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REGULAR ARTICLE

APPLYING ENVIRONMENTAL DNA METHODS TO INFORM DETECTION OF *SIMPSONAIAS AMBIGUA* UNDER VARYING WATER VELOCITIES IN A RIVER

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ABSTRACT

Conventional survey methods to find rare and endangered aquatic species can be time consuming, expensive, destructive to habitat, and limited by the physical conditions of a site. Sampling for environmental DNA (eDNA) shed by organisms into their environments can overcome these limitations, maximizing conservation resources. However, the optimal spatial sampling interval for eDNA detection is poorly known. We developed and assessed eDNA methods for application to *Simpsonaias ambigua* (Salamander Mussel), a unionid mussel that is considered at risk throughout most of its range. We developed a quantitative PCR assay and optimized methods to detect *S. ambigua* eDNA in water samples, and we experimentally determined eDNA shedding and decay rates. We used these rates to populate a previously published eDNA transport model to estimate the maximum downstream distance from the source (i.e., the location of live mussels) at which eDNA could be detected as a function of environmentally relevant source eDNA concentrations and water velocities. The model predicted that maximum detection distance varied greatly depending on source eDNA concentration and water velocity. At low eDNA concentration and water velocity (1.0 copy/mL and <0.1 m/s, respectively), eDNA will be detected only at the source, requiring spatially intensive eDNA sampling. At higher eDNA concentration and water velocity (5.0 copies/mL and 0.8 m/s, respectively), eDNA can be detected at least 10 km downstream, requiring less intensive sampling. Based on our results, we provide recommendations for the development of optimal eDNA sampling design for detecting rare or endangered species.

KEY WORDS: environmental DNA, rare or endangered mussel species, survey techniques

INTRODUCTION

Conventional survey methods (e.g., hand sampling, sediment excavation, trawling, seining) to find elusive, rare, or threatened aquatic species are limited both by the difficulty in identifying species and by the physical conditions of a site; furthermore, they can be time consuming and can damage or destroy habitats (Jerde et al. 2011; Clark et al. 2015; Andruszkiewicz et al. 2017; Closek et al. 2019). Environmental DNA (eDNA) approaches recover DNA from an environmental sample without disturbing the species of interest or their habitats. Despite the limitations of eDNA sampling (e.g., filter clogging, PCR inhibitors, transportation and preservation of water samples), eDNA methods can be more cost effective and can overcome the limitations of conventional survey methods (Rees et al. 2014; Thomsen and Willerslev 2015; Ruppert et al. 2019).

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eDNA methods have been used to detect and develop multiscale occupancy models for rare and endangered aquatic species (Dorazio and Erickson 2018; Strickland and Roberts 2019; Coghlan et al. 2021). Results from eDNA surveys support those of conventional surveys (Wilson et al. 2014; Hinlo et al. 2017; Cilleros et al. 2019), and in some cases, eDNA methods are more sensitive and effective, especially for rare species (Jerde et al. 2011; McKelvey et al. 2016; Currier et al. 2017). Although eDNA methods provide many advantages, the effective management of rare and threatened species still requires biological data (e.g., population health, sex ratios, size frequency estimates) that can be obtained only through conventional sampling approaches. Thus, a strategy that involves a combination of conventional and eDNA approaches will best achieve most conservation objectives.

Environmental DNA originates from waste products, gametes, shed body parts, or other sources, and its persistence in the environment is controlled by factors such as the rate of shedding from the organism, resuspension, decay, advection, and transport (Barnes et al. 2014; Strickler et al. 2015; Barnes and Turner 2016). Quantification of eDNA shedding and decay rates has proven to be informative when modeling eDNA presence and transport in the environment, and understanding these processes is critical for developing optimal sampling designs (Sassoubre et al. 2016; Sansom and Sassoubre 2017; Andruszkiewicz et al. 2020).

We developed and assessed eDNA methods for detecting *Simpsonaias ambigua*, the Salamander Mussel (family Unionidae). Sampling for freshwater mussels is time consuming and expensive because their benthic occurrence and burrowing habits make their detection difficult. *Simpsonaias ambigua* is small (maximum 50 mm shell length), and it occurs almost exclusively beneath large, flat stones or rock ledges, often in deep water or in turbid conditions (Howard 1915), characteristics that make detecting *S. ambigua* particularly difficult. *Simpsonaias ambigua* is listed as globally vulnerable by the International Union for Conservation of Nature Red List (Bogan et al. 2017) and endangered under Canada's Species at Risk Act (Morris and Burridge 2006), and it is a candidate for listing under the U.S. Endangered Species Act (USFWS 2011). The imperiled status of this species, along with the difficulty of its detection, provides impetus for development of sensitive, cost-effective survey methods.

Our study goals were to (1) develop a quantitative (q)PCR assay and optimize methods for detection of *S. ambigua* eDNA, (2) experimentally determine eDNA shedding and decay rates, and (3) use these rates to populate a previously published eDNA transport model to estimate the maximum downstream distance from the source (i.e., the location of live mussels) at which eDNA could be detected as a function of environmentally relevant source eDNA concentrations and water velocities in a third-order stream. Based on our results, we provide recommendations for the development of optimal eDNA sampling designs for detecting rare or endangered species.

METHODS

Simpsonaias ambigua Primer and Probe Development and Optimization in the Laboratory

We developed a qPCR assay for *S. ambigua* following guidelines in Bustin et al. (2009) and Wilcox et al. (2013), with modifications outlined below. Because there were limited sequences available in public databases, we developed primer probes by amplifying and sequencing two mitochondrial genes, cytochrome oxidase subunit I (COI, 622 bp) and NADH dehydrogenase (ND1, 599 bp), from mantle swabs of five *S. ambigua* collected from the Sydenham River (known as Jongquakamik in Nishnaabemwin [Ojibwe], Lake St. Clair drainage, Ontario, Canada). We extracted genomic DNA from mantle swabs by using the DNeasy Blood and Tissue extraction kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. We amplified COI by using Folmer et al. (1994) primers and ND1 by using Buhay et al. (2002) primers. We amplified each mitochondrial gene via PCR in a 25-μL reaction, with the following concentrations: 2.0 ng/μL of extracted genomic DNA, 0.3 mM dNTPs, 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 μM of each primer, and 1 U of Taq polymerase. We carried out a touchdown PCR for both genes, with the following amplification conditions: initial heating to 94°C for 2 min; 5 cycles of 94°C for 40 s, annealing at 50°C for 40 s, and a 90-s extension time at 72°C; 25 cycles of 94°C for 40 s; annealing at 40°C for 40 s and a 90-s extension time at 72°C; and a final extension of 10 min at 72°C. We screened all PCR products on 2% agarose gel to confirm amplification and targeted sequence size. We sent successfully amplified samples to the Aquatic Research and Monitoring Section, Ontario Ministry of Natural Resources and Forestry, Trent University, for Sanger sequencing. We edited and aligned chromatograph files of COI and ND1 sequences by using Geneious 10 (Kearse et al. 2012). Sequences were translated using the mitochondrial invertebrate genetic code to ensure the absence of stop codons. Although available *S. ambigua* sequences were limited, we designed primers by using sequences and specimens from different watersheds to ensure that this assay could be used to detect *S. ambigua* across its distributional range. We used COI sequences from the Monongahela River, Ohio River basin (voucher NCSM30607, GenBank accession number KX822666), and from five individuals from the Sydenham River (GenBank accession number MN920704). ND1 sequences originated from five individuals from the Sydenham River (GenBank accession number MN920703). All five sequenced individuals from the Sydenham River shared the same COI and ND1 haplotypes.

