthyomyzon castaneus*	1.16	2
GDRH CO1	CO1	Golden Redhorse *Moxostoma erythrurum*	0.60	9
HHCH CO1	CO1	Hornyhead Chub *Nocomis biguttatus*	2.65	27

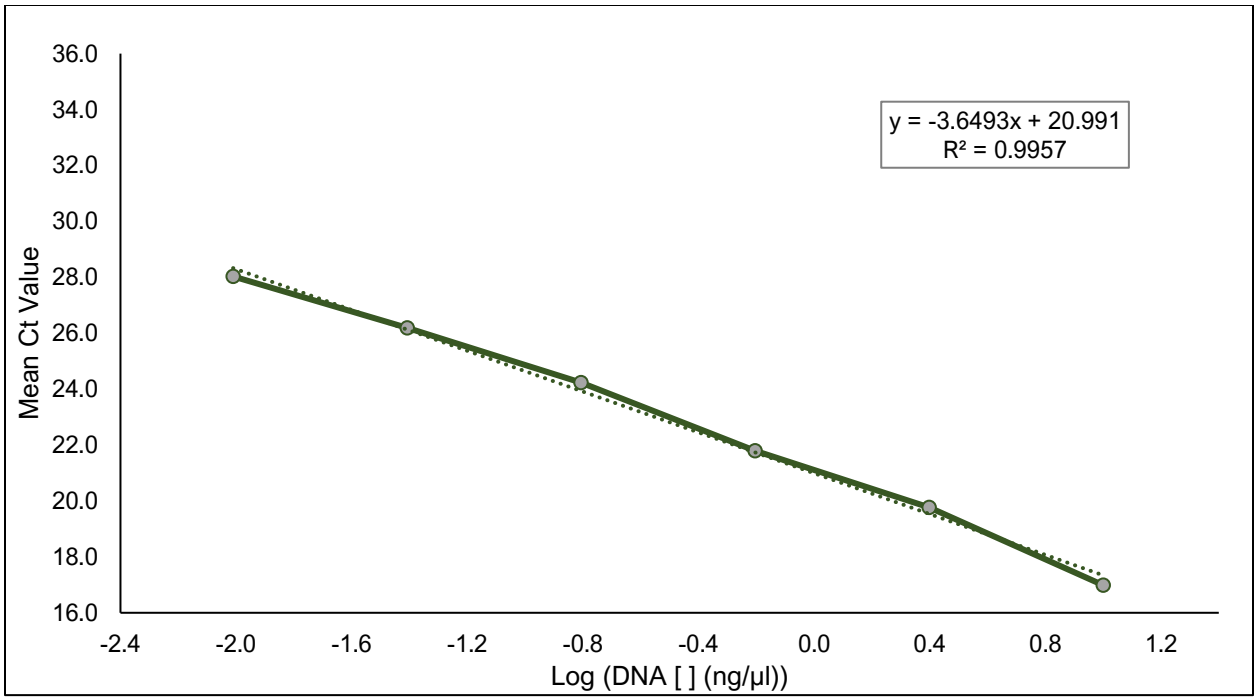


Figure 2.3 Standard curve of the designed assay for CytB gene of Northern Brook and Silver lampreys. The assay was designed to target and amplify a region of the cytochrome *b* (CytB) gene of Northern and Silver lampreys. The standard curve was created using data generated by a 6-point, 4-fold dilution series with 8 replicates per dilution to assess the efficiency and proportion of variance of the assay. The starting concentration of 10 ng/µl of tissue-extracted Northern Brook Lamprey DNA was used as the stock solution.

adenosine from the 3’ end of the probe increases the LOD and LOQ of the Bigmouth Buffalo CO1 assay.

This chapter details the design and validation of assays that target and amplify the CO1 gene of Bigmouth Buffalo, Chestnut Lamprey, Golden Redhorse, and Hornyhead Chub, and the CytB gene of Bigmouth Buffalo, Chestnut Lamprey, Golden Redhorse, Hornyhead Chub, and Northern Brook and Silver lampreys. Future users of these assays are strongly encouraged to perform further validation of these assays to confirm their specificity in the targeted sampling region, and to determine the sensitivity of the assay with the methods and techniques used. Furthermore, future research could explore comparing the sensitivity of the designed CytB and CO1 assays *in vitro* and *in situ*.

Literature Cited

Abbott C, Coulson M, Gagné N, et al (2021) Guidance on the Use of Targeted Environmental DNA (eDNA) Analysis for the Management of Aquatic Invasive Species and Species at Risk. DFO Canadian Science Advisory Secretariat Research Document 2021/019. https://waves-vagues.dfo-mpo.gc.ca/library-bibliotheque/40960791.pdf

Burd EM (2010) Validation of laboratory-developed molecular assays for infectious diseases. Clinical Microbiology Reviews 23:550–576. https://doi.org/10.1128/CMR.00074-09

COSEWIC (2020) COSEWIC guidelines for recognizing designatable units. In: Committee on the Status of Endangered Wildlife in Canada. https://cosewic.ca/index.php/en-ca/reports/preparing-status-reports/guidelines-recognizing-designatable-units.html

Docker MF (2009) A review of the evolution of nonparasitism in lampreys and update of the paired species concept. In: Brown LR, Chase SD, Mesa MG, et al. (eds) Biology, Management, and Conservation of Lampreys in North America. American Fisheries Society Symposium 72, Bethesda, pp 71–114

Forootan A, Sjöback R, Björkman J, et al (2017) Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). Biomolecular Detection and Quantification 12:1–6. https://doi.org/10.1016/j.bdq.2017.04.001

Goldberg CS, Turner CR, Deiner K, et al (2016) Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods in Ecology and Evolution 7:1299–1307. https://doi.org/10.1111/2041-210X.12595

Hubert N, Hanner R, Holm E, et al (2008) Identifying Canadian freshwater fishes through DNA barcodes. PLoS ONE 3:e2490. https://doi.org/10.1371/journal.pone.0002490

Hunter ME, Meigs-Friend G, Ferrante JA, et al (2018) Surveys of environmental DNA (eDNA): a new approach to estimate occurrence in vulnerable manatee populations. Endangered Species Research 35:101–111. https://doi.org/10.3354/esr00880

Klymus KE, Merkes CM, Allison MJ, et al (2020a) Reporting the limits of detection and quantification for environmental DNA assays. Environmental DNA 2:271–282. https://doi.org/10.1002/edn3.29

Klymus KE, Ruiz Ramos DV, Thompson NL, Richter CA (2020b) Development and testing of species-specific quantitative PCR assays for environmental DNA applications. Journal of Visualized Experiments e61825. https://doi.org/10.3791/61825

Kutyavin IV, Afonina IA, Mills A, et al (2000) 3’-Minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Research 28:655–661. https://doi.org/10.1093/nar/28.2.655

LeBlanc F, Belliveau V, Watson E, et al (2020) Environmental DNA (eDNA) detection of marine aquatic invasive species (AIS) in Eastern Canada using a targeted species-specific qPCR approach. MBI 11:201–217. https://doi.org/10.3391/mbi.2020.11.2.03

Merkes CM, Klymus KE, Allison MJ, et al (2019) Generic qPCR limit of detection (LOD) / limit of quantification (LOQ) calculator. R Script. https://github.com/cmerkes/qPCR_LOD_Calc

Minamoto T, Fukuda M, Katsuhara KR, et al (2017) Environmental DNA reflects spatial and temporal jellyfish distribution. PLoS ONE 12:e0173073. https://doi.org/10.1371/journal.pone.0173073

Ratnasingham S, Hebert PDN (2007) BOLD: the barcode of life data system. Molecular Ecology Notes 7:355–364. https://doi.org/10.1111/j.1471-8286.2007.01678.x

Thalinger B, Deiner K, Harper LR, et al (2021) A validation scale to determine the readiness of environmental DNA assays for routine species monitoring. Environmental DNA 3:823–836. https://doi.org/10.1002/edn3.189

Thermo Fisher Scientific (2022) Primer Express Software Version 3.0 Getting Started Guide

Tsuji S, Takahara T, Doi H, et al (2019) The detection of aquatic macroorganisms using environmental DNA analysis - a review of methods for collection, extraction, and detection. Environmental DNA 1:99–108. https://doi.org/10.1002/edn3.21

Wilcox TM, McKelvey KS, Young MK, et al (2013) Robust detection of rare species using environmental DNA: the importance of primer specificity. PLoS ONE 8:e59520. https://doi.org/10.1371/journal.pone.0059520

Yao Y, Nellåker C, Karlsson H (2006) Evaluation of minor groove binding probe and TaqMan probe PCR assays: influence of mismatches and template complexity on quantification. Molecular and Cellular Probes 20:311–316. https://doi.org/10.1016/j.mcp.2006.03.003

CHAPTER 3 MAPPING THE CURRENT DISTRIBUTION OF BIGMOUTH BUFFALO, GOLDEN REDHORSE, HORNYHEAD CHUB, AND CHESTNUT, NORTHERN BROOK AND SILVER LAMPREYS IN MANITOBA USING ENVIRONMENTAL DNA

3.1 Introduction

Freshwater fishes in Manitoba face many risks, many of which are common to freshwater ecosystems in Canada and worldwide (*see* Dextrase and Mandrak 2006; Dudgeon 2010). The loss and degradation of key aquatic habitats from anthropogenic actions can reduce available habitat for fish and disrupt important ecological processes (Vörösmarty et al. 2010; Fuller et al. 2015). The pollution of freshwater can impair fish health, growth, and development, and disrupt reproductive cycles (Sopinka et al. 2012; Gomes et al. 2015). The introduction of non-native species can have profound and lasting effects on freshwater ecosystems as non-native species can outcompete native species for resources, alter habitat structure, and transmit disease (Dextrase and Mandrak 2006). Furthermore, the fragmentation of freshwater habitats through the installation of barriers, such as dams, culverts, and weirs, can impede or prevent fish movement, disrupt essential migration patterns, and ultimately isolate populations (Olden 2016; Enders et al. 2019). This fragmentation can lead to a reduction in genetic connectivity in a population, which diminishes biodiversity and increases the vulnerability of a population to environmental change and stochastic events (Fraser et al. 2014; Fuller et al. 2015). Lastly, with increasing temperatures and changes in streamflow regimes due to climate change, the distribution of some freshwater fishes is expected to change as suitable habitats for each species are altered (Barbarossa et al. 2021).

In this chapter, I outline the current distribution of six freshwater fishes of conservation concern in Manitoba (i.e., Bigmouth Buffalo *Ictiobus cyprinellus,* Chestnut Lamprey *Ichthyomyzon castaneus*, Golden Redhorse *Moxostoma erythrurum,* Hornyhead Chub *Nocomis*

biguttatus, Northern Brook Lamprey *Ichthyomyzon fossor,* and Silver Lamprey *Ichthyomyzon unicuspis*) determined using environmental DNA (eDNA) probe-based assays designed and validated in Chapter 2. Documentation and historic distribution for these fishes vary (*see* Chapter 1); therefore, establishing a current understanding of the distribution of these fishes in Manitoba is essential for monitoring their response to the continued changes to their habitats.

3.2 Methods

3.2.1 Sampling Sites

Key sites for sampling were determined using a combination of published collection records of the target species (Scott and Crossman 1973; Dalton 1989; Goodchild 1990; COSEWIC 2007, 2009, 2010, 2011, 2020), the known and potential distribution of each species described in Stewart and Watkinson (2004), previous eDNA sampling and collection records for the native lampreys from Margaret Docker (University of Manitoba, unpublished), and previous collection records from Fisheries and Oceans (Doug Watkinson, Fisheries and Oceans Canada, unpublished). Protocols for sampling and analysis followed those developed by Gingera et al. (2016) and by the Docker Lab (*see* Chapter 2).

3.2.2. eDNA Sample Collection and Filtration

Any equipment used for sampling or filtration was thoroughly cleaned and decontaminated before and after use by soaking the equipment in warm, soapy water for a minimum of 5 minutes, followed by spraying the equipment with ELIMINase® decontaminant, and 70% ethanol. The equipment was rinsed after each step with distilled water. For any samples collected in Lake Manitoba, the sampling equipment was also soaked in a 10% bleach solution for 30 minutes before cleaning and decontamination after use, following AIS Permit No. 23-2022 and AIS Permit No. 24-2023. In any cases where water was transported to the lab and disposed of down the drain, 100 mL of bleach to 1 L of water was added to the drain for a

duration of 30 minutes per AIS Permit No. 23-2022. All surfaces or equipment that cannot be submerged in water were wiped down using ELIMINase® decontaminant and 70% ethanol. For drying and sterilization, the equipment was placed in a UV3 HEPA PCR workstation equipped with HEPA filtration and exposed to UV light for a minimum of 15 minutes, when possible. The sterilized equipment was stored in a contaminant-free place (e.g., UV-sterilized plastic bag, decontaminated sealed cupboard) until its next use, or in a decontaminated, sealed case. Coolers used to transport samples were rinsed with warm soapy water, then sprayed internally and externally with ELIMINase® decontaminant, and 70% ethanol.

All the samples were filtered through 1.5 μm pore 47 mm diameter glass microfibre filters (Whatman®, CAT No. 1827-047) to retain DNA. The forceps used to place and remove filters from filtering devices were sterilized before and after touching the filter by dipping them in 95–100% ethanol, and then flaming them for 5 seconds. Each filter was placed in labelled 15 mL screw-top test tubes, containing at least 7 mL of 95–100% ethanol, using sterilized forceps, to preserve the filter. Nitrile gloves were worn during the collection of the samples, and the filtration and preservation of the filters, and were changed between samples to prevent the carryover of DNA from sampling sites. All filters were stored in a freezer between -20°C and -30°C until DNA extractions were performed.

No personnel and, when possible, no non-eDNA sampling gear (apart from boats during paired sampling) entered the water before eDNA sampling to limit the risk of contamination (i.e., carry-over from other locations on boots or other gear). ELIMINase® decontaminant was sprayed onto any equipment where contamination was suspected (e.g., forceps dropped).

3.2.2.1 Sample Filtration using Vacuum Filtration

All samples collected in 2021 were collected by hand in sterilized Nalgene™ wide-mouth bottles (Thermo Scientific™) filtered in the laboratory using vacuum filtration (Eisco™ Glass Filtration Assembly, S88322). The glass filtration assembly included a 1000 mL vacuum filtration flask fit with a 300 mL funnel, attached using a 47 mm vacuum base with a sintered disk (porosity range of 40–90 μm), secured with a 47 mm clamp. The assembly was attached to a vacuum valve using silicone tubing.

Three 500 mL water samples were collected from the surface of the water at each sampling site from the bank of the water body, or when possible, off a dock, using a combination of 500 mL and 1 L sterilized Nalgene™ wide-mouth bottles. The samples were collected by hand while wearing nitrile gloves. After collection, samples were placed in a cooler on ice in sealed, labelled plastic bags and transported back to the lab for filtration. Each water sample was filtered until the filter clogged, or 500 mL of water was filtered, whichever occurred first. The total volume of water filtered was recorded. Samples were kept in sterilized plastic bags in a cooler on ice until filtration occurred, which was within 8 hours of collection.

3.2.2.2 Sample Collection and Filtration using OSMOS

All samples collected in 2023, except for samples collected in northern Manitoba, were collected and filtered using an OSMOS eDNA sampler (Halltech). The OSMOS consists of a battery-powered pump, a tripod, a telescopic pole attached to a hose, and an aluminum filter housing. The OSMOS uses negative pressure to pull water from a water body into an aluminum filter housing and over a filter paper, where eDNA and any suspended debris greater than the pore size of the filter are trapped. The OSMOS was set up according to the GEN-FISH eDNA Sampling Protocol (*see* Appendix Protocol 3.1). At each site, a negative control sample was filtered first by submerging the filter housing in a sterile plastic bag containing 1.5 L of UV-

treated bottled water. The six 2 L biological replicates were sequentially collected and filtered by submerging the filter housing 0.05–0.15 m below the surface of the water. One filter housing was used per site to minimize the risk of contamination between sites.

3.2.3 eDNA Extraction and Quantitative PCR Analysis

All DNA extractions and qPCR plating occurred in a UV3 HEPA PCR workstation. All equipment used that is not light-sensitive was UV-sterilized for a minimum of 15 minutes before starting the protocol for each site to reduce the risk of contamination of the samples. Aerosol-filtered pipette tips were used for all DNA extractions and qPCR plating to reduce the risk of contamination. All processes occurred in a laboratory with limited exposure to DNA (i.e., no genetic work on tissue-derived DNA, tissue sample processing, or preserved specimen examination within the laboratory). Workspaces, equipment, and reagent bottles were decontaminated using 70% ethanol and ELIMINase® between samples.

3.2.3.1 eDNA Extractions

The filtered eDNA was isolated and extracted from the preserved filters using the DNeasy Blood and Tissue Kit (Qiagen) following the purification of total DNA from animal tissues protocol (spin-column protocol), with modifications. To increase the total DNA yield from each filter, 450 µl of Buffer ATL and 50 µl of Proteinase K were used per filter, per tube, and the filters were incubated for 18–24 hours before the elimination of RNA using 6 µl of RNase A. After the addition of RNase A and the associated incubation, mixing and centrifugation, filters of biological replicates were processed through QIAshredder spin columns (Qiagen) and centrifuged for 2 minutes at 11,000 rpm to decrease the viscosity of the sample, filter out insoluble particles, and increase the digestion of the DNA present in the sample (Yukl et al. 2014). The liquid produced was pipetted into a new 1.5 mL tube and used in place of the filter for the remainder of the protocol. Each replicate was eluted to a final volume of 100 µl

using Buffer AE. An additional negative control (lab negative control) was included to identify potential contamination during the eDNA extraction process. The extracted DNA was eluted using Buffer AE to a final volume of 100 µl per replicate. After extraction, the OneStep™ PCR Inhibitor Removal kit (Zymo Research) was used for all biological replicates to reduce the presence of inhibitors in the samples.

3.2.3.2 Quantitative PCR Analysis

Initial qPCR analyses of the eluted DNA occurred for certain target species at each site (Table 3.3, Table 3.4). Only target species whose presence was possible at a site based on their historical distribution, previous collection records, habitat requirements, and/or suspected range expansion were assayed in 2021 due to a lack of available eDNA aliquot (*see* Chapter 1: General Introductions; *see* 3.1.1 Sampling Sites). Except for the sites sampled in Churchill, Manitoba, eluted eDNA from all sites sampled in 2023 were assayed for all target species (Table 3.5, Table 3.6). Three technical qPCR replicates were performed for each biological replicate and negative control for each species that was assayed. The analyses were performed following the "qPCR using eDNA water samples" protocol (*see* Appendix Protocol 3.1) using TaqMan™ MGB the species-specific probe-based assays designed (*see* Chapter 2) to target regions of the CO1 or CytB gene (Table 3.1). The total reaction volume per well was 15 µl, consisting of 10 µl qPCR master mix and 5 µl template. The qPCR master mix consisted of 0.3 µl forward primer (10 µM), 0.3 µl reverse primer (10 µM), 0.15 µl probe (10 µM), and 7.5 µl TaqMan™ Environmental Master Mix 2.0 (Applied Biosystems®). A no-template control (5 µl of molecular-grade water instead of eluted DNA) was included to establish the baseline fluorescence, and a positive control consisting of 5 µl of tissue-extracted DNA was used to ensure primer and probe efficacy. Both were run in triplicate in each qPCR run (i.e., on each 96-well plate).

The qPCR reactions were performed using a StepOne Plus™ Real-Time PCR System (Applied Biosystems®) or a QuantStudio3™ Real-Time PCR System (Applied Biosystems®) in 0.1 mL 96-well reaction plates with adhesive film (Applied Biosystems®, Cat. No. 4366932). The reaction plates were spun down using a mini centrifuge (VWR® Labnet PCR Mini Plate Spinner Centrifuge, MPS 1000), and then placed directly onto the sample block. The qPCR cycling parameters were set as follows: incubation at 50°C for 2 minutes; denaturation at 95°C for 10 minutes; and 45 cycles of denaturation at 95°C for 15 seconds and annealing/extension for 1 minute at set assay annealing temperature (Table 3.1).

If target DNA was detected in any negative or no-template controls (field, extraction, or qPCR negative controls), the negative or no-template controls were re-plated and analyzed using a new mixture of qPCR pre-mix. If non-target DNA was detected again in the controls, the samples were considered contaminated, and results were not considered in further analyses. Field and lab negative controls were run after the biological replicates were analyzed, and only negative controls were assayed for samples where the detection of DNA occurred in the biological replicates to minimize the total use of reagents.

3.3 Results

3.3.1 Sampling

The samples were collected from sites within and outside of the historical range of the species of interest (Table 3.2, Figure 3.1). In 2021 from 58 sites of interest (Table 3.3, Table 3.4), two sites in 2022 (*see* Chapter 4), and 69 sites in 2023 (Table 3.5, Table 3.6). Sites sampled in 2021 were sampled four times each, in May/June, July, August, and September (Table 3.3, Table 3.4). Sampling in 2023 occurred between June and September. All sampling in 2022 occurred in June. Of the 69 sites sampled in 2023, 17 of the sites were previously sampled in 2021 (Table 3.6). Comparing the data of these sites from 2021 and 2023, more detections

Table 3.1 Summary of the TaqMan probe-based assays used in this project. Each assay consists of two primers and one probe which targets a sequence on the cytochrome *c* oxidase subunit 1 (CO1) or cytochrome *b* (CytB) gene for the species of interest in the project. The optimal annealing temperature (T_a), amplicon size (base pair, bp), and probe reporter dye for each assay are given.

Species	Name	Marker	Sequence (5'-3')	T_a (°C)	Size (bp)	Dye
Bigmouth Buffalo	BMBF CO1	Forward	CCCCACTTGCGGGTAATCT	60	85	VIC
		Reverse	ATTGATGAAACTCCTGCTAGGTGAA			
		Probe	AGCTTCAGTAGACTTGACTA			
Chestnut Lamprey	CHLP CO1	Forward	GGATGAACTGTATACCCCCCTTT	58	85	FAM
		Reverse	CGAGGTGTAGGGAGAAGATTGTTAA			
		Probe	TCACACAGGAGCCTCTA			
Golden Redhorse	GDRH CO1	Forward	AGCATTCCCCCGGATGA	58	80	FAM
		Reverse	CAACTCCGGAAGAAGCCAATAA			
		Probe	CAATATAAGCTTCTGACTCCT			
Hornyhead Chub	HHCH CO1	Forward	ATCAGACACCCCTCTTTGTTTGA	57	80	FAM
		Reverse	TCCAGCAGCTAGGACAGGTAATG			
		Probe	TAACTGCTGTTCTTCTACTACTA			
Northern Brook/Silver Lamprey	NBLP CytB	Forward	TTGGAGGAGTTTTAGCTTTAGTCATG	57	90	FAM
		Reverse	GAAATTGTATGCCTCGCTGCTT			
		Probe	TATCCTAATCCTCCTAATCAT			

Table 3.2 Locations sampled in southern Manitoba in 2021, 2022 and 2023. Historical distribution based on most recent COSEWIC status reports (Dalton 1989; Goodchild 1990; COSEWIC 2009, 2010, 2020), with additional information from Stewart and Watkinson (2004). Samples were collected from 58 sites in 2021 (Table 3.3, Table 3.4), two sites in 2022 (*see* Chapter 4), and 69 sites in 2023 (Table 3.5, Table 3.6). Sites sampled in 2021 were sampled four times each, in May/June, July, August, and September (Table 3.3, Table 3.4), and sites sampled in 2023 were sampled once from June to September.

River/Lake Basin	Waterbody
Assiniboine River	Assiniboine River[1,2,3,6]
	Little Saskatchewan River[2,6]
	Oak Creek[2,6]
	Shell River[1,2,6]
Lake Manitoba	Dauphin Lake[1]
	Delta Channel[1]
	Lundar Beach[1]
Lake of the Woods	Lake of the Woods[1*,2,3, 6]
Lake Winnipeg	Brokenhead River[2,3,4]
	Hazel Creek[2,4]
	Icelandic River[1]
	Lake Winnipeg[1,2,3]
	Manigotagan River[1,2,3]
Red River	Cook's Creek[1]
	Joubert Creek[2,3,6]
	La Salle River[1,2,6]
	Pembina River
	Rat River[2,3,6]
	Red River[1,2,3,4*,6]
	Roseau River[2,3,6]
	Seine River[1,2,6]
Qu'Appelle River	Qu'Appelle River[1,2,6]

Winnipeg River	Barrier Bay[6]
	Birch River[4,5]
	Bird River[6]
	Boggy River[4]
	Dorothy Lake[6]
	Eight-Foot Falls[6]
	Eleanor Lake[6]
	Keskinen Bay[6]
	Natalie Lake[4,6]
	Nutimik Lake[6]
	Otter Falls[6]
	Pointe du Bois[6]
	Rice Creek
	Whitemouth River[2,4,5]
	Winnipeg River[2,3,4,5,6]

*Population is suspected to be extirpated.; [1]Bigmouth Buffalo; [2]Chestnut Lamprey; [3]Golden Redhorse; [4]Hornyhead Chub; [5]Northern Brook Lamprey; [6]Silver Lamprey.

Table 3.3 Detection by species using eDNA for 2021 sampling in Lake Winnipeg, Red River, and Assiniboine River basins (*see* Figure 3.1). The presence/absence of eDNA was assessed using species-specific probe-based assays designed and validated during this project. Sites were sampled four times in 2021. Historical indicates historical distribution based on recent COSEWIC status reports for each species. The samples were not assessed for the presence of the eDNA of Northern Brook and Silver lampreys due to insufficient eDNA aliquot and a lack of historical distribution in the area. Y = positive eDNA detection with detection in at least one technical replicate of one biological replicate; N = no eDNA detected. Coloured highlighting for historical data emphasizes where eDNA detection and historical distribution do not match.

a) Bigmouth Buffalo

Site Information		eDNA Detection in 2021					
Site	**Waterbody**	**May/June**	**July**	**August**	**September**	**Total**	**Historical**
BR.01	Brokenhead River	N	N	N	N	N	N
BR.02	Brokenhead River	N	N	N	N	N	N
BR.03	Brokenhead River	N	N	N	N	N	N
HC.01	Hazel Creek	N	N	N	N	N	N
RR.101	Joubert Creek	N	N	N	N	N	N
RR.102	Rat River	N	N	N	N	N	N
RR.103	Rat River	N	N	N	N	N	N
RR.104	Roseau River	N	N	N	N	N	N
RR.105	Roseau River	Y	N	N	N	Y	N
RR.106	Morris River	Y	N	N	N	Y	N
RR.107	Red River	N	N	N	N	N	Y
RR.108	La Salle River	Y	N	Y	N	Y	Y
RR.109	Red River	Y	Y	Y	N	Y	Y
RR.01	Red River	Y	N	N	Y*	Y	Y
RR.02	Red River	Y	N	Y	Y	Y	Y
RR.03	Red River	Y	N	Y	Y*	Y	Y
RR.04	Red River	Y	Y	N	N	Y	Y
SR.01	Seine River	N	Y	N	N	Y	Y
LSR.01	Little Saskatchewan River	N	N	N	N	N	N
LSR.02	Little Saskatchewan River	N	N	N	N	N	N
OC.01	Oak Creek	N	N	N	N	N	N
AR.02	Assiniboine River	Y	Y	N	N	Y	Y
AR.03	Assiniboine River	Y	Y	N	N	Y	Y
AR.04	Assiniboine River	Y	Y	N	N	Y	Y
AR.06	Assiniboine River	Y	Y	N	N	Y	Y
AR.07	Assiniboine River	N	N	N	N	N	N
AR.08	Assiniboine River	N	N	N	N	N	N
AR.09	Assiniboine River	Y	Y	N	N	Y	N

*Detectable amplification occurred between cycles 40 and 45.

b) Chestnut Lamprey

Site Information		eDNA Detection in 2021					
Site	**Waterbody**	**May/June**	**July**	**August**	**September**	**Total**	**Historical**
BR.01	Brokenhead River	Y	Y	Y	Y	Y	Y
BR.02	Brokenhead River	Y	Y	Y	Y	Y	Y
BR.03	Brokenhead River	Y	Y	Y	Y	Y	Y
HC.01	Hazel Creek	Y	Y	Y	Y	Y	Y
RR.101	Joubert Creek	Y	Y	Y	N	Y	Y
RR.102	Rat River	Y	Y	Y	Y	Y	Y
RR.103	Rat River	Y	Y	Y	Y	Y	Y
RR.104	Roseau River	Y	Y	N	Y	Y	Y
RR.105	Roseau River	Y	Y	N	Y	Y	Y
RR.106	Morris River	N	N	N	N	N	Y
RR.107	Red River	Y	Y	Y	N	Y	Y
RR.108	La Salle River	N	N	N	N	N	Y
RR.109	Red River	N	N	N	N	N	Y
RR.01	Red River	N	Y	N	N	Y	Y
RR.02	Red River	N	N	N	N	N	Y
RR.03	Red River	N	N	N	N	N	Y
RR.04	Red River	Y	N	N	N	Y	Y
SR.01	Seine River	Y	N	N	N	Y	Y
LSR.01	Little Saskatchewan River	Y	N	N	Y	Y	Y
LSR.02	Little Saskatchewan River	Y	Y	Y	Y	Y	Y
OC.01	Oak Creek	N	N	N	N	N	Y
AR.02	Assiniboine River	N	N	N	N	N	Y
AR.03	Assiniboine River	Y	N	N	N	Y	Y
AR.04	Assiniboine River	N	N	N	N	N	Y
AR.06	Assiniboine River	Y	N	Y	N	Y	Y
AR.07	Assiniboine River	N	N	N	N	N	Y
AR.08	Assiniboine River	N	N	N	N	N	Y
AR.09	Assiniboine River	N	N	N	Y	Y	Y

c) Golden Redhorse

Site Information		eDNA Detection in 2021					
Site	**Waterbody**	**May/June**	**July**	**August**	**September**	**Total**	**Historical**
BR.01	Brokenhead River	Y	N	Y	Y	Y	Y
BR.02	Brokenhead River	N	Y	N	Y	Y	Y
BR.03	Brokenhead River	N	Y	N	Y	Y	Y
HC.01	Hazel Creek	N	Y	N	Y*	Y	N
RR.101	Joubert Creek	Y	N	N	N	Y	Y
RR.102	Rat River	Y	Y	N	Y	Y	Y
RR.103	Rat River	Y	N	Y	Y	Y	Y
RR.104	Roseau River	Y	Y	N	Y	Y	Y
RR.105	Roseau River	N	Y	N	Y	Y	Y
RR.106	Morris River	N	N	N	N	N	Y
RR.107	Red River	N	N	N	N	N	Y
RR.108	La Salle River	N	N	N	N	N	Y
RR.109	Red River	N	N	N	N	N	Y
RR.01	Red River	N	N	N	N	N	Y
RR.02	Red River	N	N	N	Y	Y	Y
RR.03	Red River	N	N	N	N	N	Y
RR.04	Red River	N	N	N	N	N	Y
SR.01	Seine River	N	N	N	N	N	Y
LSR.01	Little Saskatchewan River	N	N	N	N	N	N
LSR.02	Little Saskatchewan River	N	N	N	N	N	N
OC.01	Oak Creek	N	N	N	N	N	N
AR.02	Assiniboine River	N	N	N	N	N	Y
AR.03	Assiniboine River	N	N	N	N	N	Y
AR.04	Assiniboine River	N	N	N	N	N	Y
AR.06	Assiniboine River	N	N	N	N	N	Y
AR.07	Assiniboine River	N	N	N	N	N	N
AR.08	Assiniboine River	N	N	N	N	N	N
AR.09	Assiniboine River	N	N	N	N	N	N

*Detectable amplification occurred between cycles 40 and 45.

d) Hornyhead Chub

Site Information		eDNA Detection in 2021					
Site	**Waterbody**	**May/June**	**July**	**August**	**September**	**Total**	**Historical**
BR.01	Brokenhead River	Y	N	Y	Y	Y	Y
BR.02	Brokenhead River	Y	N	Y	Y	Y	Y
BR.03	Brokenhead River	Y	N	Y	Y	Y	Y
HC.01	Hazel Creek	Y	N	Y	Y	Y	Y
RR.101	Joubert Creek	N	N	N	N	N	N
RR.102	Rat River	N	N	N	N	N	N
RR.103	Rat River	N	N	N	N	N	N
RR.104	Roseau River	N	N	N	N	N	N
RR.105	Roseau River	N	N	N	N	N	N
RR.106	Morris River	N	N	N	N	N	N
RR.107	Red River	N	N	N	N	N	N
RR.108	La Salle River	N	N	N	N	N	N
RR.109	Red River	N	N	N	N	N	N
RR.01	Red River	N	N	N	Y	Y	Y
RR.02	Red River	N	N	N	Y	Y	Y
RR.03	Red River	N	N	N	N	N	Y
RR.04	Red River	N	N	N	N	N	Y
SR.01	Seine River	N	N	N	N	N	N
LSR.01	Little Saskatchewan River	N	N	N	N	N	N
LSR.02	Little Saskatchewan River	N	N	N	N	N	N
OC.01	Oak Creek	N	N	N	N	N	N
AR.02	Assiniboine River	N	N	N	N	N	N
AR.03	Assiniboine River	N	N	N	N	N	N
AR.04	Assiniboine River	N	N	N	N	N	N
AR.06	Assiniboine River	N	N	N	N	N	N
AR.07	Assiniboine River	N	N	N	N	N	N
AR.08	Assiniboine River	N	N	N	N	N	N
AR.09	Assiniboine River	N	N	N	N	N	N

Table 3.4 Detection by species using eDNA for 2021 sampling in the Winnipeg River basin (*see* Figure 3.1). The presence/absence of eDNA was assessed using species-specific probe-based assays designed and validated during this project. Sites were sampled four times in 2021. Historical indicates historical distribution based on the most recent status reports for each species. Bigmouth Buffalo and Golden Redhorse eDNA were not detected in any samples collected in the Winnipeg River basin, thus tables of their data are not included. The samples in the Winnipeg River basin were not assessed for the presence of Chestnut Lamprey eDNA due to insufficient eDNA aliquot and a lack of historical distribution in the area. Y = positive eDNA detection with detection in at least one technical replicate of one biological replicate; N = no eDNA detected.

a) Hornyhead Chub

Site Information		eDNA Detection in 2021					
Site	**Waterbody**	**May/June**	**July**	**August**	**September**	**Total**	**Historical**
EFF	Eight Foot Falls	N	N	N	N	N	N
PB	Winnipeg River	N	N	N	N	N	N
KB	Keskinen Bay	N	N	N	N	N	N
WRA	Winnipeg River	N	N	Y	N	Y	N
WRB	Winnipeg River	N	N	N	N	N	N
LB	Winnipeg River	N	N	Y	Y	Y	N
BR	Winnipeg River	N	N	Y	Y	Y	Y
Nu	Nutimik Lake	N	N	N	N	N	N
BB	Barrier Bay	N	N	N	N	N	N
DLA	Dorothy Lake	N	N	N	N	N	N
DLB	Dorothy Lake	N	N	Y*	N	Y*	N
OF	Otter Falls	N	N	N	N	N	N
ELA	Eleanor Lake	N	N	N	N	N	N
ELB	Eleanor Lake	N	N	N	N	N	N
Na	Natalie Lake	N	N	N	N	N	N
WM.01	Whitemouth River	Y	N	Y	Y	Y	Y
WM.02	Whitemouth River	Y	Y	Y	Y	Y	Y
WM.03	Whitemouth River	Y	Y	Y	Y	Y	Y
WM.04	Whitemouth River	Y	Y	Y	Y	Y	Y
WM.05	Whitemouth River	Y	Y	Y	Y	Y	Y
WM.06	Whitemouth River	Y	Y	Y	Y	Y	Y
WM.07	Whitemouth River	Y	Y	Y*	Y	Y	Y
WM.08	Whitemouth River	Y	Y	Y	Y	Y	Y
WM.09	Whitemouth River	Y	Y	Y	Y	Y	Y
BCR.01	Birch River	Y	N	Y*	Y*	Y	Y
BCR.02	Birch River	Y	Y	Y	Y	Y	Y
BCR.03	Birch River	Y	Y	Y	Y	Y	Y
BCR.04	Birch River	Y	Y	Y	Y	Y	Y
BGR.01	Boggy River	Y	Y	Y	Y	Y	Y

b) Northern Brook and Silver lampreys**

Site Information		eDNA Detection in 2021					
Site	**Waterbody**	**May/June**	**July**	**August**	**September**	**Total**	**Historical**
Silver Lamprey**							
EFF	Eight Foot Falls	N	N	N	Y	Y	Y
PB	Pointe du Bois	N	Y	N	N	Y	Y
KB	Keskinen Bay	N	N	N	N	N	Y
WRA	Winnipeg River	Y	N	N	N	Y	Y
WRB	Winnipeg River	N	N	N	N	N	Y
LB	Winnipeg River	Y	N	Y	Y	Y	Y
BR	Winnipeg River	Y	Y	N	N	Y	Y
Nu	Nutimik Lake	N	N	Y	N	Y	Y
BB	Barrier Bay	N	N	Y	N	Y	Y
DLA	Dorothy Lake	N	N	N	N	N	Y
DLB	Dorothy Lake	N	N	N	N	N	Y
OF	Otter Falls	N	N	N	N	N	Y
ELA	Eleanor Lake	N	N	N	N	N	Y
ELB	Eleanor Lake	N	N	N	N	N	Y
Na	Natalie Lake	N	Y	N	N	Y	Y
Northern Brook Lamprey**							
WM.01	Whitemouth River	Y	N	N	Y	Y	Y
WM.02	Whitemouth River	Y	Y	Y	Y	Y	Y
WM.03	Whitemouth River	Y	Y	Y	Y	Y	Y
WM.04	Whitemouth River	Y	Y	Y	Y	Y	Y
WM.05	Whitemouth River	Y	Y	Y	Y	Y	Y
WM.06	Whitemouth River	Y	Y	Y	Y	Y	Y
WM.07	Whitemouth River	Y	Y	Y	Y	Y	Y
WM.08	Whitemouth River	Y	Y	Y	Y	Y	Y
WM.09	Whitemouth River	Y	Y	Y	Y	Y	Y
BCR.01	Birch River	Y	N	Y	Y	Y	Y
BCR.02	Birch River	Y	Y	Y	Y	Y	Y
BCR.03	Birch River	Y	Y	Y	Y	Y	Y
BCR.04	Birch River	Y	Y	Y	Y	Y	Y
BGR.01	Boggy River	Y	Y	Y	Y	Y	N

*Detectable amplification occurred between cycles 40 and 45.

**Northern Brook and Silver lampreys cannot be distinguished genetically; however, detection of Northern Brook Lamprey or Silver Lamprey eDNA was hypothesized based on previous collection records (COSEWIC 2020).

Table 3.5 Detection by species using eDNA for 2023 sampling (*see* Figure 3.1). The presence/absence of eDNA was assessed using species-specific probe-based assays designed and validated during this project. Sites were sampled once between June and September. BMBF = Bigmouth Buffalo; CHLP = Chestnut Lamprey; GDRH = Golden Redhorse; HHCH = Hornyhead Chub; NBLP/SVLP = Northern Brook Lamprey/Silver Lamprey. Y = positive eDNA detection with detection in at least one technical replicate of one biological replicate; N = no eDNA detected. Site codes highlighted yellow indicate sites that were sampled in 2021 (*see* Table 3.3, Table 3.4).