We designed all primers and probes by using Primer3 v.0.4.0 (Koressaar and Remm 2007; Untergasser et al. 2012). We carried out in silico testing of all primer–probe sets for specificity against 35 mussel species present in Ontario (Table 1). Table A1 provides a list and the properties of two

Table 2. Mussel species tested for cross-amplification of the cytochrome oxidase subunit I gene (COI) (SamCOI_1) and the NADH dehydrogenase gene (ND1) (SamND_1) by using primers developed for *Simpsonaias ambigua*. Cycle quantification value (C_q) is presented for each species that yielded amplification after 40 cycles; a dash (—) indicates no amplification. See Table A1 for additional information about the primers. All tissue samples were collected from Ontario by the Great Lakes Laboratory for Fisheries and Aquatic Sciences, Fisheries and Oceans Canada.

		C_q	
Species	Common name	SamCOI_1	SamND1
Alasmidonta marginata	Elktoe	—	—
Alasmidonta undulata	Triangle Floater	37.35	—
Alasmidonta viridis	Slippershell Mussel	—	—
Amblema plicata	Threeridge	39.15	—
Anodontoides ferussacianus	Cylindrical Papershell	—	—
Cambarunio iris	Rainbow	—	—
Cyclonaias pustulosa	Pimpleback	—	—
Cyclonaias tuberculata	Purple Wartyback	—	—
Elliptio complanata	Eastern Elliptio	—	—
Epioblasma rangiana	Northern Riffleshell	—	—
Epioblasma triquetra	Snuffbox	—	—
Eurynia dilatata	Spike	—	—
Fusconaia flava	Wabash Pigtoe	—	—
Lampsilis cardium	Plain Pocketbook	38.34	—
Lampsilis fasciola	Wavyrayed Lampmussel	—	—
Lasmigona complanata	White Heelsplitter	—	—
Lasmigona compressa	Creek Heelsplitter	—	—
Lasmigona costata	Fluttedshell	—	—
Ligumia recta	Black Sandshell	—	—
Obliquaria reflexa	Threehorn Wartyback	38.24	39.79
Obovaria subrotunda	Round Hickorynut	—	—
Actinonaias ligamentina	Mucket	—	—
Pleurobema sintoxia	Round Pigtoe	38.37	—
Potamilus alatus	Pink Heelsplitter	—	—
Potamilus fragilis	Fragile Papershell	38.81	—
Ptychobranchus fasciolaris	Kidneshell	—	—
Pyganodon grandis	Giant Floater	—	—
Quadrula quadrula	Mapleleaf	—	—
Sagittunio nasutus	Eastern Pondmussel	37.00	39.66
Simpsonaias ambigua	Salamander Mussel	20.10	25.42
Strophitus undulatus	Creeper	—	—
Toxolasma parvus	Liliput	—	—
Truncilla donaciformis	Fawnsfoot	—	—
Truncilla truncata	Deertoe	38.48	—
Utterbackia imbecillis	Paper Pondshell	—	
Paetulunio fabalis	Rayed Bean	—	—

COI and two ND1 primer–probe sets that we designed and tested.

To determine the most sensitive primer–probe combination, we optimized the assays by testing final primer concentrations of 0.3, 0.6, and 0.9 μM per reaction and final probe concentrations of 0.15 and 0.25 μM. Throughout this study, we set up all qPCRs in an isolated UV workstation with a set of dedicated pipettes. Before setting up reactions, we decontaminated the workstation with hydrogen peroxide and 15 min of ultraviolet (UV) light exposure. The qPCRs for

both genes were carried out using 2 μL of extracted genomic DNA in 20-μL reactions containing the following final concentrations: 1× TaqMan™ Environmental Master Mix 2.0 (Applied Biosystems™, Waltham, MA, USA), 0.3–0.9 μM of each primer, and 0.15–0.25 μM of probe with a ZEN/Iowa Black FQ quencher (IDT, Coralville, IA, USA). Two no template controls (NTCs) were run for each qPCR plate by using 2 μL of molecular grade water (Sigma-Aldrich, St. Louis, MO, USA) instead of genomic DNA. The amplification conditions were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 10 s and annealing at 60°C for 1 min.

Subsequently, we performed in vitro testing of the COI and ND1 primer–probe sets that had the greatest DNA sequence mismatches with nontarget species (SamND_1 and SamCOI_1, see Table A1) against the same 35 mussel species found in Ontario (Table 1). We used approximately 2 ng/μL of genomic DNA of each species to carry out the qPCR reactions.

We determined the limit of detection (LOD, the minimum number of copies in a sample that can be detected accurately) following Hunter et al. (2017) to provide a conservative estimation of LOD. The limit of quantification (LOQ) determines the ability of an assay to precisely quantify the number of DNA copies. In this study, the LOQ was defined as the lowest standard concentration with a coefficient of variation below 35% (Klymus et al. 2020). To calculate LOD and LOQ, and to determine eDNA concentrations from environmental samples, we prepared standard curves consisting of 1:10 serial dilutions of the gBlock oligo from 1 to 1×10^7 copies per reaction. The gBlock Gene Fragments (IDT) consisted of a 471-bp sequence that started with a 40-bp sequence of randomly chosen nucleotides, followed by a 150-bp COI sequence, a 20-bp sequence of randomly chosen nucleotides, and 261-bp ND1 sequence; therefore, the same gBlock was used with all COI and ND1 primers. For LOD and LOQ calculation, we ran each standard 12 times in the same plate.