Site Code	Waterbody	BMBF	CHLP	GDRH	HHCH	NBLP/SVLP
AR.03	Assiniboine River	N	N	N	N	N
AR.04	Assiniboine River	N	N	N	N	N
AR.06	Assiniboine River	N	N	N	N	N
AR.07	Assiniboine River	N	N	N	N	N
AR.08	Assiniboine River	N	Y	N	N	N
AR.09	Assiniboine River	Y	N	N	N	N
AR.10	Assiniboine River	N	Y	N	N	N
BCR.02	Birch River	N	N	N	Y	Y
BCR.03	Birch River	N	N	N	Y	Y
BGR.01	Boggy River	N	N	N	Y	Y
BR.02	Brokenhead River	N	Y	Y	Y	N
BR.04	Brokenhead River	N	Y	Y	Y	N
BRD.01	Bird River	N	N	N	N	N
BRD.02	Bird River	N	N	N	N	Y
CC.02	Cook's Creek	N	N	Y*	N	N
DP.01	Dauphin Lake	N	N	N	N	N
DV.01	Devil's Creek	N	N	N	N	N
IC.01	Icelandic River	N	N	N	N	N
IC.02	Icelandic River	N	Y	Y*	N	N
LE.01	Lee River	N	N	N	N	
LE.02	Lee River	N	N	N	N	Y
LMB.01	Lake Manitoba	Y	N	N	N	N
LOW.01	Lake of the Woods	N	N	N	N	N
LP.01	Lake Winnipegosis	N	N	N	N	N
LS.01	La Salle River	N	Y	N	N	N
LS.02	La Salle River	N	N	N	N	N
LSR.03	Little Saskatchewan River	N	Y	N	N	N
LSR.04	Little Saskatchewan River	N	N	N	N	N
LWPG.01	Lake Winnipeg	N	N	N	N	N
LWPG.02	Lake Winnipeg	N	Y	Y*	N	N
LWPG.03	Lake Winnipeg	N	Y	Y*	N	N
MT.01	Manigotagan River	Y*	N	Y	N	N
NU.01	Nutimik Lake	N	N	N	N	Y
OC.01	Oak Creek	N	N	N	N	N
PM.01	Pembina River	N	N	N	N	N
PM.02	Pembina River	N	N	Y*	N	N
PM.03	Pembina River	N	N	N	N	N
QU.01	Qu'Appelle River	N	N	N	N	N
RC.01	Rice Creek	N	N	N	N	N
RC.02	Rice Creek	N	N	N	Y	N
RR.01	Red River	N	N	N	N	N
RR.02	Red River	N	N	N	N	N
RR.03	Red River	N	N	N	N	N
RR.04	Red River	N	Y	N	N	N
RR.05	Red River	N	N	N	N	N
RR.06	Red River	Y*	N	N	N	N
RR.109	Red River	N	N	N	N	N
RR.110	Roseau River	N	Y	Y	N	N

*Detectable amplification occurred between cycles 40 and 45.

Table 3.5. continued.

Site Code	Waterbody	BMBF	CHLP	GDRH	HHCH	NBLP/SVLP
SH.01	Shell River	N	Y	N	N	N
SH.02	Shell River	N	Y	N	N	N
SR.02	Seine River	N	N	N	N	N
SR.03	Seine River	N	N	N	Y	N
SR.04	Seine River	N	Y	N	N	N
WD.01	Whitemud River	N	N	N	N	N
WD.02	Whitemud River	N	N	N	N	N
WM.07	Whitemouth River	N	N	N	Y	N
WM.08	Whitemouth River	N	N	Y*	Y	Y
WM.11	Whitemouth River	N	N	N	Y	Y

*Detectable amplification occurred between cycles 40 and 45.

Table 3.6 Summary of results using qPCR and designed eDNA assay for Silver and Northern Brook lampreys and eDNA samples collected in the Churchill River and Hudson Bay in July 2023 (*see* Figure 3.1). Of the six species of interest, as only Silver Lamprey is historically known to occur in the area, samples were only assessed using the NBLP CytB assay given the limited aliquot available. No detections of Silver Lamprey eDNA were noted for any of these samples. Y = positive eDNA detection; N = no eDNA detected.

Site Code	Waterbody	SVLP/NBLP
CHR.01	Churchill River	N
CHR.02	Churchill River	N
CHR.03	Churchill River	N
CHR.04	Churchill River	N
HB.01	Hudson Bay	N
HB.02	Hudson Bay	N
HB.03	Hudson Bay Estuary	N

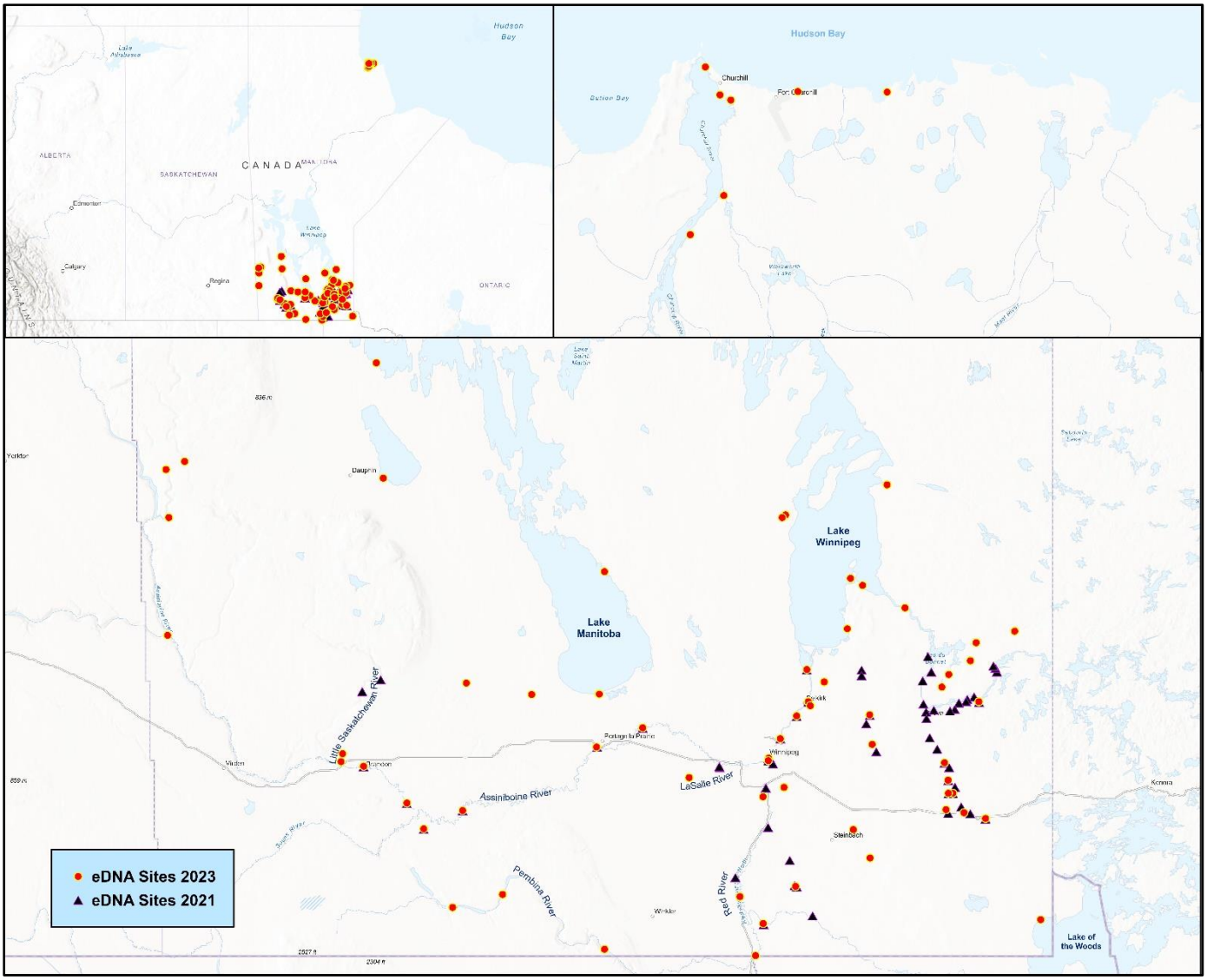
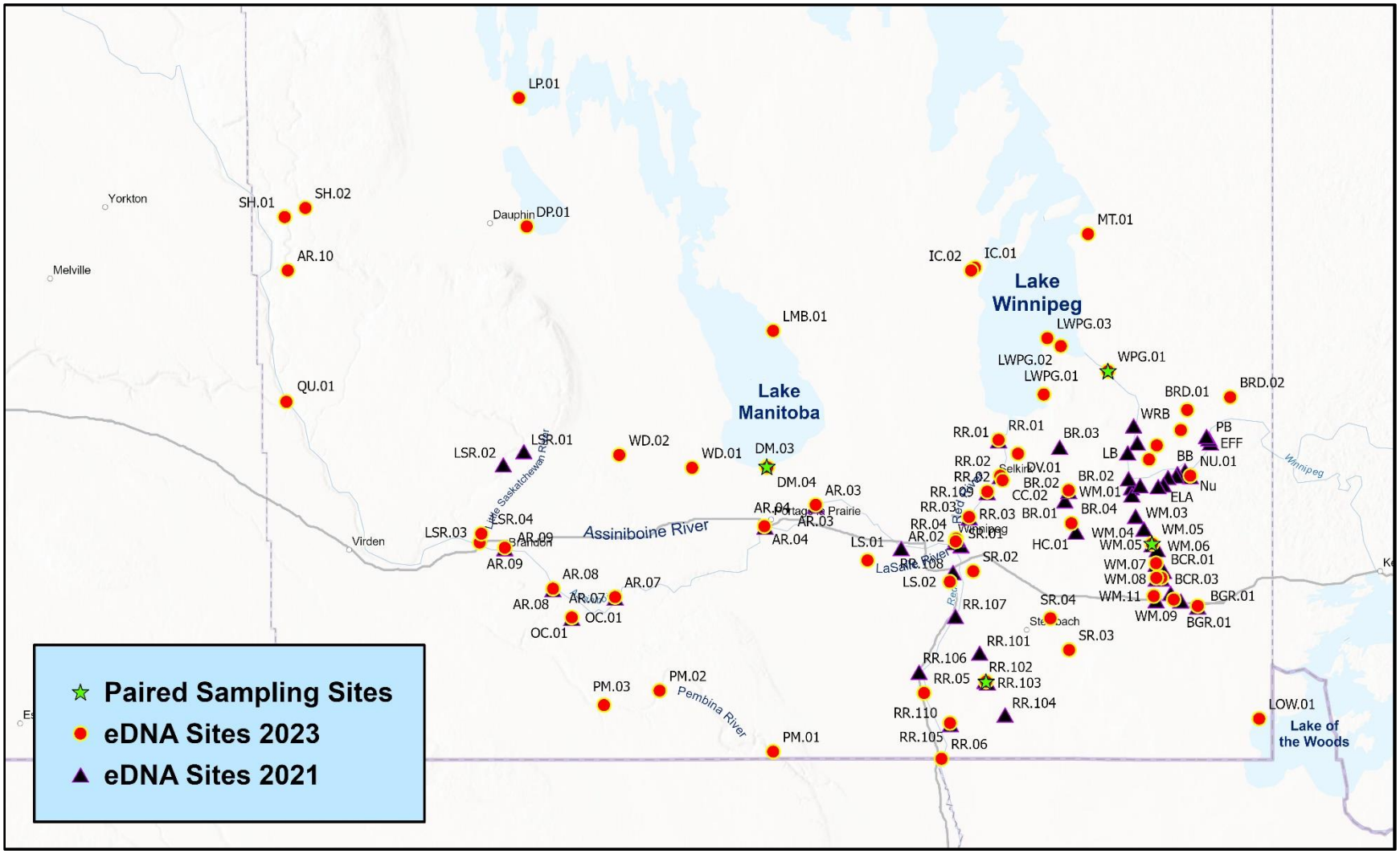


Figure 3.1 a) Sites sampled for eDNA in Manitoba in 2021 and 2023; b) Sites sampled for eDNA in Manitoba in 2021 and 2023 with site codes.

a) Orange circle markers indicate sites sampled for eDNA in 2023 (apart from Delta Marsh, which was sampled in 2022). Purple triangles indicate sites sampled in 2021 for eDNA. The top left view shows all sites sampled. The top right view highlights sites sampled in and around Churchill, Manitoba. The lower view shows sites sampled in southern Manitoba.



b) Orange circle markers indicate sites sampled for eDNA in 2023 (apart from Delta Marsh (DM.03, DM.04), which were sampled in 2022). Purple triangles indicate sites sampled in 2021 for eDNA. Greens stars indicate sites that were paired sampled using eDNA and electrofishing (*see* Chapter 4).

occurred in 2021 versus 2023 (Table 3.3, Table 3.4, Table 3.5, Table 3.6). In 2021, Bigmouth Buffalo eDNA was detected at sites AR.03, AR.04, AR.06, RR.01, RR.02, RR.03 and RR.04, but it was only detected at site AR.09 of the samples sampled both in 2021 and 2023 (Table 3.3, Table 3.5). Chestnut Lamprey eDNA was also detected more frequently in 2021 versus 2023, with detections at sites AR.03, AR.06, AR.09 and RR.01 in 2021, but only AR.08 and RR.04 in 2023 (Table 3.3, Table 3.5). This was also noted for Hornyhead Chub, which was detected in the Red River at sites RR.01 and RR.02 in 2021, but not in 2023 (Table 3.3, Table 3.5).

3.3.2 Clean Procedure

Minor contamination of field negative controls was noted in a few samples. If the contamination was distinguishable from positive eDNA detection (i.e., consistently detectable amplification of eDNA in biological replicates cycles before that of the negative control by a difference of at least four cycles between detectable amplification), positive eDNA detection was accepted. In cases where there was no distinguishable positive eDNA detection from the contamination, samples were omitted from analysis for the species where contamination occurred.

3.3.3 Bigmouth Buffalo

Bigmouth Buffalo eDNA was detected in 14 of the sites sampled in 2021, and four of the sites sampled in 2023 (Figure 3.2). Most Bigmouth Buffalo eDNA detections occurred in samples collected in 2021 (Table 3.3, Table 3.5), and in samples collected in May/June (Table 3.3). Bigmouth Buffalo eDNA was most present in the Red River and Assiniboine River (Table 3.3, Table 3.5).

3.3.4 Chestnut Lamprey

Chestnut Lamprey eDNA was detected in 18 of the sites sampled in 2021, and 14 of the sites sampled in 2023 (Figure 3.3). Of the 18 sites where Chestnut Lamprey eDNA was detected

in 2021, eDNA was detected most in samples collected in May/June (16/18), then July (12/18), followed by September (11/18) and August (10/18), respectively (Table 3.3).

3.3.5 Golden Redhorse

Golden Redhorse eDNA was detected in 10 of the sites sampled in 2021, and 10 of the sites sampled in 2023 (Figure 3.4). Golden Redhorse eDNA was mostly detected in tributaries of larger waterbodies, such as the Brokenhead, Rat and Roseau rivers (Table 3.3, Table 3.5). Golden Redhorse eDNA occurred in Hazel Creek in samples collected in 2021 (Table 3.3).

3.3.6 Hornyhead Chub

Hornyhead Chub eDNA was detected in 24 of the sites sampled in 2021 and 10 sites of the sites sampled in 2023 (Figure 3.5). In both years, Hornyhead Chub eDNA was most prevalent in the Whitemouth, Birch, Boggy, and Brokenhead rivers. Novel detections of Hornyhead Chub occurred in the Boggy River and in some areas of the Winnipeg River systems (Table 3.4) in 2021. Hornyhead Chub were detected outside their known range in 2023 in Rice Creek and Seine River (Table 3.5).

3.3.7 Northern Brook Lamprey/Silver Lamprey

The eDNA of Northern Brook and Silver lampreys was detected in samples collected at 22 of the sites sampled in 2021, and 8 of the sites sampled in 2023 (Figure 3.6). The historical distribution of Northern Brook Lamprey in Manitoba includes the Whitemouth and Birch rivers and Hazel Creek ((COSEWIC 2020). Silver Lamprey have historically been found in the Red, Rat, Assiniboine, Winnipeg, and Nelson rivers (COSEWIC 2020). Based on these records, detections of eDNA using the designed eDNA assay that detects the eDNA of Northern Brook and Silver lampreys were classified depending on their historical range. Consistent eDNA detection, which was presumed to be Northern Brook Lamprey, occurred in the Whitemouth,

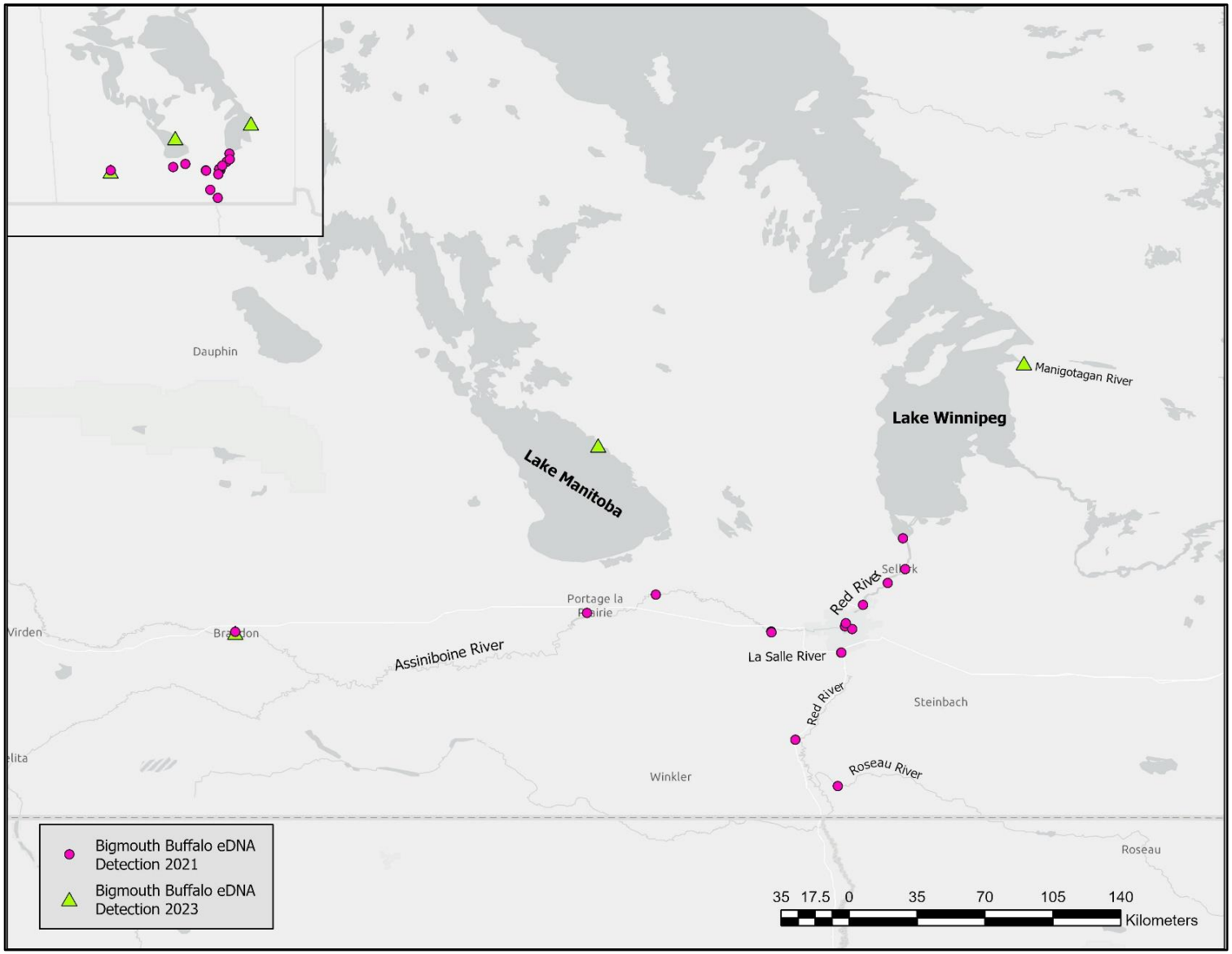


Figure 3.2 Detections of Bigmouth Buffalo eDNA in Manitoba at sites sampled for eDNA in 2021 and 2023. Pink circle markers indicate sites where Bigmouth Buffalo eDNA was detected in 2021. Green triangle markers indicate positive detections of Bigmouth Buffalo eDNA in samples collected in 2023.