The primer–probes SamND_FWD1: 5′-ACTAGGGCTTAGTGGCATTCC, SamND_RVS1: 5′-AGGGCGAGTATAGTTATTGGGG, and SamND_Probe1: 5′-AACCCGCAGCAGACGCCTTG showed the highest specificity of all tested primer–probe sets (Table 1), with *S. ambigua* DNA being detected at quantification cycle (Cq) = 25.42. Cross-amplification was observed for nontarget species *Obliquaria reflexa* (Cq = 39.79) and *Sagittunio nasutus* (Cq = 39.66); however, this was above the Cq threshold (Cq = 38; see below) despite 2 ng/μL of template DNA, which is a high concentration of nontarget DNA to test for cross-reactivity. This ND1 assay also showed good efficiency across six standard curves, with an average efficiency of 94% and $R^2 > 0.99$. Therefore, we used this primer–probe set in all subsequent eDNA qPCR assays. We tested a temperature gradient between 55 and 62°C for annealing temperature, and the optimal temperature was 60°C. The optimized primer and probe concentrations for SamND1 were 0.9 and 0.25 μM, respectively.

Optimization and Testing of eDNA Detection in the Field

We optimized filter pore size and the volume of water filtered in the field by collecting water samples from a site on the Sydenham River that supports a population of *S. ambigua* (site LSC-SRY-05 in Fig. 1). We collected and filtered water samples with an OSMOS eDNA backpack sampler (Halltech, Guelph, ON, Canada) during two consecutive days in October 2019 (mean water depth, 3.4 m; mean discharge, 5.97 m^3/s; real-time hydrometric data for Florence Station; wateroffice.ec.gc.ca). Filtering in the field instead of in the laboratory allowed us to filter larger volumes (1–10 L in the field; <500 mL in the laboratory) and to store, refrigerate, and transport filters instead of large volumes of water. We tested three different cellulose nitrate filter pore sizes (0.45, 0.80, and 1.00 μm) and two water volumes (1 and 10 L) to determine which pore size–volume combination was optimal for eDNA capture in the field. We collected water samples at the river surface (Currier et al. 2017) from the bank or by wading in the midchannel, depending on the width and depth of the river. When sampling by wading, we placed the filter housing upstream from the surveyors to avoid contamination. We decontaminated reusable filter housings by soaking them for 10 min in a 10% bleach solution and thoroughly rinsing them with water between samples. We discarded nitrile gloves and decontaminated the forceps after collecting each sample. We collected two field replicate samples for each pore size–volume combination. We did not take field blanks because all samples were taken at the same location and the main goal was to test the volume of water that we were able to filter by using different pore sizes before the filters clogged. After filtration, we placed all filters in 5-mL transport polypropylene tubes (Thermo Fisher Scientific, Waltham, MA, USA), stored them in a cooler with ice, and froze them at −20°C within 12 h. We stored filters at −80°C and conducted DNA extraction within a week of collection.

We extracted DNA from filters by using the DNeasy Blood and Tissue extraction kit (Qiagen) following the manufacturer's protocol, with the following modifications. We completed DNA extractions in a separate room from the qPCR instrument and cleaned bench surfaces with hydrogen peroxide. We placed all pipettes under UV light for 3 min before extractions. We doubled the volume of buffer ATL and proteinase K, and we extended the incubation in buffer ATL and proteinase K to 16–24 h at 56°C. After incubation, we added 400 μL of buffer AL and 400 μL of 100% molecular grade ethanol to obtain a 1:1:1 volume ratio (buffer ATL plus proteinase K:buffer AL:ethanol). The final elution volume with buffer AE was 100 μL. We extracted a DNA extraction blank with each set of samples to check for contamination during the extraction process. Inhibition of qPCR is common in eDNA detection from environmental samples (for review, see Goldberg et al. 2016); therefore, we tested for inhibition by diluting samples 1:10 and 1:100. An increase in eDNA concentration with an increase in the

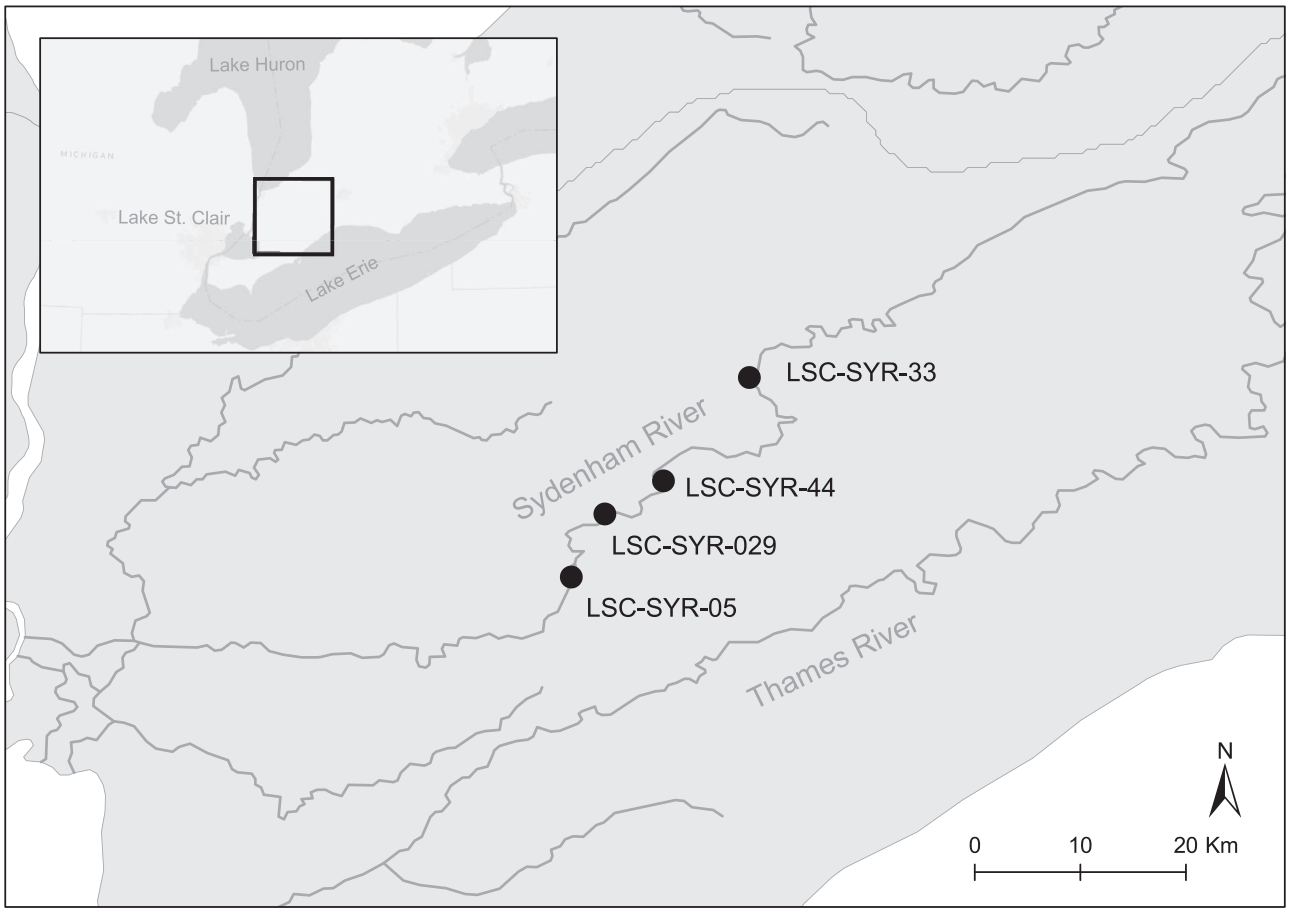

Figure 1. Map of sites sampled for *Simpsonaias ambigua* environmental DNA (eDNA) in the Sydenham River. Sample site numbers increase in an upstream direction. Inset map shows the location of the Sydenham River in Ontario, Canada.

dilution factor would indicate inhibition affected eDNA detection and quantification. We carried out all qPCR reactions as described previously.

We field tested the primer–probe sets by collecting three field replicate 2-L water samples with an OSMOS eDNA sampler (Halltech) as described previously at four sites along a 45-river kilometer (rkm) reach of the Sydenham River (Fig. 1) during two consecutive days in March 2020 (mean water depth, 3.95 m; mean discharge, 14.93 m^3/s; real-time hydrometric data for Florence Station; wateroffice.ec.gc.ca). Although 1 L was the optimal volume (see previous text and Results), we collected 2-L samples to maximize detection probabilities. The distance between adjacent sites ranged from 7 to 25 rkm. A qualitative survey conducted in 2018 and 2019 in this reach detected 43 live *S. ambigua* within a 12-rkm reach between sites LSC-SYR-29 and LSC-SYR-05 (I. Porto-Hannes, unpublished data). No live *S. ambigua* were found at LSC-SYR-44, one live individual was found at LSC-SYR-33, and no live individuals were reported upstream of LSC-SYR-33 (LGLUD 2020). We filtered water samples through a 0.8-µm cellulose nitrate filter (see previous text and Results), and we stored and extracted all filters and subjected DNA to qPCR as described previously.

We tested for PCR inhibition in field samples in two ways. First, we diluted extracted DNA 1:1, 1:2, and 1:10 and quantified DNA concentration by qPCR with and without the addition of 0.4 mg/mL (final concentration) bovine serum albumin (BSA), which can overcome inhibition in environmental samples (Kreader 1996). Second, we spiked extractions with a known concentration of DNA. We prepared spiked replicates of six samples by adding to each sample 2.0 µL of a 10,000 copies/ml DNA standard to 2.0 µl of each sample's eluate. We then compared DNA concentrations from qPCR reactions against expected DNA concentrations based on spiking. A decrease in DNA detection was observed in only one of the spiked samples; therefore, we ran each environmental sample six times using 5 µl of 1:1 extracted DNA and adding 0.4 µl of BSA per reaction (final concentration, 0.4 mg/mL) to increase the probability of *S. ambigua* eDNA detection. We ran a standard curve and NTC as described previously for each plate of samples. We pooled standard curves with efficiency >90% across plates to calculate DNA concentrations in unknown samples. We considered a sample quantifiable if at least three of six qPCR replicates amplified at a $Cq \leq 35$ cycles (LOQ).

Estimation of eDNA Shedding and Decay Rates

We performed an experiment to estimate eDNA shedding and decay rates for *S. ambigua* in tap water. Because eDNA decay is influenced by many environmental variables and differs between environmental water and tap water (Sassoubre et al. 2016; Sansom and Sassoubre 2017), we also estimated eDNA decay in environmental water from the Sydenham River. We used eDNA decay rates determined from environmental water in the model for eDNA downstream transport (see subsequent text).

We acquired 60 juvenile *S. ambigua* (mean shell length, 12.56 ± 3.00 mm; mean wet mass, 0.19 ± 0.10 g) from the Genoa National Fish Hatchery, U.S. Fish and Wildlife Service, Genoa, Wisconsin, USA; juveniles were raised from brood stock from the Chippewa River, Wisconsin. Mussels were shipped to our laboratory, and upon arrival, we placed them in a continuously aerated 40-L tank with gravel substrate (median diameter D50 of 0.01 m) and filled with tap water treated with AmQuel (number-31261, Kordon, Hayward, CA, USA) to neutralize chlorine, chloramine, and ammonia. The tank was continuously aerated with air stones (5 cm × 10 cm) connected to an air pump (model AAPA15L, ActiveAQUA, Petaluma, CA, USA). We maintained the tank at room temperature (22 ± 1°C) for the duration of the acclimation and experimental periods. We exposed tanks to indirect sunlight through a window and artificial lights in the laboratory. We fed mussels by adding 2.0 mL of algae to the tank (Shellfish Diet 1800, Reed Mariculture, Campbell, CA, USA) every 2 d. We allowed mussels to acclimate for 4 wk before the experiments.

Determination of experimental mussel density and sample volume.— We conducted a pilot study to determine the optimal number of mussels and sample volume needed to detect eDNA with our SamND1 assay in the experiments. We established six 20-L tanks, three containing 15 L of environmental water (Sydenham River) and three containing 15 L of tap water treated as described previously. We collected environmental water from the Sydenham River in 3.78-L acid-washed plastic containers and stored them on ice in coolers during transportation to the laboratory. Each set of three tanks included one tank with two *S. ambigua*, one tank with 18 *S. ambigua*, and one control tank with no mussels. From each tank containing mussels, we collected water samples of 100, 500, 1,000, and 3,000 mL 48 h after the initiation of the experiment. We collected replicate samples of each volume in 1-L polycarbonate bottles that previously were acid washed (10% HCl), neutralized in $NaHCO_3$, and rinsed with deionized water. We filtered samples in the laboratory over 47-mm-diameter polycarbonate filters (EMD, Millipore, Germany) with a pore size of 0.40 μm for 100-, 500-, and 1,000-mL samples and a pore size of 1.2 μm for the 3,000-mL samples. We also collected and filtered 500 mL of water from the control tanks and a filtration control consisting of 200 mL of molecular grade water (Sigma-Aldrich). We placed all filters in 5-mL transport polypropylene tubes (Thermo Fisher Scientific) and stored them at −80°C until DNA extraction. We extracted DNA from the filters and subjected DNA to PCR as described previously.