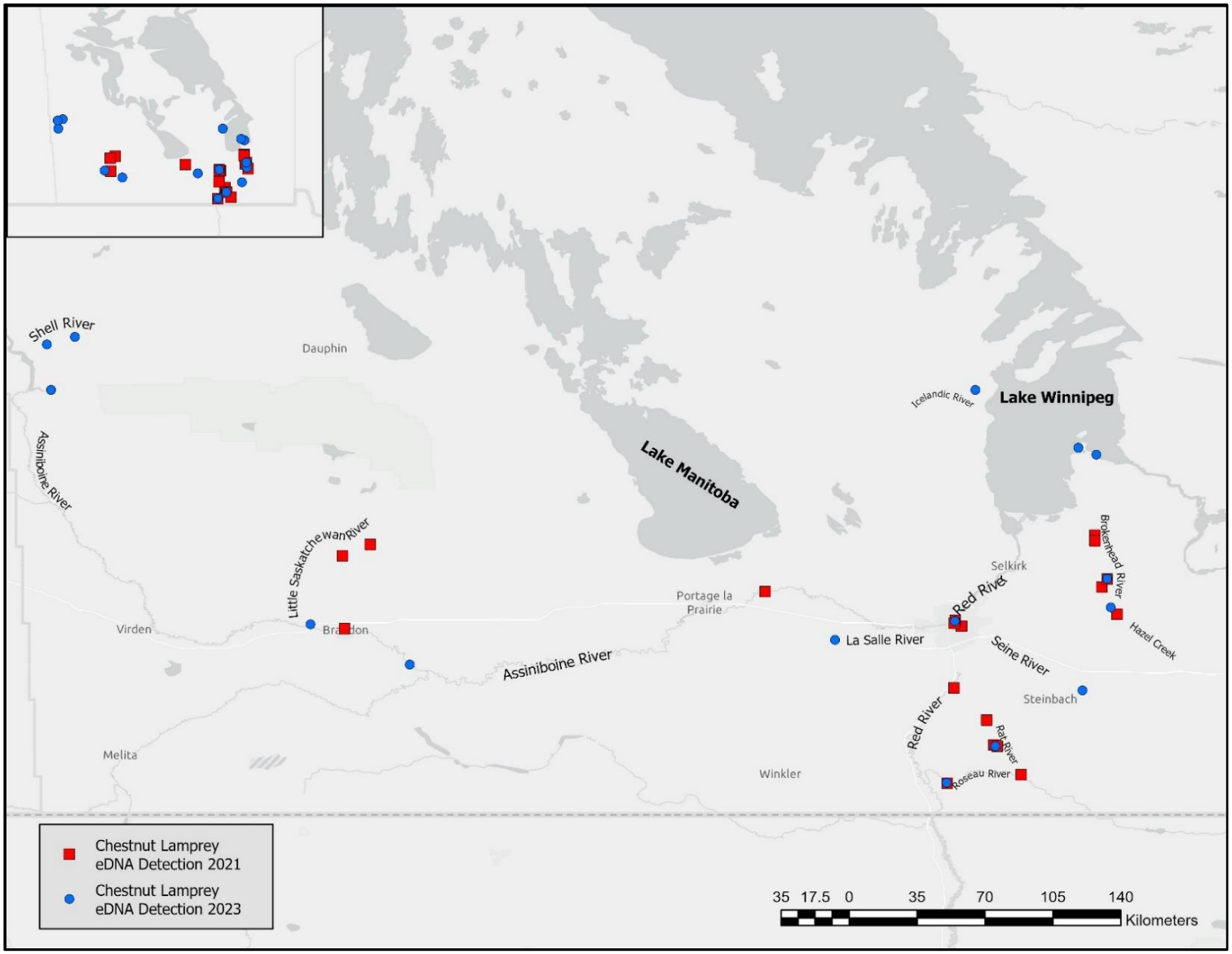


Figure 3.3 Detections of Chestnut Lamprey eDNA in Manitoba at sites sampled for eDNA in 2021 and 2023. Red square markers and blue circle markers indicate sites where Chestnut Lamprey eDNA was detected in 2021 and 2023, respectively.

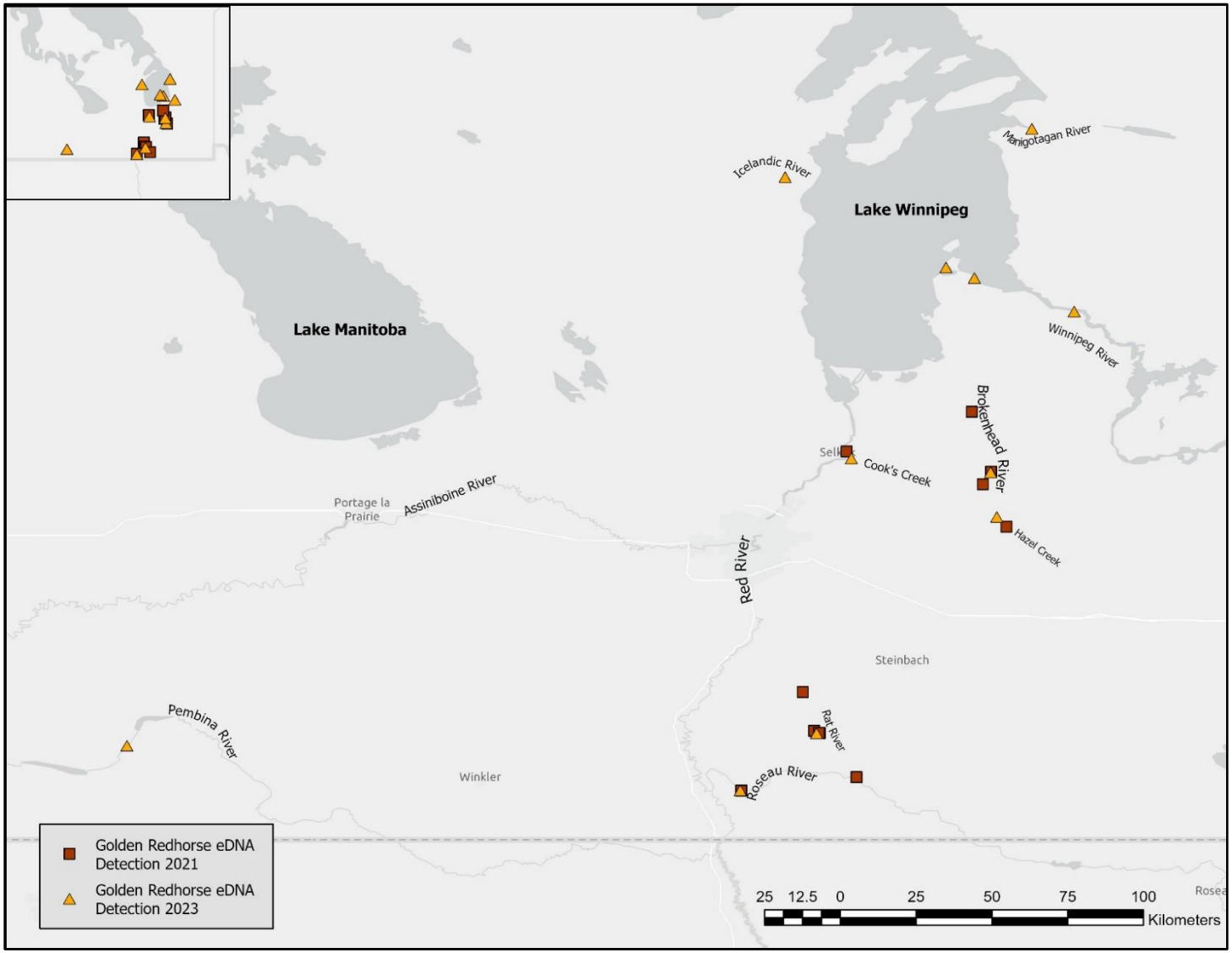


Figure 3.4 Detections of Golden Redhorse eDNA in Manitoba at sites sampled for eDNA in 2021 and 2023. Brown square markers indicate sites where Golden Redhorse eDNA was detected in 2021. Gold triangle markers indicate positive detections of Golden Redhorse eDNA in samples collected in 2023.

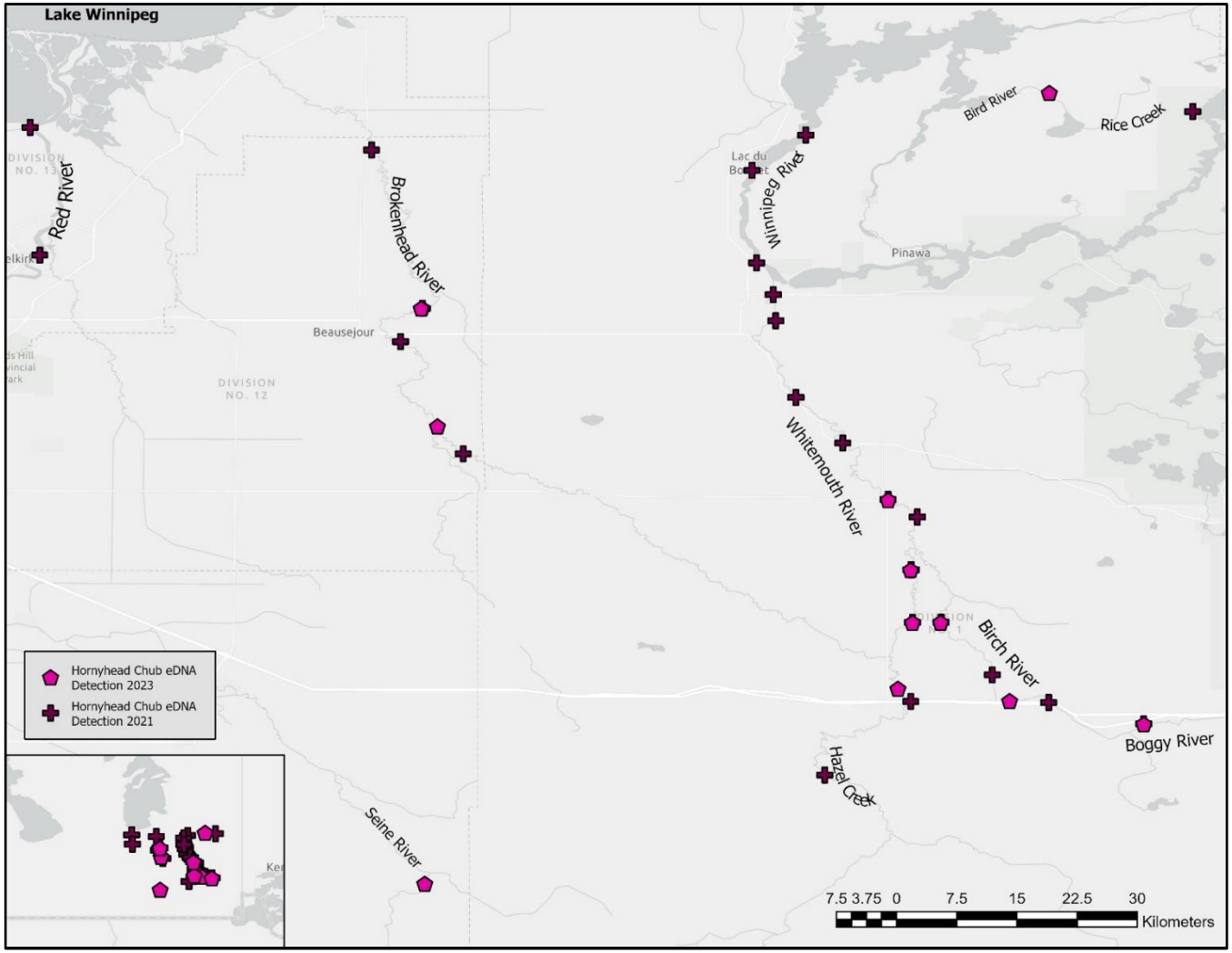


Figure 3.5 Detections of Hornyhead Chub eDNA in Manitoba at sites sampled for eDNA in 2021 and 2023. Pink hexagon markers indicate sites where Hornyhead Chub eDNA was detected in 2021. Dark pink cross markers indicate positive detections of Hornyhead Chub eDNA in samples collected in 2023.

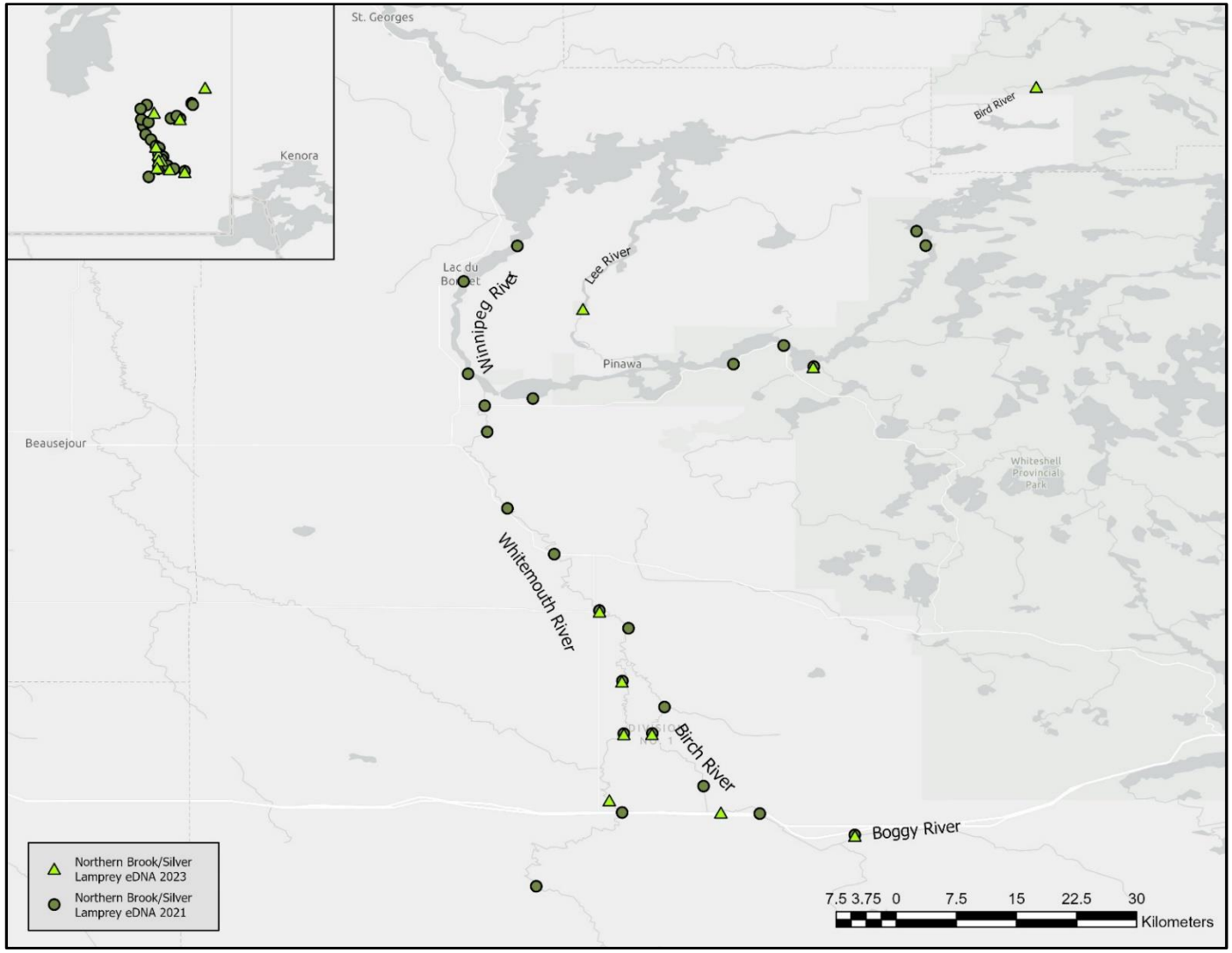


Figure 3.6 Detections of Northern Brook and/or Silver Lamprey eDNA in Manitoba at sites sampled for eDNA in 2021 and 2023. Neon green triangle markers indicate sites where Northern Brook and/or Silver Lamprey eDNA was detected in 2021. Dark green circle markers indicate positive detections of Northern Brook and/or Silver Lamprey eDNA in samples collected in 2023.

Birch, and Boggy rivers in both years, except for one sampling event in 2021 in the Birch River where there was no detection of eDNA (Table 3.4). Suspected Silver Lamprey eDNA was detected in both years throughout the Winnipeg River system (Table 3.4, Table 3.5). The eDNA of Northern Brook and Silver lampreys was not detected in samples collected in northern Manitoba in the Churchill River and the Hudson Bay (Table 3.6), or in western Manitoba (Table 3.5).

3.4 Discussion

3.4.1 Sampling Method

In 2021, samples were collected in four rounds (May/June, July, August, and September) to increase the likelihood of detection and see if migratory patterns could be detected. However, this method was very costly and time-consuming, and minimally increased the number of detections that occurred (Table 3.3, Table 3.4). Most positive eDNA detections were a result of samples collected in late Spring/early Summer or early Fall. This could be due to samples being collected during or shortly after spawning or during the migration of some of these fishes to the waterbodies where they overwinter (*see* Chapter 1). Future sampling using eDNA to detect these species should occur during periods with the highest likelihood of detection based on biotic (e.g., spawning, migration, increased activity) and abiotic factors (e.g., slower flow rates and shallower depths, water temperature) (i.e., May – July; September – October) to reduce the number of consumables and time required to process samples while increasing the likelihood of detection (*see* Chapter 1).

Some sites were sampled both in 2021 and 2023. Of these sampling events, more detections occurred in 2021 than in 2023 (Table 3.3, Table 3.5). Most notably, for Bigmouth Buffalo, Chestnut Lamprey and Hornyhead Chub, eDNA of these species was detected at some sites in 2021, but not in 2023 (Table 3.3, Table 3.5). More sampling events occurred in 2021 per

site than in 2023; however, different sampling methods were also used (*see* 3.2 Methods). In 2021, samples were collected by hand in sterilized Nalgene™ wide-mouth bottles and filtered using vacuum filtration in the lab, whereas samples in 2023 were collected and filtered using the OSMOS. This difference in sampling methods could have contributed to false negative detections (*see* below) in eDNA samples, as the sample filtration method has been reported to affect the yield of eDNA (Piggott 2016; Furlan et al. 2016; Song et al. 2020).

3.4.2 Sample Inhibition

Many sites within the known range of the species of interest resulted in a lack of eDNA detection (Table 3.2, Table 3.3, Table 3.4, Table 3.5). Inhibition of qPCR reactions can occur in eDNA samples and result in failed or delayed amplification of the target DNA (Goldberg et al. 2016). Inhibition can be identified in samples by including internal positive controls (IPC) or internal amplification controls (IAC) in qPCR reactions or prevented using inhibitor removal treatments or by diluting DNA (Goldberg et al. 2016; Klymus et al. 2020). False negatives due to qPCR inhibition could be another explanation for the lack of detection but are not expected, as the eluted DNA was subjected to an inhibitor removal treatment after extraction, and the qPCR master mix (TaqMan™ Environmental Master Mix 2.0, Applied Biosystems) used is moderately resistant to inhibitors (Minogue et al. 2014). However, inhibitor removal kits can lead to decreased DNA yield and detection (Goldberg et al. 2016; Loeza-Quintana et al. 2021) and could have resulted in false negative detections in this study. Other biotic or abiotic factors or a lack of assay sensitivity could be to blame for the lack of detection of eDNA in these samples (*see* Chapter 1). Additional surveying of the target species could help confirm the nature of these lack of detections (i.e. if they are true or false negatives).