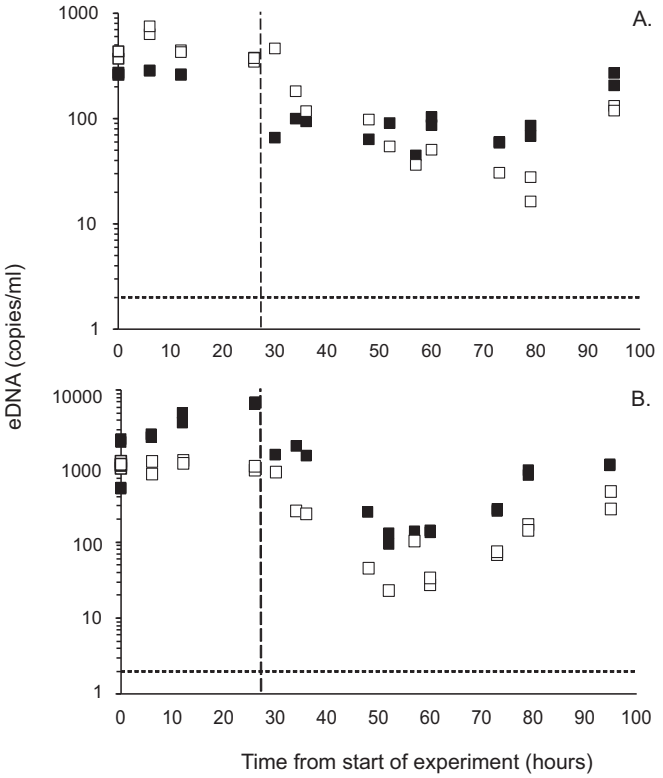

Figure 2. *Simpsonaias ambigua* environmental DNA (eDNA) concentration over time in tap water at (A) low mussel density (4 mussels [0.76 g wet mass]/15 L) and (B) high mussel density (13 mussels [2.47 g wet mass]/15 L). Solid and filled symbols represent two replicate tanks within each density treatment. The vertical dashed line indicates the time at which mussels were removed from the tanks. The horizontal dot-dashed line represents DNA limit of detection (LOD; 2.15 copies/mL in 500-mL sample or 10.76 copies/μL).

Shedding and decay rates in treated tap water.— We established five 20-L tanks, each containing 15 L of tap water treated as described previously. We established two high-density tanks, each containing 13 mussels (approximate total wet mass, 2.47 g); two low-density tanks, each containing four mussels (approximate total wet mass, 0.76 g); and one control tank containing no mussels. We placed an air stone in each tank to provide mixing. Mussels were not fed for 6 h before, and for the duration of, the experiment, and they were allowed to acclimate for 18 h before beginning the experiment. We collected duplicate 500-mL water samples from each tank at the beginning of the experiment (T_0) and every 6–7 h for the next 26 h ($N = 4$ after T_0; Fig. 2). We collected samples in 1-L polycarbonate bottles cleaned as described previously. From these samples, we determined whether eDNA concentration reached a steady state where eDNA concentration did not change over two consecutive time periods. To estimate eDNA decay rate, we removed mussels from the

tanks after 26 h and collected duplicate water samples every 3–4 h within two 12-h periods over the next 2 d (i.e., 30–60 h after T_0, $N = 7$; Figs. 2, 3), and at three time points over the following 2 d (i.e., 73–95 h after T_0; Fig. 2).

Immediately after collection, we filtered water samples through a 0.45-μm cellulose nitrate filter (Whatman™ type WCN cellulose nitrate membranes, GE Healthcare, Chicago, IL, USA) by using 47-mm magnetic funnels (magnetic filter funnels, Pall Corporation, Port Washington, NY, USA). We also filtered a control consisting of 200 mL of molecular grade water coincident with filtering of samples at each time point. We placed filters in 5-mL transport polypropylene tubes and stored them immediately at −80°C until DNA extraction. We extracted DNA from the filters and subjected DNA to PCR as described previously.

Decay rates in environmental water.— We established a 37-L tank containing 19 L of environmental water from the Sydenham River. We collected environmental water using 3.78-L plastic containers that were previously acid washed. All water samples were kept in coolers with ice until arrival at the laboratory. We placed eight *S. ambigua* (mean individual wet mass, 0.19 ± 0.10 g) in the tank when water reached room temperature (22°C). We used air stones to completely mix the water; we did not add substrate to this tank to avoid potential eDNA capture by sediments. We left mussels in the tank for 24 h to allow the eDNA concentration to reach a steady state then removed all mussels from the tank. Three of the eight mussels died during the first 24 h; however, because this experiment was designed to estimate eDNA decay rates only, death of the mussels is not expected to influence our estimates. We collected duplicate water samples from the tank immediately after the mussels were removed from the tank (T_0), every 3–4 h within two 12-h periods over the next 2 d (1.5–34.5 h after T_0; $N = 8$), two times per day for 1 d (47.0–52.5 h after T_0; $N = 2$), one sample every 24 h for 2 d (71.5–95.5 h after T_0), and once 11 d after T_0 (263.5 h; Fig. 4a,b).

Immediately after collection, we filtered water samples, including filtration controls, over a 47-mm-diameter 0.45-μm cellulose nitrate filter as described previously. We stored and extracted all filters and subjected DNA to PCR as described previously.

Data analysis.— We calculated eDNA shedding and decay rates based on a completely mixed batch reactor model:

$$V\frac{dC}{dt} = S - kC\mathrm{V}$$

where V is the volume of the tank (mL), C is the eDNA concentration (copies/mL), t is the time since the start of the experiment (h), S is the eDNA shedding rate (copies/h), and k is the first-order decay-rate constant (/h) (Sassoubre et al. 2016; see subsequent for k calculation). This model assumes that the tank is well mixed and that the decay is first order (linear decay over time). At steady state, $dC/dt = 0$, therefore

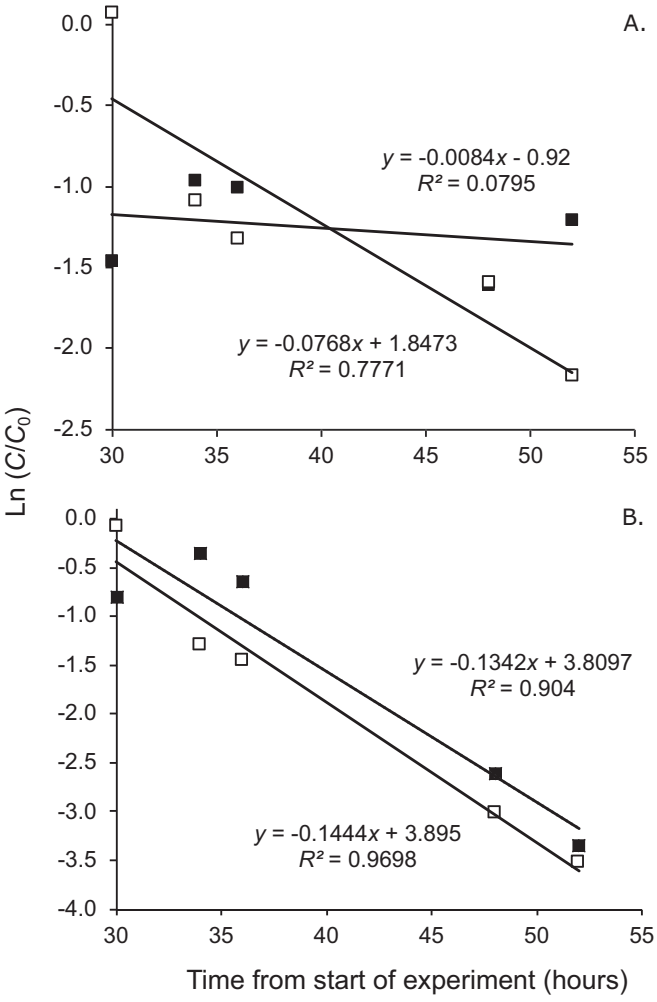

Figure 3. Linear decay of DNA concentration (ln (C/C_0)) over time in tap water at (A) low mussel density (4 mussels [0.76 g wet mass]/15 L) and (B) high mussel density (13 mussels [2.47 g wet mass]/15 L). Solid and filled symbols represent two replicate tanks within each density treatment.

$S = kCV$. We used a t-test to determine whether there was a difference in shedding rates between replicates and experimental tanks with tap water.

We calculated the k value after removal of the mussels, when $S = 0$ and therefore $dC/dt = -kC$. We determined k by fitting the data to a linear decay on a plot of $\ln(C/C_0)$ versus time (t) (Fig. 4c). In tap water, C_0 was the mean eDNA concentration until reaching steady state ($T_0 - T_{26}$). In environmental water, C_0 was the eDNA concentration at the time mussels were removed from the tank, because the aim was to calculate only the decay rate. We modeled eDNA decay in environmental water with nine regression models (Table 2) by using GInaFiT (Geeraerd et al. 2005), a software package designed to model the decay of bacteria over time and has also been used to model eDNA decay (Andruszkiewicz et al. 2020). We tested all models from T_0 until the end of the experiment (263.5 h). We chose the best-fit model based on the greatest R^2 and adjusted R^2.

We compared k values (i.e., the slope representing eDNA decay over time) among different experimental treatments in

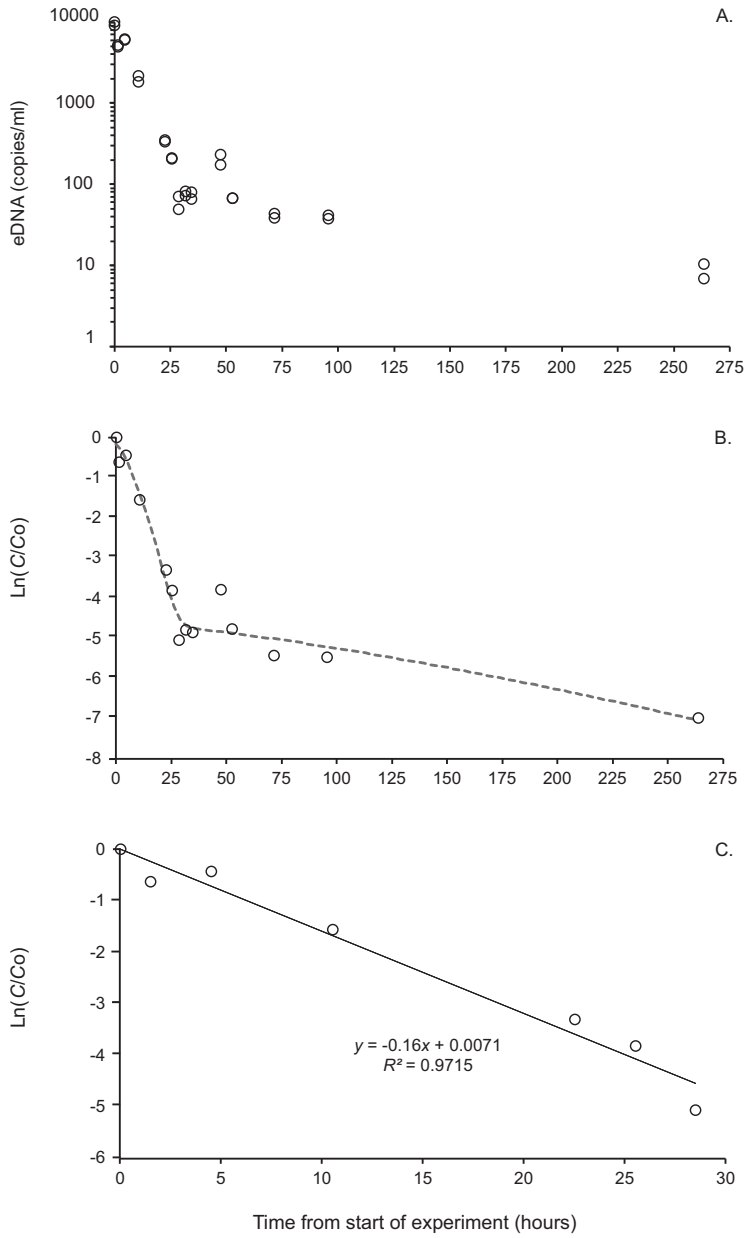

Figure 4. (A) *Simpsonaias ambigua* environmental DNA (eDNA) concentration over time in environmental water. The horizontal dot-dashed line represents the DNA limit of detection (LOD; 2.15 copies/mL in 500-mL sample or 10.76 copies/μL). (B) Linear decay of eDNA concentration (ln (C/C_0)) for the duration of the experiment and (C) during the first 28.5 h, which were used to calculate the decay-rate constant (k).

tap water (low density vs. high density) with analysis of covariance (ANCOVA) by using R v. 3.6.2 (R Core Team 2011). Before conducting the ANCOVA, we confirmed that the data met the assumptions of linearity, homogeneity of regression slopes, normality of residuals, and homogeneity of variance.

eDNA Transport Model

To evaluate eDNA sampling intervals, we modeled downstream transport of eDNA for a range of realistic source eDNA concentrations and water velocities by using the one-dimensional plug-flow reactor model of Sansom and Sassoubre (2017):

$$C = C_{bed} e^{\frac{-kx}{u}}$$

where C is eDNA concentration (copies/mL) at a given distance downstream from the source, C_{bed} is a hypothetical value based on lab and field observations and represents the expected eDNA concentration originating from the source, k is the first-order decay-rate constant (/h), x is the downstream distance (km) from the source, and u is the water velocity (km/h). We populated the model as follows. For C_{bed}, we modeled two hypothetical eDNA concentrations: 1.0 and 5.0 copies/mL. These values are based on reported eDNA concentrations for other unionid species in Ontario (<0.5–10 copies/mL; *Quadrula quadrula*,

Table 2. Regression models evaluated to describe environmental DNA decay in environmental water.