3.4.3 Novel and Notable Detections

3.4.3.1 Bigmouth Buffalo

Bigmouth Buffalo eDNA was notably absent from the Qu'Appelle River basin, where declines in Bigmouth Buffalo have been reported due to the degradation, and in some cases, elimination of spawning habitat through water management practices to control flooding (COSEWIC 2009; Fisheries and Oceans Canada 2021). This suggests that there may be no movement of Bigmouth Buffalo of Bigmouth Buffalo from Manitoba to Saskatchewan. A lack of recruitment of Bigmouth Buffalo to Saskatchewan from Manitoba has also been reported (Bryshun 2023), strongly indicating that impermeable barriers for Bigmouth Buffalo exist, separating Saskatchewan and Manitoban populations of Bigmouth Buffalo.

Novel detections of Bigmouth Buffalo in a region of the Assiniboine River near Brandon, Manitoba, occurred using eDNA. These detections could indicate an isolated population of Bigmouth Buffalo that could be restricted to a small section of the Assiniboine River due to habitat fragmentation after the installation of the Portage Diversion Dam and various dams along the Assiniboine River, as Bigmouth Buffalo are long-lived (Lackmann et al. 2023). If this population of Bigmouth Buffalo is isolated, it is at great risk of inbreeding depression due to a lack of gene flow (Blanchet et al. 2010; Fraser et al. 2014; Vandervelde 2022). Bigmouth Buffalo eDNA was also noted in the Roseau River, which was not previously reported within the known range of Bigmouth Buffalo. More research is needed to determine the state of Bigmouth Buffalo in the Roseau River and the Assiniboine River near Brandon, Manitoba, which could indicate further expansion, severe fragmentation of populations, or recruitment failure of Bigmouth Buffalo in Manitoba.

Overall, a lack of detection of Bigmouth Buffalo eDNA and high threshold cycle (i.e., the number of cycles needed for the fluorescence generated by the reaction to surpass the

background fluorescence) values were observed throughout this research. A lack of sensitivity of the designed Bigmouth Buffalo CO1 assay due to an unintentional addition of an adenosine nucleotide to the 3' end of the probe of the Bigmouth Buffalo CO1, which was added by human error at some point during the design or ordering process. This additional nucleotide likely contributed to low detections of Bigmouth Buffalo eDNA due to the changes in the characteristics of the Bigmouth Buffalo probe caused by the addition of the adenosine (Thermo Fisher Scientific 2022). As the probe is the element of the assay which is responsible for identifying the quantity of target eDNA present in the sample (*see* Chapter 1, Section 1.4 Environmental DNA), altered effectiveness of the probe is likely to result in a lack of sensitivity of the assay overall. This error was discovered in the late stages of this project and could not be rectified within the time constraints of this project. Therefore, to confirm the presence or absence of Bigmouth Buffalo eDNA in the samples collected, further research is needed to re-assay the samples using the assay with the altered probe (i.e., without the additional adenosine), or by using the designed Bigmouth Buffalo CytB assay.

3.4.3.2 Chestnut Lamprey

Few recent detections of Chestnut Lamprey have been reported in western Manitoba before this research (COSEWIC 2010). Using eDNA, this research confirmed the presence of Chestnut Lamprey on the border of Manitoba and Saskatchewan in the Shell and Qu'Appelle rivers. Chestnut Lamprey were not detected in Devil's Creek, Oak Creek, Whitemouth River, Winnipeg River, or Lake of the Woods despite historical collections within these waterbodies (COSEWIC 2010). The last reported collections of Chestnut Lamprey in these waterbodies were in 1983, 2004, 1990, 1991, and 1970, respectively, and therefore, could indicate a restriction of the range of Chestnut Lamprey (*see* COSEWIC 2010). If vouchers of these specimens are available, the re-examination of these specimens and genetic identification would be useful to

confirm their identity as Chestnut Lamprey. Notable detections of Chestnut Lamprey eDNA were made in the Brokenhead River, where Chestnut Lamprey eDNA was almost always detected, which is likely used by adult Chestnut Lamprey for spawning, and larval lamprey as habitat (Collerone 2014). Chestnut Lamprey were detected outside of their known range (COSEWIC 2010) in the Roseau River and the Icelandic River (Table 3.5). This research provides current distribution data for Chestnut Lamprey in Manitoba and creates a baseline from which more research can and should be done. Most importantly, more research is needed to determine the status of the populations of Chestnut Lamprey and any risks these populations may be facing.

3.4.3.3 Golden Redhorse

The distribution of Golden Redhorse in Manitoba has been largely unmonitored since their discovery in Manitoba in 1984, shortly before their last assessment in 1989 (Franzin et al. 1986; Goodchild 1990). Because of this, many novel detections of Golden Redhorse using eDNA occurred during this study. Most historical records of the distribution of Golden Redhorse in Manitoba report their presence near the Lockport Dam in the Red River (Scott and Crossman 1973; Franzin et al. 1986; Goodchild 1990); however, Golden Redhorse eDNA was not detected in this region during this research. This could be because Golden Redhorse no longer occur in this region or occur in such low numbers that it was not possible to capture their eDNA due to the high levels of water turnover from the dam (Curtis et al. 2021). With this research, widespread distribution of Golden Redhorse in Manitoba was determined, with detections of Golden Redhorse in southern Manitoba, such as in the Rat River, and the north-eastern region of Lake Winnipeg in the Manigotagan River. If these detections are the result of range expansion, like Bigmouth Buffalo, Golden Redhorse could be at risk of isolated populations and reduced gene flow (COSEWIC 2010). However, these detections could also indicate previously

unreported populations, and highlight the need for further research to assess the status of the Golden Redhorse in Manitoba.

3.4.3.4 Hornyhead Chub

The status of Hornyhead Chub in Manitoba has gone largely unchecked since its last status report in 1988 but has been assumed to be stable (Dalton 1989; Stewart and Watkinson 2004). This research confirms the limited, but seemingly stable, distribution of Hornyhead Chub in Manitoba using eDNA in the Winnipeg River system and the Brokenhead River with the nearly consistent detection of Hornyhead Chub in these waterbodies. However, determining the state of Hornyhead Chub within these waterbodies is essential in assessing its well-being in Manitoba and safeguarding its future by protecting its necessary habitat as the Hornyhead Chub is suspected to play an important role in the lifecycle of other Leuciscid fishes, such as the Endangered Carmine Shiner (*see* Chapter 1, Section 1.2 Hornyhead Chub). Further research should investigate the importance, if any, that the Hornyhead Chub has in the survival of other at-risk species, such as the Carmine Shiner in Manitoba (Vives 1990), as their continued persistence may be critical for the survival and recovery of other species.

3.4.3.5 Northern Brook Lamprey/Silver Lamprey

One of the limitations of eDNA can be its specificity, especially in the case of paired species, like Northern Brook and Silver lampreys, where significant and consistent genetic differences that can be used to create species-specific assays are not yet known (*see* Chapter 1, Section 1.3). In this case, historical records can be used to make hypotheses as to which species a detection may be associated with. Because of this, novel detections of Northern Brook Lamprey or Silver Lamprey cannot be said with as much certainty, as an eDNA detection using the assay designed in this research indicates the detection of either species. However, with this research, we report more frequent detection of, presumably, Northern Brook Lamprey, in the Whitemouth,

Birch and Boggy rivers using eDNA than previously reported in their last status report (COSEWIC 2020). This could be due to a lack of sensitivity of the previous assay used, an increase in population size, a combination of the latter, or other factors. Additional monitoring of these species is necessary, and future research should investigate the possibility of designing an eDNA assay that can distinguish between the two species.

Historical collection records state the presence of Silver Lamprey in the Assiniboine, Shell, Rat, Red and Seine rivers, Lake of the Woods, and Joubert Creek. We could not detect any Silver Lamprey eDNA in any of these waterbodies, which could indicate extirpation of Silver Lamprey in these waterbodies, as the last observations of Silver Lamprey in these waterbodies were in 2002, 1991, 1986, 1974, 1974, 1970 and 1976, respectively, although faint eDNA detections were reported of Northern Brook Lamprey or Silver Lamprey eDNA in the Whitemud and Seine rivers, and in two Assiniboine River tributaries during sampling efforts occurring between 2015 and 2017 (*see* COSEWIC 2020). It is also possible that these previous records result from the misidentification of Chestnut Lamprey as Silver Lamprey (Stewart and Watkinson 2004; COSEWIC 2020). As with Chestnut Lamprey, if voucher specimens of these collections exist, re-examination of these specimens morphologically and genetically to confirm the identify of the specimens would assist in clarifying the historical range of Silver Lamprey in Manitoba, as genetically-identified Chestnut Lamprey without bicuspid inner lateral teeth (a distinguishing morphological characteristic of Chestnut Lamprey from Silver Lamprey) have been found in Manitoba (COSEWIC 2020). If aliquots of the eDNA samples where Silver Lamprey or Northern Brook Lamprey eDNA was detected in the Whitemud and Seine rivers, and tributaries of the Assiniboine River assessed are still available, future research should sequence the amplicons of the eDNA to determine the origin of the eDNA.

3.5 Conclusion

The degradation and loss of aquatic habitat through the construction of dams, urbanization, agriculture, and other methods can directly impact populations of freshwater fishes (Fraser et al. 2014; Fuller et al. 2015). Furthermore, climate change is expected to have various impacts on freshwater ecosystems, all of which can affect further the distribution and abundance of these fishes (Pandit et al. 2017; Barbarossa et al. 2021). Addressing these risks to freshwater ecosystems and fishes requires coordinated conservation and management efforts, aimed at protecting and restoring freshwater habitat, including controlling invasive species, implementing climate adaptation strategies, and promoting sustainable water resource management practices. In this chapter, I assessed the current distribution of six freshwater fishes of conservation concern in Manitoba using eDNA: Bigmouth Buffalo, Chestnut Lamprey, Golden Redhorse, Hornyhead Chub, Northern Brook Lamprey, and Silver Lamprey. More research is needed to investigate the discrepancies between historical collection records and eDNA collection records reported with this research. Furthermore, additional research is needed before the assessment of these species to identify the threats, if any, Bigmouth Buffalo, Chestnut Lamprey, Golden Redhorse and Hornyhead Chub face, and what factors may be contributing to the changes in their distribution.

Literature Cited

Barbarossa V, Bosmans J, Wanders N, et al (2021) Threats of global warming to the world's freshwater fishes. Nature Communications 12:1701. https://doi.org/10.1038/s41467-021-21655-w

Blanchet S, Rey O, Etienne R, et al (2010) Species-specific responses to landscape fragmentation: implications for management strategies. Evolutionary Applications 3:291–304. https://doi.org/10.1111/j.1752-4571.2009.00110.x

Bryshun R (2023) An evaluation of Bigmouth Buffalo (*Ictiobus cyprinellus*) and Common Carp (*Cyprinus carpio*) association within the Qu'Appelle River system and its implications for species conservation. PhD thesis, University of Saskatchewan, Saskatoon

Collerone S (2014) Distribution and habitat of larval and spawning Silver (*Ichthyomyzon unicuspis*), Chestnut (*I. castaneus*), and Northern Brook (*I. fossor*) lampreys in southeastern Manitoba. Honour's thesis, University of Manitoba, Winnipeg

COSEWIC (2007) COSEWIC assessment and update status report on the Northern Brook Lamprey *Ichthyomyzon fossor* (Great Lakes – Upper St. Lawrence populations and Saskatchewan – Nelson population) in Canada. Committee on the Status of Endangered Wildlife in Canada, Ottawa

COSEWIC (2009) COSEWIC assessment and status report on the Bigmouth Buffalo *Ictiobus cyprinellus* (Great Lakes - Upper St. Lawrence populations and Saskatchewan - Nelson River populations) in Canada. Committee on the Status of Endangered Wildlife in Canada, Ottawa

COSEWIC (2010) COSEWIC assessment and status report Chestnut Lamprey *Ichthyomyzon castaneus* (Great Lakes - Upper St. Lawrence populations and Saskatchewan-Nelson River populations) in Canada. Committee on the Status of Endangered Wildlife in Canada, Ottawa

COSEWIC (2011) COSEWIC assessment and status report on the Silver Lamprey *Ichthyomyzon unicuspis* (Great Lakes - Upper St. Lawrence populations and Saskatchewan - Nelson Rivers populations) in Canada. Committee on the Status of Endangered Wildlife in Canada, Ottawa

COSEWIC (2020) COSEWIC assessment and status report on the Northern Brook Lamprey *Ichthyomyzon fossor* (Great Lakes - Upper St. Lawrence populations and Saskatchewan - Nelson River populations) and the Silver Lamprey *Ichthyomyzon unicuspis* (Great Lakes - Upper St. Lawrence populations) in Canada. Committee on the Status of Endangered Wildlife in Canada, Ottawa

Curtis AN, Tiemann JS, Douglass SA, et al (2021) High stream flows dilute environmental DNA (eDNA) concentrations and reduce detectability. Diversity and Distributions 27:1918–1931. https://doi.org/10.1111/ddi.13196

Dalton KW (1989) Status of the Hornyhead Chub, *Nocomis biguttatus*, in Canada. Canadian Field-Naturalist 103:180–185

Dextrase AJ, Mandrak NE (2006) Impacts of alien invasive species on freshwater fauna at risk in Canada. Biol Invasions 8:13–24. https://doi.org/10.1007/s10530-005-0232-2

Dudgeon D (2010) Prospects for sustaining freshwater biodiversity in the 21st century: linking ecosystem structure and function. Current Opinion in Environmental Sustainability 2:422–430. https://doi.org/10.1016/j.cosust.2010.09.001

Enders EC, Charles C, Watkinson DA, et al (2019) Analysing habitat connectivity and home ranges of Bigmouth Buffalo and Channel Catfish using a large-scale acoustic receiver network. Sustainability 11:3051. https://doi.org/10.3390/su11113051

Fisheries and Oceans Canada (2021) Management plan for the Bigmouth Buffalo (*Ictiobus cyprinellus*) in Canada (Saskatchewan-Nelson River populations). Fisheries and Oceans Canada, Ottawa

Franzin WG, Parker BR, Harbicht SM (1986) A first record of Golden Redhorse *Moxostoma erythrurum*, from the Red River in Manitoba, Canada. Canadian Field-Naturalist 100:270–271

Fraser DJ, Debes PV, Bernatchez L, Hutchings JA (2014) Population size, habitat fragmentation, and the nature of adaptive variation in a stream fish. Proc R Soc B 281:20140370. https://doi.org/10.1098/rspb.2014.0370

Fuller MR, Doyle MW, Strayer DL (2015) Causes and consequences of habitat fragmentation in river networks. Annals of the New York Academy of Sciences 1355:31–51. https://doi.org/10.1111/nyas.12853

Furlan EM, Gleeson D, Hardy CM, Duncan RP (2016) A framework for estimating the sensitivity of eDNA surveys. Molecular Ecology Resources 16:641–654. https://doi.org/10.1111/1755-0998.12483

Gingera TD, Steeves TB, Boguski DA, et al (2016) Detection and identification of lampreys in Great Lakes streams using environmental DNA. Journal of Great Lakes Research 42:649–659. https://doi.org/10.1016/j.jglr.2016.02.017

Goldberg CS, Turner CR, Deiner K, et al (2016) Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods in Ecology and Evolution 7:1299–1307. https://doi.org/10.1111/2041-210X.12595

Gomes AD, Tolussi CE, Ribeiro CDS, et al (2015) The role of ovarian steroids in reproductive plasticity in *Hoplias malabaricus* (Teleostei: Characiformes: Erythrinidae) in tropical reservoirs with different degrees of pollution. General and Comparative Endocrinology 222:1–10. https://doi.org/10.1016/j.ygcen.2014.10.008

Goodchild CD (1990) Status of the Golden Redhorse, *Moxostoma erythrurum*, in Canada. Canadian Field-Naturalist 104:103–111

Klymus KE, Ruiz Ramos DV, Thompson NL, Richter CA (2020) Development and testing of species-specific quantitative PCR assays for environmental DNA applications. Journal of Visualized Experiments e61825. https://doi.org/10.3791/61825

Lackmann AR, Sereda J, Pollock M, et al (2023) Bet-hedging Bigmouth Buffalo (*Ictiobus cyprinellus*) recruit episodically over a 127-year timeframe in Saskatchewan. Canadian Journal of Fisheries and Aquatic Sciences 80:313–329. https://doi.org/10.1139/cjfas-2022-0122

Loeza-Quintana T, Crookes S, Li PY, et al (2021) Environmental DNA detection of endangered and invasive species in Kejimkujik National Park and Historic Site. Genome 64:172–180. https://doi.org/10.1139/gen-2020-0042

Minogue TD, Rachwal PA, Hall AT, et al (2014) Cross-institute evaluations of inhibitor-resistant PCR reagents for direct testing of aerosol and blood samples containing biological warfare agent DNA. Applied and Environmental Microbiology 80:1322–1329. https://doi.org/10.1128/AEM.03478-13

Olden JD (2016) Challenges and opportunities for fish conservation in dam-impacted waters. In: Conservation of Freshwater Fishes. Cambridge University Press, Cambridge

Pandit SN, Maitland BM, Pandit LK, et al (2017) Climate change risks, extinction debt, and conservation implications for a threatened freshwater fish: Carmine Shiner (*Notropis percobromus*). Science of The Total Environment 598:1–11. https://doi.org/10.1016/j.scitotenv.2017.03.228

Piggott MP (2016) Evaluating the effects of laboratory protocols on eDNA detection probability for an endangered freshwater fish. Ecology and Evolution 6:2739–2750. https://doi.org/10.1002/ece3.2083

Scott W, Crossman E (1973) Freshwater Fishes of Canada. Fisheries Research Board of Canada, Ottawa

Song JW, Schultz MT, Casman EA, et al (2020) A probabilistic model for designing and assessing the performance of eDNA sampling protocols. Molecular Ecology Resources 20:404–414. https://doi.org/10.1111/1755-0998.13113

Sopinka NM, Fitzpatrick JL, Taves JE, et al (2012) Does proximity to aquatic pollution affect reproductive traits in a wild-caught intertidal fish? Journal of Fish Biology 80:2374–2383. https://doi.org/10.1111/j.1095-8649.2012.03281.x

Stewart KW, Watkinson DA (2004) The Freshwater Fishes of Manitoba. University of Manitoba Press, Winnipeg

Thermo Fisher Scientific (2022) Primer Express Software Version 3.0 Getting Started Guide

Vandervelde C (2022) Population Structure of Bigmouth Buffalo (*Ictiobus cyprinellus*) across Canada and the United States. MSc thesis, University of Manitoba, Winnipeg

Vives SP (1990) Nesting ecology and behavior of Hornyhead Chub *Nocomis biguttatus*, a keystone species in Allequash Creek, Wisconsin. The American Midland Naturalist 124:46–56. https://doi.org/10.2307/2426078

Vörösmarty CJ, McIntyre PB, Gessner MO, et al (2010) Global threats to human water security and river biodiversity. Nature 467:555–561. https://doi.org/10.1038/nature09440

Yukl SA, Kaiser P, Kim P, et al (2014) Advantages of using the QIAshredder instead of restriction digestion to prepare DNA for droplet digital PCR. BioTechniques 56:194–196. https://doi.org/10.2144/000114159

CHAPTER 4 COMPARISON OF TRADITIONAL VERSUS ENVIRONMENTAL DNA SAMPLING TO DETECT SPECIES OF INTEREST

4.1. Introduction

Aquatic ecosystems are some of the most important, yet most imperilled, ecosystems on Earth, and assessing the distribution and abundance of the species in these ecosystems is an essential step in the effective monitoring of the well-being of their populations (Gangloff et al. 2016). However, the detection of certain freshwater fishes can be challenging when targeting elusive, rare, and small-bodied fishes as they can evade methods traditionally used to survey fishes (e.g., electrofishing, seines, and gill nets), leaving the status of these species largely unchecked (Castañeda et al. 2021).