Model	R^2	R^2 adjusted
Double Weibull (Coroller et al. 2006)	0.967	0.957
Biphasic + Shoulder (Geeraerd et al. 2005)	0.966	0.951
Biphasic (Cerf 1977)	0.964	0.953
Weibul + Tail (Albert and Mafart 2005)	0.901	0.871
Log-linear + Shoulder + Tail (Geeraerd et al. 2005)	0.901	0.871
Log-linear + Tail (Geeraerd et al. 2005)	0.899	0.881
Weibul (Mafart et al. 2002)	0.839	0.81
Weibul Fixed parameter (0.5) (Mafart et al. 2002)	0.768	0.726
Log-linear + Shoulder (Geeraerd et al. 2005)	0.723	0.672
Log-linear Regression (Bigelow and Esty 1920)	0.487	0.444

Ptychobranchus fasciolaris, and *Lampsilis fasciola*; Currier et al. 2017); however, given *S. ambigua*'s LOD, shedding rates (see Results), and low population densities in the Sydenham River, we used lower values of eDNA concentration. For k, we used the value 0.164/h, as estimated in environmental water (see Results). For x, we used values from 0 to 10 km, and for u we used values from 0 to 3 km/h (0.00–0.83 m/s), which are within the range of observed water velocity in the Sydenham River (I. Porto-Hannes, unpublished data). The model of Sansom and Sassoubre (2017) assumes no additional eDNA inputs downstream of the hypothetical initial source.

RESULTS

Simpsonaias ambigua Primer and Probe Development and Optimization in the Laboratory

Amplification efficiency was >90% for all the COI and ND1 primers developed for *S. ambigua*, but the specificity of primer pair SamND_1 was highest (Table 1). Cross-amplification was observed for eight nontarget species for SamCOI_I and two nontarget species for SamND1, but all values for nontarget species were above the *Cq* threshold ($Cq = 38$), indicating no significant cross-reactivity with other mussel species (Table 1). All primer pairs amplified *S. ambigua* DNA from individuals from Wisconsin (juveniles used in experiments) and Ontario, suggesting that these assays can be used to detect *S. ambigua* across its distributional range. The LOD and LOQ of primer pair SamND1 was 10.76 copies/μL (95% confidence interval: 7.47–15.51 copies/μL; $Cq \leq 38$) and 50 copies/μL ($Cq \leq 35$), respectively.

Optimization and Testing of eDNA Detection in the Field

We determined the optimal filter size was 0.80 μm, based on detection of *S. ambigua* eDNA in one of two field replicates and three of four qPCR replicates for each sample volume (Table 3). One field replicate of a 0.45-μm filter and sample volume of 10 L resulted in eDNA detection in two of four qPCR replicates, but no DNA was detected when the sample volume was 1 L. There was no detection of eDNA with a 1.0-μm filter for either sample volume. There was no evidence of contamination in any field, filtration, or extraction blanks, and all qPCR NTCs showed no amplification.

Most detections of *S. ambigua* eDNA were observed at site LSC-SYR-05, which is downstream of the reach of the Sydenham River that appears to support the largest populations of the species. However, we detected eDNA in only two of three field replicates and two to three qPCR replicates at this site. At sites LSC-SYR-29 and LSC-SYR-44, we detected eDNA in only one of three field replicates and one qPCR replicate. We did not detect eDNA at site LSC-SYR-33, which appears to support only small populations of *S. ambigua* and may be near the upstream limit of the species in the river (see previous text).

Estimation of eDNA Shedding and Decay Rates

Determination of experimental mussel density and sample volume.— We detected eDNA in all tanks with mussels (2 and 18 *S. ambigua*) and all sample volumes (100–3,000 mL). No DNA was detected in control tanks with no mussels.

Shedding and decay rates in treated tap water.— Shedding rate was significantly higher in the high-density tanks than in the low-density tanks ($t_{7.74} = -2.59$, $P = 0.033$; Fig. 2 and Table 4). However, there was considerable variation among replicates, particularly in the low-density tanks, where shedding rate differed significantly between tanks ($t_3 = -5.90$,

Table 2. Results from the October 2019 field experiment to test the effects of filter pore size and water volume on detection of *Simpsonaias ambigua* eDNA. Amplification is the number of qPCR replicates within each field replicate in which *S. ambigua* environmental DNA was detected. *Cq* is the quantification cycle. NA = not applicable.

Filter size (μm)	Volume (L)	Field replicate	Amplification	Mean (range) *Cq*
0.45	1	1	0/4	NA
		2	0/4	NA
	10	1	2/4	38.10 (36.47–39.72)
		2	0/4	NA
0.80	1	1	0/4	NA
		2	3/4	37.87 (36.23–39.21)
	10	1	3/4	38.12 (37.13–38.75)
		2	0/4	NA
1.00	1	1	0/4	NA
		2	0/4	NA
	10	1	0/4	NA
		2	0/4	NA

Table 2. Environmental DNA shedding and decay-rate constants (k) for *Simpsonaias ambigua* over time in tap water at low mussel density [LD: 4 mussels [0.76 g wet mass]/15 L) and high mussel density [HD: 13 mussels [2.47 g wet mass]/15 L). Propagated error for shedding rate was calculated for each tank over four time points from T_0 to $T = 26$ h. Standard error for k was calculated over five time points from $T = 30$ h to $T = 52$ h. Within a column, values with different lowercase letters are significantly different (shedding rate, copies/h/mussel: t-test; k: analysis of covariance). Asterisk (*) indicates LD tank 1 did not follow first-order kinetics.