In the last decade, environmental DNA (eDNA) surveying has emerged as a promising, non-invasive solution to this problem in aquatic ecosystems (Nevers et al. 2018; Hempel et al. 2020). Instead of capturing the target organism, eDNA involves collecting and assessing genetic material (i.e., DNA) shed by a target organism into its environment. When detection rates are compared, eDNA frequently outperforms other methods of fish surveying (e.g., netting, electrofishing), particularly when surveying populations at low densities (Pilliod et al. 2013; Hinlo et al. 2017; Nevers et al. 2018; Piggott et al. 2021). However, the detectability of eDNA can be affected by environmental and biological factors, leading to false-negative results (i.e., the target species is present, but their DNA is not detected) (Hunter et al. 2019) and false-positive results (i.e., the DNA of a target species is detected, but the species is not present) (Kamoroff and Goldberg 2018), as the detection of a target species can be affected by the production of eDNA, the state of eDNA, the transport of eDNA and the degradation of eDNA (Jo et al. 2019).

The work outlined in this chapter aimed to validate the efficacy of the designed eDNA assays to detect five species of underappreciated fishes of conservation concern in Manitoba, Canada: Bigmouth Buffalo (*Ictiobus cyprinellus*), Chestnut Lamprey (*Ichthyomyzon castaneus*), Golden Redhorse (*Moxostoma erythrurum*), Hornyhead Chub (*Nocomis biguttatus*) and Northern Brook Lamprey (*Ichthyomyzon fossor*). Additional information about the designed assays can be found in Chapter 2.

4.2 Methods

4.2.1 Sampling Sites

All paired sampling performed during this project occurred alongside Fisheries and Oceans Canada (*see* Chapter 1.5 Ethical Statement and Permits). Paired sampling locations were determined using published collection records and distributions of each species (Dalton 1989; Goodchild 1990; Stewart and Watkinson 2004; COSEWIC 2009, 2010), previous eDNA sampling (Litke 2021) and collection records from M. Docker (University of Manitoba, unpublished) and Fisheries and Oceans (Doug Watkinson, Fisheries and Oceans Canada, unpublished). At each paired sampling site, eDNA sampling sites were defined as covering a minimum of 250 m, as eDNA can be detected at least 240 m from the target organism in freshwater (Jane et al. 2015). When areas surveyed using electrofishing were greater than 250 m, multiple samples were collected from multiple stations at the site with a distance of at least 250 m between each station. One paired sampling event occurred at a site where at least one species of interest could be captured to validate the designed species-specific TaqMan MGB probe-based assays (*see* Chapter 2). Sites were sampled in June 2022 (Delta Channel, Manitoba), and between July and September of 2023 (Figure 4.1; Table 4.1).

Table 4.1 The sites sampled using paired environmental DNA and capture-based sampling (electrofishing). Collected specimens were identified in the field, and either fin clippings and photographs, or whole specimens were collected to confirm identity in-lab.

Site Code	Waterbody	Latitude	Longitude	Sampling Date	Sampling Method	Target Species Captured
DM.03	Delta Channel	50.1104	-98.1859	21/06/2022	Boat electrofisher	20 Bigmouth Buffalo
DM.04	Delta Channel	50.1059	-98.1849	21/06/2022	Boat electrofisher	
WPG.01	Winnipeg River	50.5664	-96.1816	17/08/2023	Boat electrofisher	3 Golden Redhorse
RR.102	Rat River	49.3173	-96.9449	07/09/2023	Backpack electrofisher	3 Chestnut Lamprey; 1 Golden Redhorse
WM.05	Whitemouth River	49.8758	-95.9062	13/09/2023	Backpack electrofisher	6 Northern Brook Lamprey, 6 *Ichthyomyzon* sp. ammocoetes; 18 Hornyhead Chub

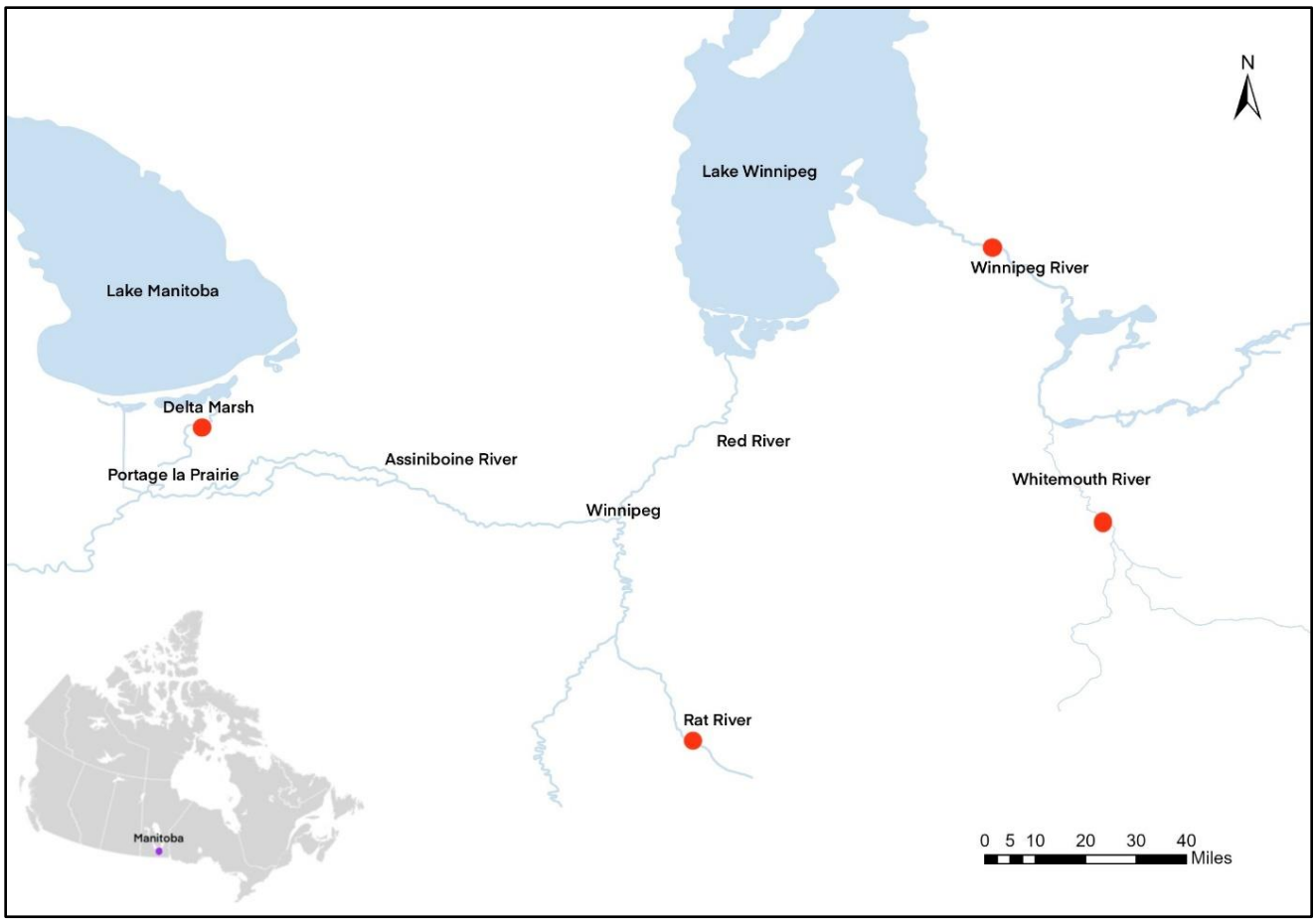


Figure 4.1 Locations where paired sampling using environmental DNA and capture-based sampling (boat/backpack electrofishing) occurred. Red markers indicate the four sites where paired sampling occurred for each of the target species: Bigmouth Buffalo (Delta Marsh), Rat River (Chestnut Lamprey), Winnipeg River (Golden Redhorse) and Whitemouth River (Northern Brook and Silver lampreys and Hornyhead Chub). All sampling was conducted in Manitoba, Canada. Figure generated by Sarah Glowa.

4.1.2 Sample Collection, Filtration, and Preservation

Two methods of sample collection and filtration were used to collect eDNA samples: hand collection of samples in sterilized Nalgene™ wide-mouth bottles, with 0.01% benzalkonium chloride (BAC; Thermo Scientific™ Benzalkonium chloride 50 w/w aqueous solution) for on-site preservation of water samples, followed by vacuum filtration in June 2022; and 2) an OSMOS automated eDNA sampler in 2023 (*see* Chapter 3).

eDNA samples in June 2022 were collected by hand and filtered using vacuum filtration because the OSMOS sampler was unavailable. The collection protocol followed that described in Chapter 3; however, recent studies suggest the addition of BAC to a final concentration of 0.01% to any water samples not filtered on-site to suppress eDNA degradation (Yamanaka et al. 2017; Sales et al. 2019; Takahara et al. 2020; Jo et al. 2021); thus, BAC was added to collected water samples and the negative control. The addition of BAC also complies with the permitting requirements (Permit No. 23-2022) for water collection where aquatic invasive species (AIS) are present, as on-site preservation of water samples should kill any AIS present in the samples, such as zebra mussel (*Dreissena polymorpha*) veligers.

Before the collection of water samples, a field negative control was collected by pouring 500 mL of water from UV-sterilized store-bought bottled water into one of the Nalgene™ wide-mouth bottles, then pouring the water back into the store-bought bottles, while wearing UV-sterilized nitrile gloves. Next, 0.5 mL of 10% BAC was added to the bottle (final concentration of 0.01% BAC) to check for potential contamination in the prepared BAC solution. The field negative control was filtered first using the vacuum filtration glass assembly, followed by the field sample replicates, to identify potential contamination in the filtration glass assembly. The

preservation and storage of the field negative control water sample and filter follow the same protocol outlined for the field sample replicates.

Three 1 L water samples were collected below the surface of the water at each sampling site from the side of a boat using sterilized 1 L Nalgene™ wide-mouth bottles. The samples were collected by hand while wearing UV-sterilized nitrile gloves after the collection of the field negative control. Once samples were collected, 1 mL of 10% BAC was added to each sample, for a final concentration of 0.01% BAC in each sample. Samples were placed in a cooler on ice and filtered within 8 hours of collection. Each water sample was filtered until the filter clogged, or 1 L of water was filtered, whichever occurred first. The total volume of water filtered was recorded. All equipment and surfaces were thoroughly cleaned between the filtration of each site (*see* Chapter 3).

All samples collected using the OSMOS sampler in 2023 followed the procedures outlined in Chapter 3. All cleaning procedures, eDNA extractions, and qPCR analysis for both methods of sampling followed protocols outlined in Chapter 3.

4.1.3 Electrofishing

All electrofishing occurred after the collection and preservation of eDNA samples. Two methods of electrofishing were used: backpack and boat. The voltage, hertz, duty cycle and effort varied by sampling event (*see* Appendix Table 4.1). Fish were captured using a boat electrofisher (Smith-Root electrofishing boat; model, SR-20EH; Smith-Root, Vancouver, WA, USA) in the Winnipeg River and Delta Channel. Captured fish were held in a holding tank filled with ambient water before identifying and collecting fin clippings for genetic testing or euthanizing and preserving whole specimens by submerging the specimen in a mixture of 99% ethanol and tricaine mesylate (MS-222). Fish were stunned using the backpack electrofisher (Smith-Rooth

backpack electrofisher; model LR-24; Smith-Root, Vancouver, WA, USA), and subsequently netted and placed in a bucket filled with ambient water. Once identified, fin clippings were collected from some fishes for genetic testing. In the case where fin clipping or field identification were not possible, whole specimens were euthanized and preserved by submerging the specimen in a mixture of 99% ethanol and MS-222. All non-euthanized fishes were released.

4.3 Results

All eDNA samples were tested for the presence of Bigmouth Buffalo, Chestnut Lamprey, Golden Redhorse, Hornyhead Chub, and Northern Brook and Silver lampreys using the designed TaqMan probe-based assays (Table 4.2). Negative controls collected during sampling confirmed sampling and filtration gear was void of target species eDNA before sampling, as no amplification of target species eDNA occurred in any field negative controls analyzed using qPCR.

4.3.1. Delta Marsh

In 2022, paired sampling of eDNA and electrofishing was conducted alongside the Fisheries and Oceans biologists for Bigmouth Buffalo in the Delta Channel, Manitoba, to validate the designed eDNA assay for Bigmouth Buffalo. The boat electrofishing resulted in the capture of 20 individual Bigmouth Buffalo, which were all released (Table 4.3). Fin clippings were taken from the right pectoral fin of the fish to identify which have been captured and for future genetic analyses. DNA was subsequently extracted from these tissue samples and used to validate the developed CO1 assay for Bigmouth Buffalo (*see* Chapter 2).

Of the target species, only Bigmouth Buffalo eDNA was detected in Delta Marsh using the designed assays (Table 4.3), albeit below the defined threshold (Figure 4.2), confirming the specificity of the eDNA assay but highlighting its lack of sensitivity. During electrofishing, Common Carp (*Cyprinus carpio*), Yellow Perch (*Perca flavescens*), and Freshwater Drum

(*Aplodinotus grunniens*) were present and visually identified, but not captured (*see* Appendix Table 4.1).

4.3.2 Rat River

Three Chestnut Lamprey and one Golden Redhorse were captured and identified in the field during backpack electrofishing in the Rat River and kept as voucher specimens to confirm identification and genetic analyses (Table 4.3). In line with these physical captures, Chestnut Lamprey and Golden Redhorse were detected from the collected water samples; 5/9 technical replicates amplified Chestnut Lamprey eDNA, and 9/9 technical replicates amplified Golden Redhorse eDNA using the respective designed CO1 assays.

Table 4.2 A summary of the TaqMan™ probe-based assays used in this project. Each assay consists of two primers and one probe which targets a sequence on the cytochrome *c* oxidase subunit 1 (CO1) or cytochrome *b* (CytB) gene for the species of interest in the project. The optimal annealing temperature (T_a), amplicon size (base pair, bp), and probe reporter dye for each assay are given. *See* Chapter 2 for assay validation information.

Species	Name	Marker	Sequence (5'-3')	T_a (°C)	Size (bp)	Dye
Bigmouth Buffalo	BMBF CO1	Forward	CCCCACTTGCGGGTAATCT	60	85	VIC
		Reverse	ATTGATGAAACTCCTGCTAGGTGAA			
		Probe	AGCTTCAGTAGACTTGACTA			
Chestnut Lamprey	CHLP CO1	Forward	GGATGAACTGTATACCCCCCTTT	58	85	FAM
		Reverse	CGAGGTGTAGGGAGAAGATTGTTAA			
		Probe	TCACACAGGAGCCTCTA			
Golden Redhorse	GDRH CO1	Forward	AGCATTCCCCCGGATGA	58	80	FAM
		Reverse	CAACTCCGGAAGAAGCCAATAA			
		Probe	CAATATAAGCTTCTGACTCCT			
Hornyhead Chub	HHCH CO1	Forward	ATCAGACACCCCTCTTTGTTTGA	57	80	FAM
		Reverse	TCCAGCAGCTAGGACAGGTAATG			
		Probe	TAACTGCTGTTCTTCTACTACTA			
Northern Brook/Silver Lamprey	NBLP CytB	Forward	TTGGAGGAGTTTTAGCTTTAGTCATG	57	90	FAM
		Reverse	GAAATTGTATGCCTCGCTGCTT			
		Probe	TATCCTAATCCTCCTAATCAT			

Table 4.3 A comparison of detection of target species using quantitative PCR and designed species-specific TaqMan MGB probe-based eDNA assays versus capture-based sampling using boat and backpack electrofishing and netting. Three biological replicates were collected per site and run in triplicate, resulting in nine technical replicates per site. The proportion of detection of eDNA in the nine technical replicates per site assayed for the given species is provided. A value of "0/9" indicates a lack of eDNA detection in any technical replicates. "Captured" indicates the total number of fish per species caught and subsequently vouchered, photographed and/or sampled (i.e., fin clipping taken from fish) at a given site during electrofishing.

Site Information		Bigmouth Buffalo		Golden Redhorse		Hornyhead Chub		Chestnut Lamprey		Northern Brook/Silver Lamprey	
Site Code	Waterbody	eDNA	Captured	eDNA	Captured	eDNA	Captured	eDNA	Captured	eDNA	Captured
DM.03*	Delta Channel	***	20	0/9	0	0/9	0	0/9	0	0/9	0
DM.04*	Delta Channel	***		0/9		0/9		0/9		0/9	
WPG.01	Winnipeg River	0/9	0	**5/9**	**3**	0/9	0	0/9	0	0/9	0
RR.102	Rat River	0/9	0	**9/9**	**1**	0/9	0	**5/9**	**3**	0/9	0
WM.05	Whitemouth River	0/9	0	0/9	0	**3/9**	**10**	0/9	0	**2/9**	**12****

*Combined capture records as fish travelled between stations during electrofishing.

** Six Northern Brook Lamprey and six *Ichthyomyzon* spp. ammocoetes.

***Below threshold amplification noted.

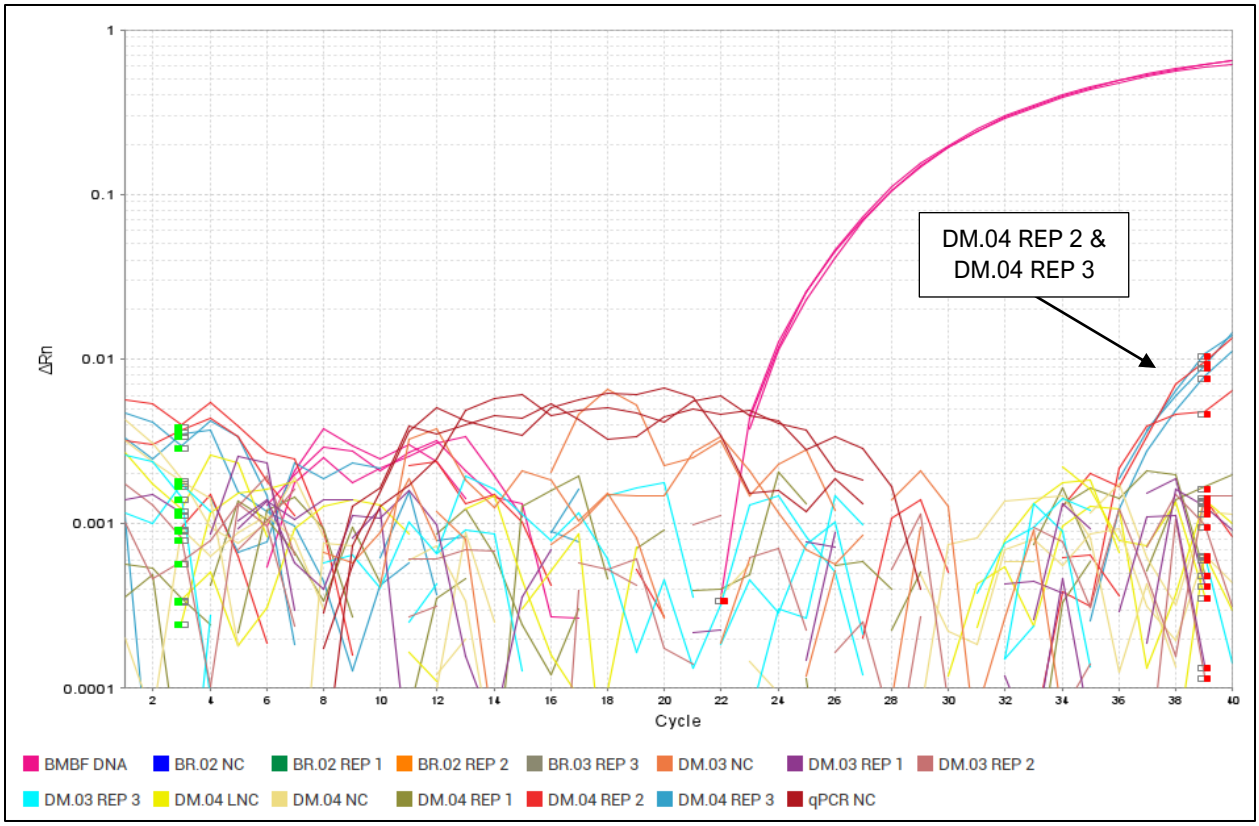


Figure 4.2 The amplification plot for paired eDNA sample analysis of samples collected and analyzed for Bigmouth Buffalo at Delta Marsh, Manitoba, using the designed CO1 assay. The amplification plot was generated using the QuantStudio™ Design & Analyze Software v1.5.2, using raw qPCR data of samples collected from sites DM.03 and DM.04. Three biological replicates (REP 1, REP 2, REP 3) were collected at each site and preserved on-site using a final concentration of 0.01% benzalkonium chloride. A qPCR negative control of molecular grade water was included (qPCR NC) to ensure the sterility of the reagents used. Three replicates of Bigmouth Buffalo DNA (BMBF DNA) were included to ensure the proper function of the assay. Note the late-stage amplification of DM.04 REP 2 and DM.04 REP 3, indicating eDNA is present, but at low concentrations.

4.3.3. Whitemouth River

Six Northern Brook Lamprey, 18 Hornyhead Chub, and six *Ichthyomyzon* sp. ammocoetes (presumably Northern Brook Lamprey but unidentifiable to species) were captured using backpack electrofishing and identified in the field within the Whitemouth River (Table 4.3). All captured lampreys were euthanized and kept as vouchers for further in-lab identification and future genetic analyses, as distinguishable morphological characteristics of larval lampreys (i.e., ammocoetes) can be ambiguous or impossible (Neave et al. 2007). Of the 18 Hornyhead Chub captured, six were euthanized and kept as vouchers for confirmation of identification and genetic analyses (*see* Appendix Table 4.1). Of the target species, only the eDNA of Hornyhead Chub (in 3/9 technical replicates) and Silver/Northern Brook lampreys (in 2/9 technical replicates) was detected using the designed species-specific assays (Table 4.3).

4.3.4. Winnipeg River, Pine Falls

Two Golden Redhorses were captured during boat electrofishing in the Winnipeg River outside of the 250 m radius of the location where the paired eDNA samples were collected (Figure 4.3). However, Golden Redhorse eDNA was amplified (5/9 technical replicates) in the paired eDNA samples (Table 4.3). Fin clippings from the right pectoral fin of both captured Golden Redhorse were taken to confirm their identity using genetic analyses. Photographs of the fish were taken instead of keeping them as vouchers.

4.4 Discussion and Conclusion

Using designed species-specific TaqMan MGB probe-based assays, we performed paired eDNA and capture-based sampling for Bigmouth Buffalo, Chestnut Lamprey, Golden Redhorse, Hornyhead Chub, and Northern Brook Lamprey in Manitoba, Canada, and confirmed that eDNA can be a non-invasive supplement to capture-based sampling for these species in Manitoba. Results often show eDNA as more sensitive at detecting aquatic target species, especially rare

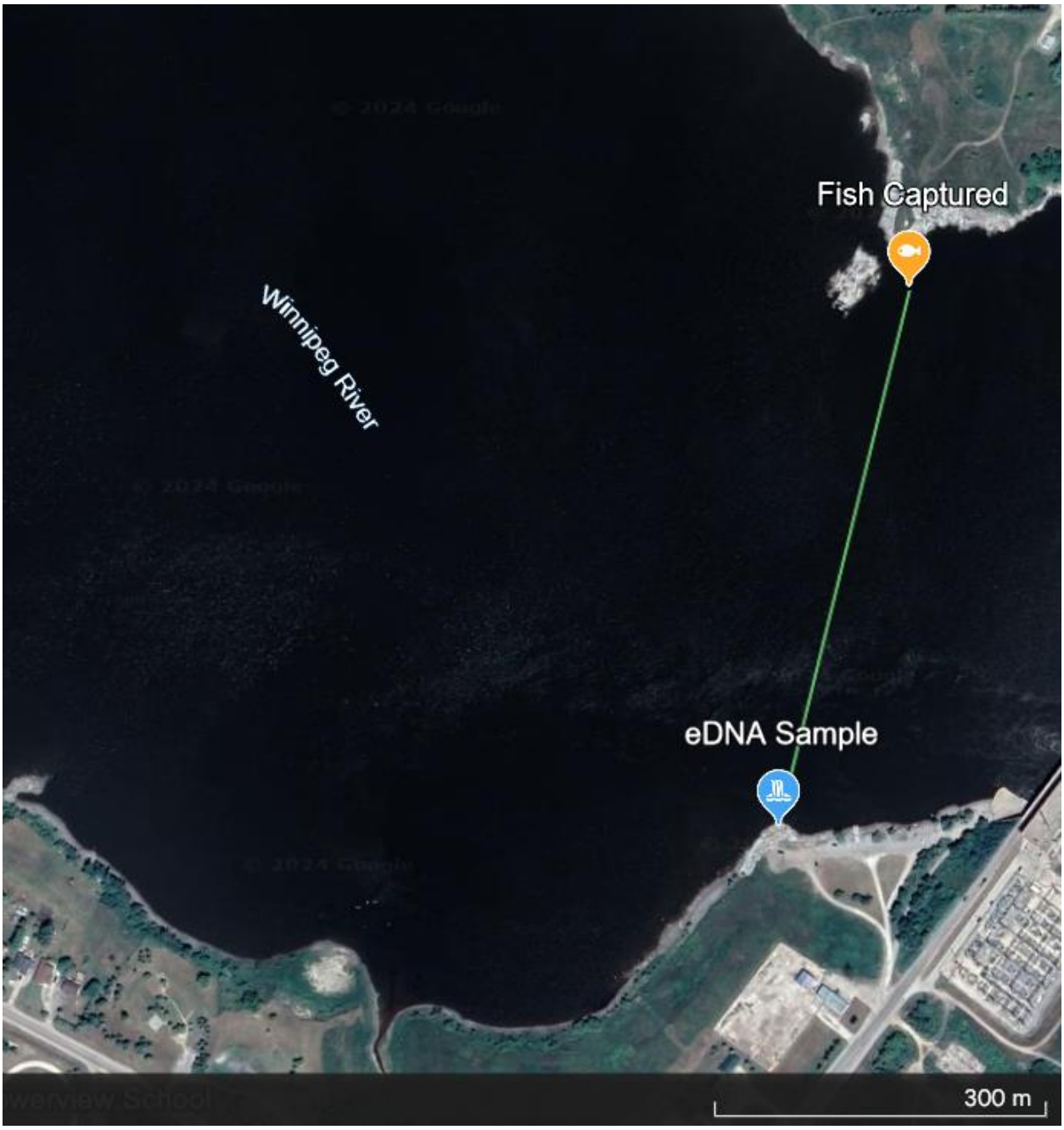


Figure 4.3 Distance between paired eDNA sampling location and capture of Golden Redhorse using boat electrofisher in Winnipeg River, Pine Falls, Manitoba. The distance between each site was estimated to be roughly 500 m by measuring the distance between the two locations using Google Earth™.

species, than capture-based methods (Smart et al. 2015; Balasingham et al. 2018; Lugg et al. 2018; Piggott et al. 2021). As paired sampling in this study focused on the validation of the designed assays and capture-based sampling was performed until the target species were caught, a comparison of both methods cannot be made using this data. Furthermore, as timing permitted for one session of capture-based sampling per target species, comparisons between catch-per-unit-effort were not possible.

As all capture-based sampling performed was done until target species were captured and in ways that increased the likelihood of capturing target species (e.g., shocking substrate with electrofishing probe to stun lamprey ammocoetes), this study displays a biased example of a comparison of capture-based and eDNA sampling. More accurately, this study highlights the importance of incorporating paired eDNA and capture-based sampling in the validation of designed eDNA assays to increase the confidence of eDNA detections.

Stations were defined as a 250 m radius surrounding the location where the eDNA sample was detected, as eDNA can be detected at least 240 m from its source in freshwater (Jane et al. 2015). Further investigation of the travel and persistence suggests that eDNA may be able to withstand transportation of greater distances, ranging from 1,700 m downstream without significant degradation to up to 1100 km in warmer months (Wacker et al. 2019; Xia et al. 2024). This allows for the detection of eDNA further away from the target species, which was suspected in this study, as Golden Redhorses were captured ~ 500 m away from the site of positive eDNA detection.

Above-threshold amplification of Bigmouth Buffalo eDNA did not occur during paired sampling, despite capturing 20 Bigmouth Buffalo during paired sampling. A few reasons have been hypothesized as causes for this lack of detection. First, the Bigmouth Buffalo eDNA

samples were collected by hand and preserved on-site using BAC. Potential inhibition of the samples caused by residual BAC may have resulted in the insufficient amplification of the Bigmouth Buffalo eDNA present in the samples, as BAC is known to inhibit enzymes, including proteinases (Jaganathan and Boopathy 2000; Zhang et al. 2011; Sabatini and Pashley 2015), which are an essential step in the extraction eDNA by causing the of lyses of cells and nuclei and freeing DNA (Goldenberger et al.). Thus, incomplete or inefficient extraction of Bigmouth Buffalo eDNA from the filters caused by residual BAC could explain the lack of Bigmouth Buffalo eDNA detection during paired sampling. Although BAC has been reported as successfully preserving eDNA (Yamanaka et al. 2017; Takahara et al. 2020; Jo et al. 2021), it is also known to have cytotoxic and genotoxic effects and can cause DNA strand breaks (Deutschle et al. 2006; Ye et al. 2011; Lavorgna et al. 2016), DNA degradation and ultimately, a lack of eDNA detection. Lastly, low detections of Bigmouth Buffalo were noticed during distribution sampling and are suspected to be due to an unintentional addition of adenosine to the 3' end of the probe of the Bigmouth Buffalo assay (*see* Chapter 3). This could have caused a lack of sensitivity of the Bigmouth Buffalo assay. Further research could investigate re-assaying the samples using the assay with the altered probe (i.e., without the additional adenosine) to narrow down if the sampling method, the probe, inhibition or a combination of all caused the lack of sufficient amplification of Bigmouth Buffalo eDNA in the samples collected.

Therefore, with this study, I emphasized the importance of performing paired eDNA and capture-based sampling to validate eDNA sampling methods and designed eDNA assays *in situ*. Capture-based sampling further provides opportunities to collect tissue that can be used to extract high-quality DNA for *in vitro* validation of eDNA assays and as positive controls during qPCR analyses.

Literature Cited

Balasingham KD, Walter RP, Mandrak NE, Heath DD (2018) Environmental DNA detection of rare and invasive fish species in two Great Lakes tributaries. Molecular Ecology 27:112–127. https://doi.org/10.1111/mec.14395

COSEWIC (2009) COSEWIC assessment and status report on the Bigmouth Buffalo *Ictiobus cyprinellus* (Great Lakes - Upper St. Lawrence populations and Saskatchewan - Nelson River populations) in Canada. Committee on the Status of Endangered Wildlife in Canada, Ottawa

COSEWIC (2010) COSEWIC assessment and status report Chestnut Lamprey *Ichthyomyzon castaneus* (Great Lakes - Upper St. Lawrence populations and Saskatchewan-Nelson River populations) in Canada. Committee on the Status of Endangered Wildlife in Canada, Ottawa

Dalton KW (1989) Status of the Hornyhead Chub, *Nocomis biguttatus*, in Canada. Canadian Field-Naturalist 103:180–185

Deutschle T, Porkert U, Reiter R, et al (2006) In vitro genotoxicity and cytotoxicity of benzalkonium chloride. Toxicology in Vitro 20:1472–1477. https://doi.org/10.1016/j.tiv.2006.07.006

Goldenberger D, Ritzler M, Altwegg M (1995) A simple “universal” DNA extraction procedure using SDS and proteinase K is compatible with direct PCR amplification. Genome Research 4:368–370

Goodchild CD (1990) Status of the Golden Redhorse, *Moxostoma erythrurum*, in Canada. Canadian Field-Naturalist 104:103–111

Hinlo R, Furlan E, Suitor L, Gleeson D (2017) Environmental DNA monitoring and management of invasive fish: comparison of eDNA and fyke netting. Management of Biological Invasions 8:89–100. https://doi.org/10.3391/mbi.2017.8.1.09

Hunter ME, Ferrante JA, Meigs-Friend G, Ulmer A (2019) Improving eDNA yield and inhibitor reduction through increased water volumes and multi-filter isolation techniques. Scientific Reports 9:5259. https://doi.org/10.1038/s41598-019-40977-w

Jaganathan L, Boopathy R (2000) Distinct effect of benzalkonium chloride on the esterase and aryl acylamidase activities of butyrylcholinesterase. Bioorganic Chemistry 28:242–251. https://doi.org/10.1006/bioo.2000.1169

Jane SF, Wilcox TM, Mckelvey KS, et al (2015) Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. Molecular Ecology Resources 15:216–227. https://doi.org/10.1111/1755-0998.12285

Jo T, Murakami H, Yamamoto S, et al (2019) Effect of water temperature and fish biomass on environmental DNA shedding, degradation, and size distribution. Ecology and Evolution 9:1135–1146. https://doi.org/10.1002/ece3.4802

Jo T, Sakata MK, Murakami H, et al (2021) Universal performance of benzalkonium chloride for the preservation of environmental DNA in seawater samples. Limnology and Oceanography: Methods 19:758–768. https://doi.org/10.1002/lom3.10459

Kamoroff C, Goldberg CS (2018) Environmental DNA quantification in a spatial and temporal context: a case study examining the removal of brook trout from a high alpine basin. Limnology 19:335–342. https://doi.org/10.1007/s10201-018-0551-5

Lavorgna M, Russo C, D'Abrosca B, et al (2016) Toxicity and genotoxicity of the quaternary ammonium compound benzalkonium chloride (BAC) using *Daphnia magna* and *Ceriodaphnia dubia* as model systems. Environmental Pollution 210:34–39. https://doi.org/10.1016/j.envpol.2015.11.042

Litke BM (2021) Investigating the use of environmental DNA to map the distribution of Chestnut Lamprey *Ichthyomyzon castaneus* in Manitoba. Honour's thesis, University of Manitoba, Winnipeg

Lugg WH, Griffiths J, Van Rooyen AR, et al (2018) Optimal survey designs for environmental DNA sampling. Methods in Ecology and Evolution 9:1049–1059. https://doi.org/10.1111/2041-210X.12951

Neave FB, Mandrak NE, Docker MF, Noakes DL (2007) An attempt to differentiate sympatric *Ichthyomyzon* ammocoetes using meristic, morphological, pigmentation, and gonad analyses. Canadian Journal of Zoology 85:549–560. https://doi.org/10.1139/Z07-032

Nevers MB, Byappanahalli MN, Morris CC, et al (2018) Environmental DNA (eDNA): a tool for quantifying the abundant but elusive Round Goby (*Neogobius melanostomus*). PLoS ONE 13:e0191720. https://doi.org/10.1371/journal.pone.0191720

Piggott MP, Banks SC, Broadhurst BT, et al (2021) Comparison of traditional and environmental DNA survey methods for detecting rare and abundant freshwater fish. Aquatic Conservation: Marine and Freshwater Ecosystems 31:173–184. https://doi.org/10.1002/aqc.3474

Pilliod DS, Goldberg CS, Arkle RS, Waits LP (2013) Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. Canadian Journal of Fisheries and Aquatic Sciences 70:1123–1130. https://doi.org/10.1139/cjfas-2013-0047

Sabatini C, Pashley DH (2015) Aging of adhesive interfaces treated with benzalkonium chloride and benzalkonium methacrylate. European Journal of Oral Sciences 123:102–107. https://doi.org/10.1111/eos.12168

Sales NG, Wangensteen OS, Carvalho DC, Mariani S (2019) Influence of preservation methods, sample medium and sampling time on eDNA recovery in a neotropical river. Environmental DNA 1:119–130. https://doi.org/10.1002/edn3.14

Smart AS, Tingley R, Weeks AR, et al (2015) Environmental DNA sampling is more sensitive than a traditional survey technique for detecting an aquatic invader. Ecological Applications 25:1944–1952. https://doi.org/10.1890/14-1751.1

Stewart KW, Watkinson DA (2004) The Freshwater Fishes of Manitoba. University of Manitoba Press, Winnipeg

Takahara T, Taguchi J, Yamagishi S, et al (2020) Suppression of environmental DNA degradation in water samples associated with different storage temperature and period using benzalkonium chloride. Limnology and Oceanography: Methods 18:437–445. https://doi.org/10.1002/lom3.10374

Wacker S, Fossøy F, Larsen BM, et al (2019) Downstream transport and seasonal variation in freshwater pearl mussel (*Margaritifera margaritifera*) eDNA concentration. Environmental DNA 1:64–73. https://doi.org/10.1002/edn3.10

Xia Z, Gu J, Wen Y, et al (2024) eDNA-based detection reveals invasion risks of a biofouling bivalve in the world's largest water diversion project. Ecological Applications 34:e2826. https://doi.org/10.1002/eap.2826

Yamanaka H, Minamoto T, Matsuura J, et al (2017) A simple method for preserving environmental DNA in water samples at ambient temperature by addition of cationic surfactant. Limnology 18:233–241. https://doi.org/10.1007/s10201-016-0508-5

Ye J, Wu H, Zhang H, et al (2011) Role of benzalkonium chloride in DNA strand breaks in human corneal epithelial cells. Graefe's Archive for Clinical and Experimental Ophthalmology 249:1681–1687. https://doi.org/10.1007/s00417-011-1755-0

Zhang C, Tezel U, Li K, et al (2011) Evaluation and modeling of benzalkonium chloride inhibition and biodegradation in activated sludge. Water Research 45:1238–1246. https://doi.org/10.1016/j.watres.2010.09.037

Appendices

Appendix Protocol 3.1. GEN-FISH eDNA Sampling Protocol for Backpack System use of OSMOS unit.

GEN-FISH eDNA Sampling Protocol
OSMOS unit – Backpack System use

Overview: The automated OSMOS eDNA sampler is a portable backpack that allows for high-throughput in-field water collection and filtration. The rate of filtration varies with turbidity and pre-filters can be incorporated as needed. Three 0.5–1 L water samples plus a field blank can be filtered in 20–40 minutes.

A. Materials

1. **OSMOS backpack,** which includes quick connect inlet port for the hose and hanging apparatus for the tripod bracket
2. **OSMOS batteries and charger**
3. **Sterilized aluminum filter housings**, one per site
4. **Tripod** with backpack hanging bracket attached
5. **Tripod swivel** with opening for securing telescopic pole
6. **Telescopic pole attached to the hose**
7. **6 ft outlet hose** for water runoff
8. **Deionized water or sealed bottled water (500 mL)**; minimum of three per site for field control
9. **Sterilized large plastic bag** for field control water
10. **Nitrile gloves,** 1–2 pairs per site.
11. **Sharpies and ethanol-proof markers.**
12. **Labeling tape**
13. **Sterile metal forceps.** These are easily sterilized in the field with ethanol
    i. **Sterile 15 mL tubes filled with 70–95% ethanol** for dipping forceps. Minimum one per sampling day and can be reused
    ii. **Lighter,** such as a barbecue lighter.
14. If using **liquid preservative (ethanol or RNAlater)**:
    i. **Sterile screw-top tubes (skirted or non-skirted, DNase and RNase free)**, prefilled and prelabeled $^2/_3$ with molecular-grade ethanol (preferably 99%, no less than 95%) or RNAlater. Four per site, plus unlabeled backups.
    ii. **Storage boxes** or **falcon tube rack** to hold tubes upright.
15. If using **liquid-free preservative (self-indicating silica beads):**
    i. **Coin envelopes** prelabeled. Four per site, plus extras.
    ii. **Plastic specimen bags,** sterile, prefilled with silica and used to store coin envelopes. Three bags per site (#1 for negative control, #2 for water samples and #3 for keeping together both bags #1 and #2 from a single site).
    iii. **Sampler spoons,** sterile, to fill specimen bags with silica. One per site. One scoop holds ~15 g of silica

iv. **Self-indicating silica,** prefilled or can fill specimen bags in the field. ~150 grams per site. ~30 grams for negative control specimen bag #1, ~90-100 grams for water replicate specimen bag #2. Extra to top up as needed as silica becomes saturated.

16. **ELIMINase or sodium hypochlorite solution;** concentrated bleach contains chlorine concentration of 5.25–8% or 52.5–80 g/L or 52,500–80,000 ppm; for example, one part 5% bleach to nine parts cold water will make a 10% bleach solution and the concentration of chlorine in the solution is 5,000 ppm, which is ideal for high-level disinfection. Bleach solutions at 10, 20, or 50 % can be made daily and stored in an opaque container away from light. ~1 L of bleach solution per site or 30 mL ELIMINase. ELIMINase is non-toxic and does not leave behind harmful residues but all ELIMINase waste and bleach must be rinsed and the wastewater stored for safe disposal. If you opt to use bleach you will need to bring enough deionized water to be able to submerge the equipment as well as a large container to store waste bleach for safe disposal after sampling
17. **Paper towels.**
18. **Filter membranes,** four per site, plus backups.

B. Cleaning Procedures (see *Equipment Sanitation Flowchart*)

Given the sensitivity of the eDNA techniques, avoiding contamination of field samples is critical:

- All sampling equipment must be kept separate from other field gear (e.g., nets, clothing, truck bed).
- Samples can be easily contaminated. Anything that has come into contact with fish or bodies of water containing fish (e.g., clothing, gear, vehicle) can be a potential source of contamination.
- Always wear fresh gloves while handling sterile items and samples.
- When in doubt, change your gloves.
- Keep contaminated, used, or dirty field materials bagged and separate from clean equipment to minimize chances of contamination.
- If fish collection, sampling, or electrofishing is also to be conducted, **collect biological water samples for eDNA before other sampling or measuring water chemistry.**
- Ideally, water samples should be collected downstream from the edge of the water body, without entering the water. This will prevent the sediments from being disturbed (which can increase the amount of clay and sand collected in the water) and will also reduce the possibility of contamination from boots that can carry over DNA from site to site. If you need to enter the water, **always stay downstream of the water collection locale, and do not touch the inside of the water collection bottles.**

C. Field sampling preparation

1. Prepare field sampling bags for each site in a freshly sanitized area, free of contaminant DNA. This is preferably a closed indoor space away from sources of contaminants such as: fish DNA, areas where dissections occur, or where PCRs are conducted. Use gloves and follow the flowchart instructions provided in the ***Equipment Sanitation Flowchart.***

2. If you have access to a PCR workstation with UV light irradiation equipment, field sampling bags can be UV-treated and then used.
3. Wearing gloves, wash all aluminum filter housings (including all interior/exterior) with warm soap and water. Wipe down filter housings with 95% ethanol and ELIMINase, and then rinse off. UV-treat filter housings for a minimum of 15 minutes.
4. In the decontaminated UV hood (using sterile gloves and forceps), place the filter membrane in between the filter screen and the rubber gasket.
5. Before reassembly of the filter housing, lightly lubricate the O-ring to prevent friction. Using the brush provided, dab a small amount of silicone along the O-ring evenly in 3 or 4 places. Then with a gloved hand gently rub the silicone along the O-ring. NOTE: be careful not to get the silicone onto the inside of the housing, as this will introduce contaminants.
6. Reassemble the filter housing and place it in a sterile plastic sandwich bag (one filter housing per bag).
7. All items can be packed into a larger container or tote bag, with extra bags to keep waste (e.g., gloves) that can be disposed of when returned to the lab or field station.
8. Install a charged lithium battery into the OSMOS unit.

D. Labels

There should be a minimum of two sets of labels for samples per site (i.e., both on the bottles as well as the outside of the bag) to ensure samples are identifiable in case labelling peels or rubs off. Bottles can be labelled ahead of time to save time in the field if desired (we recommend this). Additional information, such as the name or initials of the collector, can be included.

Minimum requirements for sample labels for each 2 mL tube, 15 mL tube, or sample container:

- the site name or abbreviation for the site
- sub-sample information to distinguish between a blank/negative control and a biological water sample
- the replicate number for biological water samples
- date sampled

E. Prepare filter storage containers

Preparing filter storage ahead of time will save time in the field and prevent contamination.

- **Ethanol or RNAlater**
    1. Label the appropriate number of tubes following the conventions above.
    2. Prefill tubes $^{2}/_{3}$ – $^{3}/_{4}$ full with DNA stabilizer.
    3. Store upright in boxes or racks, also labelled.
- **Self-indicating silica**
    1. Label the appropriate number of coin envelopes following the conventions above.
    2. Place three water sample coin envelopes into a plastic specimen bag, and the envelope for control into a secondary plastic bag
    3. Prefill specimen bag for three water samples with ~90–100 g (seven spoon-scoops) of silica

4. Prefill specimen bag for one control sample with ~30 g (two spoon-scoops) of silica
5. Store specimen bags in a secondary bag for each site.

F. Sample collection process

1. Upon arrival at the sampling site, locate your desired field sampling location: even/level surface, ground conducive for securing tripod, etc.
2. Prepare the tripod by first unclipping the strap that holds the legs together. Extend the tripod legs by turning the thumbscrews in a counter-clockwise direction and simply extend the leg assembly. Retighten the thumbscrew by turning in a clockwise direction.
3. You will notice a bracket along the top of the tripod. This is where the OSMOS unit will be hung during operations. Place the tripod in such a way that the hanging bracket is facing AWAY from your water source. Secure the tripod by stepping onto the pedals of the legs to securely plant them into the soil.
4. Attach the tripod swivel to the treaded bolt at the top of the tripod. NOTE: the swivel does not make use of the threads.
5. Thread the hose end of the pole through the opening of the tripod and pivot the pole clamp that will secure the pole. Be sure to insert the hose from the water side of the pivot assembly. Carefully continue to thread the hose through the opening.
6. Insert the end of the pole into the opening and secure it by turning the lever clockwise.
7. Connect the end of the hose to the quick connect inlet port on the OSMOS backpack.
8. Open the OSMOS unit and turn the operating switch to the ON position. You will hear a series of beeps as the unit powers up.
9. Close the lid on the OSMOS and SECURE THE LATCHES.
10. Hang the OSMOS from the red rubberized handle at the top of the backpack onto the hanging bracket of the tripod.
11. Connect the outlet hose to the OSMOS runoff located at the bottom of the unit. You will see a Quick Connect facing in one direction and a brass hose barb facing in the other direction. The outlet hose is simply pushing onto the brass hose barb. Make sure the end of the hose will discharge in a safe location (i.e., downstream of your sample site).
12. Before handling the filter housing, put on new nitrile gloves. If sampling occurs during colder temperatures, place extra-large nitrile gloves over insulated finger gloves to prevent freezing of hands.
13. Carefully remove the sterilized filter housing assembly from the individual plastic bag (this should have been assembled ahead of time and should already contain the filter membrane).
14. Attach the filter housing assembly to the end of the telescopic pole. Using both hands, hold the filter housing and pull back on the quick-connect collar located on the pole. Insert the housing into the collar and release the collar. MAKE SURE THAT THE FILTER HOUSING ASSEMBLY IS SECURELY ATTACHED. You will hear a distinct click and the filter housing assembly will sit perfectly perpendicular to the quick-connect collar surface. If improperly attached, the filter housing will appear tilted. It may take more than one attempt to successfully attach the filter housing assembly.

15. Loosen the extension locks on the pole and telescope the pole to the desired length to reach the water source.
16. Using the swivel lock handle, hold the pole with one hand while turning the swivel lock counter-clockwise to loosen it. With the swivel lock loosened, you will now be able to gently lower the pole angle and filter housing into the water. Before the pole reaches the bottom of the waterbody (natural or bottle), turn the swivel lock clockwise to lock the vertical position of the pole into place.
17. Filter your negative control by pouring 1.5 L of water from the sealed bottles into one of the large sterilized plastic bags. Place the filter housing unit into the plastic bag and make sure to keep the water inlet surface submerged throughout the running of the negative control.
18. The first screen on the OSMOS will display "SETTINGS….CHANGE CONTINUE". Press the "ENT" button to go through the settings.
19. The next screen will allow you to set the volume of water to filter. The default is 2.0 L. You can change this value by pressing the UP or DOWN buttons.
20. The next screen allows you to set the number of pre-filters that you are using. Use the UP and DOWN arrows to set this number and press the "ENT" button to continue. In most cases with clear water, you will not be using pre-filters, so you can leave this value as ZERO.
21. The remaining five screens have correct default settings for standard use; simply select "ENT" for the remaining prompts.
22. The final screen will allow you to start your run by pressing "ENT".
23. The pump will run automatically until the pre-set filtration amount has been processed. At this time a very loud and persistent beep will be heard. Remove the filter housing assembly from the sample and invert the filter housing. Press the "ENT" button for the OSMOS system to begin the purge process to clear the lines of remaining water.
24. Once the purging step is completed the OSMOS pump will shut off automatically. The display screen will show relevant sampling values for your records.
25. Carefully disconnect the filter housing from the pole by pulling back the quick-connect collar. Hold the filter housing assembly with your other hand while disconnecting the quick release.
26. Separate the final stage of the filter housing assembly from the pre-filter stage/inlet stage.
27. **Sterilize Forceps**: Dip your metal forceps into your 15 mL tube of ethanol, holding the pincers of the forceps facing down so ethanol drips off the tips of the forceps and does not run down to the portion you're holding. Then flame them with your lighter to ensure sterility. Using these flamed sterile forceps, carefully remove the rubber gasket and remove the filter membrane from the filter screen.
28. Place the filter membrane into the appropriate preservation medium (vial with ethanol/RNAlater or envelope with silica beads).
29. Flame/re-sterilize your forceps and place a new filter membrane into the filter housing. Put the rubber gasket over the filter membrane and reassemble the filter housing for use.

30. Repeat steps #13–28 for each biological water sample replicate. Exclude step #17 (instructions for the negative control) and instead submerge the filter housing assembly in the body of water to collect field samples. Gather your desired number of replicate samples (ideally three) and make sure to submerge the filter housing assembly in the same spot for all samples.
31. When finished sampling, disconnect your setup in reverse order from the assembly steps (steps #2–15).
32. Ensure each sample is labeled correctly (see **D. Labels**, above). Remove gloves and place them in the waste bag.
33. Upon arrival at the lab, place samples in -20°C to -30°C freezer for storage.
34. Discard waste from field collection in the appropriate disposal.

Notes and Precautions

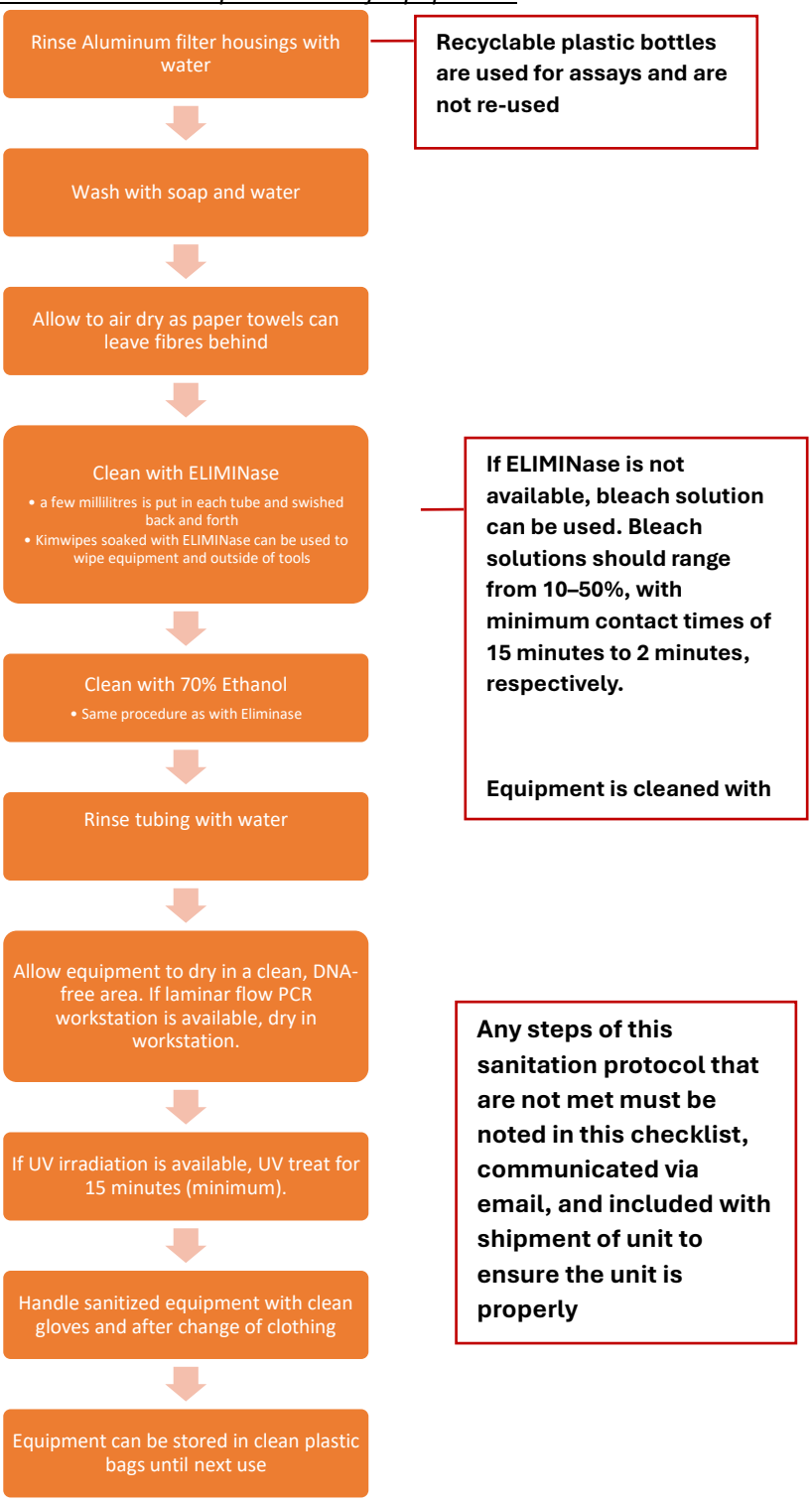
- If you're going to be doing any other sampling in the water, please make sure that you take the biological water samples for eDNA first, followed by any other sampling in the water body. If you're going to be taking any water chemistry parameters, this should take place last so you're not carrying over contaminants from one site to another; cleaning sensitive water parameter equipment may not be possible in the field.
- Dress appropriately for the weather. Insulated, waterproof gloves are a must during colder months. Bare hands can easily get frozen/frostbite when dealing with lower water temperatures and handling metal.
- Be cautious when transporting equipment down to water level, as muddy and rocky surfaces can be very slippery. Always have someone assisting you in the field.
- It is helpful to have two separate bin containers, one labelled "clean" and one "dirty", to make sure sterile filter housing assemblies are not confused with used, contaminated ones.
- Since the tripod swivel cannot be secured directly to the threads, it is recommended to have someone hold the tripod and filter housing assembly in position while running the sample, to maintain constant depth.
- Alternative sanitizing methods for forceps:
    - Dipping in a 50% bleach solution for 1–2 minutes, rinsing with deionized water, and wiped dry
    - Sprayed with ELIMINase and wiped dry.

Storage of Filters (Lab)

- Liquid preservative (ethanol or RNAlater): Filters that are preserved in ethanol can be stored at room temperature, but we suggest storing filters at -20 to -30°C.
- Liquid-free preservative (self-indicating silica beads): Filters that are preserved in silica can be stored at room temperature in a cool, dry location and away from light. The suggested location would be a cabinet or cupboard in a dry room.

Equipment Sanitation Flowchart

The procedure developed by the Docker Lab (University of Manitoba) is to be completed before and after sampling and before returning the OSMOS unit. All cleaning steps must be performed while wearing gloves and in a space free of active fish dissections, PCR, or DNA amplification. *Check when each step is complete. Be prepared to submit this checklist upon return of equipment.*



Appendix Table 4.1. Complete collection record for paired sampling events using boat and backpack electrofishing by location sampled.

a) Rat River

Site	Date	Common Name	Scientific Name	Count	Volt (V)	Hertz (Hz)	Duty Cycle (%)	Effort (s)
Rat River	07-Sep-23	Unidentified	N/A	95	200,300,400	30,35	15	965
Rat River	07-Sep-23	Burbot	*Lota lota*	24	200,300,400	30,35	15	965
Rat River	07-Sep-23	Common Shiner	*Luxilus cornutus*	28	200,300,400	30,35	15	965
Rat River	07-Sep-23	Sauger	*Sander canadensis*	2	200,300,400	30,35	15	965
Rat River	07-Sep-23	Golden Redhorse	*Moxostoma erythrurum*	2	200,300,400	30,35	15	965
Rat River	07-Sep-23	River Darter	*Percina shumardi*	5	200,300,400	30,35	15	965
Rat River	07-Sep-23	Rock Bass	*Ambloplites rupestris*	10	200,300,400	30,35	15	965
Rat River	07-Sep-23	Johnny Darter	*Etheostoma nigrum*	5	200,300,400	30,35	15	965
Rat River	07-Sep-23	Blackside Darter	*Percina maculata*	4	200,300,400	30,35	15	965
Rat River	07-Sep-23	Chestnut Lamprey	*Ichthyomyzon castaneus*	3	150	60	25	2328

b) Whitemouth River

Site	Date	Common Name	Scientific Name	Count	Volt (V)	Hertz (Hz)	Duty Cycle (%)	Effort (s)
Whitemouth River	13-Sep-23	Rock Bass	*Ambloplites rupestris*	20	300	30	15	2202*
Whitemouth River	13-Sep-23	Blackside Darter	*Percina maculata*	21	300	30	15	2202*
Whitemouth River	13-Sep-23	Central Mudminnow	*Umbra limi*	2	300	30	15	2202*
Whitemouth River	13-Sep-23	Carmine Shiner	*Notropis percobromus*	1	300	30	15	2202*
Whitemouth River	13-Sep-23	Hornyhead Chub	*Nocomis biguttatus*	18	300	30	15	2202*
Whitemouth River	13-Sep-23	Johnny Darter	*Etheostoma nigrum*	5	300	30	15	2202*
Whitemouth River	13-Sep-23	Longnose Dace	*Rhinichthys cataractae*	3	300	30	15	2202*
Whitemouth River	13-Sep-23	Northern Pike	*Esox lucius*	1	300	30	15	2202*
Whitemouth River	13-Sep-23	River Darter	*Percina shumardi*	2	300	30	15	2202*
Whitemouth River	13-Sep-23	Northern Brook Lamprey	*Ichthyomyzon fossor*	6	400	60	25	2202*

*Combined sampling time, over ~200 m area.

c) Pine Falls

Site	Date	Common Name	Scientific Name	Count	Volt (V)	Hertz (Hz)	Duty Cycle (%)	Effort (s)
Pine Falls	17-Aug-23	Spottail Shiner	*Hudsonius hudsonius*	6	300	30	15	**
Pine Falls	17-Aug-23	Emerald Shiner	*Notropis atherinoides*	3	300	30	15	**
Pine Falls	17-Aug-23	Walleye	*Sander vitreus*	2	300	30	15	**
Pine Falls	17-Aug-23	Logperch	*Percina caprodes*	1	300	30	15	**
Pine Falls	17-Aug-23	Yellow Perch	*Perca flavescens*	9	300	30	15	**
Pine Falls	17-Aug-23	Sander sp.	*Sander* sp.	1	300	30	15	**
Pine Falls	17-Aug-23	Golden Redhorse	*Moxostoma erythrurum*	2	300	30	15	**
Pine Falls	17-Aug-23	Shorthead Redhorse	*Moxostoma macrolepidotum*	3	300	30	15	**
Pine Falls	17-Aug-23	Freshwater Drum	*Aplodinotus grunniens*	1	300	30	15	**
Pine Falls	17-Aug-23	White Sucker	*Catostomus commersonii*	1	300	30	15	**

** Boat electrofishing; effort not recorded.

d) Delta Marsh

Site	Date	Common Name	Scientific Name	Count	Volt (V)	Hertz (Hz)	Duty Cycle (%)	Effort (s)
Delta Marsh	21-Jun-22	Bigmouth Buffalo	*Ictiobus cyprinellus*	20	300	30	15	**

** Boat electrofishing; effort not recorded.