	DNA shedding rate			
Treatment	Copies/h ± propagated error	Copies/h/mussel	Copies/h/g	k ± SE
LD (tank 1)	$3.73 \times 10^4 \pm 7.36 \times 10^4$	9.33×10^3a	4.91×10^4	$8.36 \times 10^{-3} \pm 1.64 \times 10^2$*
LD (tank 2)	$5.49 \times 10^5 \pm 2.38 \times 10^5$	1.37×10^5b	7.22×10^5	$7.68 \times 10^{-2} \pm 2.37 \times 10^2$a
HD (tank 1)	$7.49 \times 10^6 \pm 5.42 \times 10^6$	5.76×10^5c	3.03×10^6	$1.34 \times 10^{-1} \pm 2.53 \times 10^2$a
HD (tank 2)	$2.25 \times 10^6 \pm 3.89 \times 10^5$	1.73×10^5c	9.11×10^5	$1.44 \times 10^{-1} \pm 1.47 \times 10^2$a

$P = 0.009$); shedding rate did not differ significantly between tanks in the high-density treatment ($t_3 = 2.21$, $P = 0.113$). eDNA concentration increased in all tanks after 57 h because we tipped the tanks to obtain samples, resulting in unintended resuspension of eDNA from the substrate (Fig. 2). For this reason, we did not consider data points beyond 57 h.

The eDNA k value in both high-density tanks and one low-density tank appeared to follow first-order kinetics (Fig. 3). The eDNA decay in the other low-density tank did not follow first-order kinetics, so we excluded this tank from further statistical analysis. The eDNA k did not differ between treatments (ANCOVA: $F_{1,2} = 2.398$, $P = 0.137$), indicating that there was no effect of mussel density on the k. The mean value of k across all three tanks was 0.12 ± 0.06/h. There was no evidence of contamination in any control tanks or filtration and extraction blanks, and all qPCR NTCs showed no amplification.

Decay rates in environmental water.— Throughout the duration of the experiment (T_0 to 263.5 h), eDNA decay in environmental water was best described by a double Weibull model (Table 2). DNA continued to be detected 10 d (263.5 h) after mussels were removed from the tank (Fig. 4). Between T_0 and 28.5 h, eDNA decay followed first-order kinetics (i.e., linear decay over time) (Fig. 4b, c). An increase in eDNA copies/mL was observed at 48 h (Fig. 4a); however, we did not include this point in the k calculations because it does not fall within the linear decay period. From T_0 to 28.5 h, the k in environmental water was 0.164 ± 0.0124/h. There was no evidence of contamination in any control tanks or filtration and extraction blanks, and all qPCR NTCs showed no amplification.

eDNA Transport Model

The maximum predicted downstream distance at which eDNA could be detected (LOD = 10.76 copies/μL or 0.54 copies/mL from a 2-L water sample) varied greatly depending on the source eDNA concentration and water velocity (Fig. 5). When source eDNA concentration was 1.0 copy/mL, detection was predicted at 10 km only at high water velocity (>0.6 m/s), and higher detection (i.e., approaching 1.0 copy/mL) was predicted only at distances less than ~2.0 km. By contrast, when source eDNA concentration was 5.0 copies/mL, detection was predicted at 10 km at lower velocity (~0.2 m/s) and higher detection (greater than ~1.0 copy/mL) was predicted across a much wider range of distance and velocity.

DISCUSSION

Effective use of eDNA methods requires pilot studies that can help optimize the assay and eDNA capture methods (Goldberg et al. 2016). In our study, filter pore size was an important factor that influenced eDNA detection. Detection was greatest with a 0.8-μm filter. This is consistent with other studies that found this pore size to be optimal (Deiner et al. 2018; Li et al. 2018). We filtered larger volumes of water (e.g., 2 L) in the field than in the laboratory to increase detection probabilities; however, given the sediment loads present in the Sydenham during the spring (e.g., LSC-SYR-05, total suspended solids [TSS] for March of 76 mg/L), filtering was challenging (see subsequent text). In other systems, increasing the volume of water may not be possible, because this would likely lead to increased PCR inhibition; therefore, we recommend that the sample volume be optimized for each aquatic system.

We observed greater eDNA detection in the fall (October 2019, 16.67% amplification rate) than in the spring (March 2020, 9.72% amplification rate; see Tables 3 and 5), as has been noted by others (Troth et al. 2021). Our sample volumes differed between seasons, so it is difficult to directly compare eDNA detection. However, lower detection in spring may be expected for several reasons. The suspended sediment load was higher in the spring than in the fall (mean TSS for the Sydenham River in spring of 56.7 mg/L; fall, 14.87 mg/L), which limited the amount of water that we could filter in the spring. Higher discharge in the spring (spring, 14.93 m^3/s; fall, 5.97 m^3/s) also could have contributed to a diluted eDNA signal, as reported in other studies (Balasingham et al. 2017; Curtis et al. 2021; Gasparini et al. 2020). Lastly, because *S. ambigua* is gravid and releases glochidia in the fall (I. Porto-Hannes, unpublished data), release of glochidia may

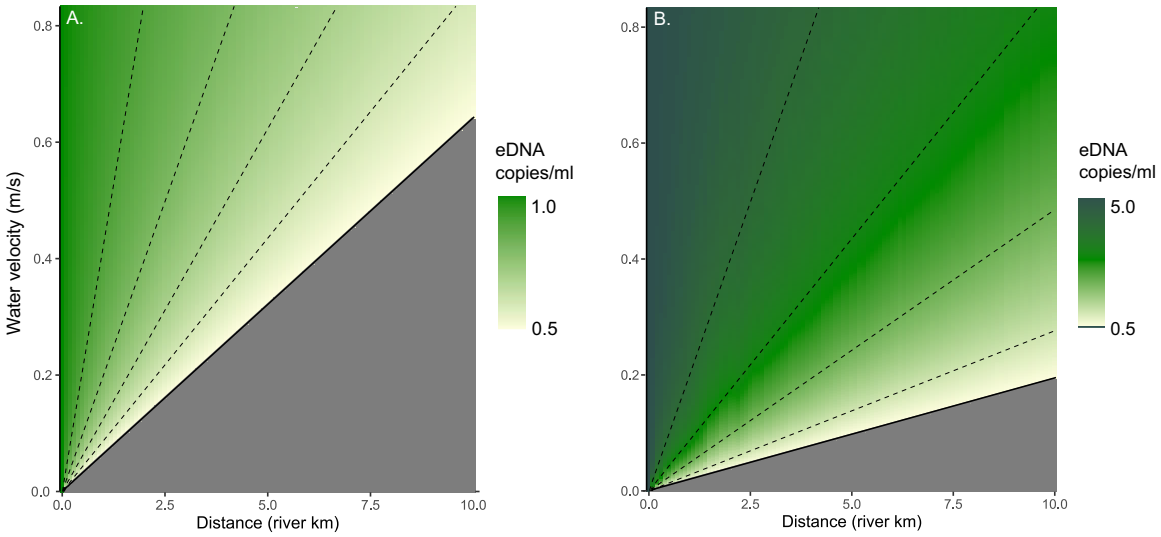

Figure 5. Color gradient graphs showing predictions of the environmental DNA (eDNA) transport model of eDNA concentration as a function of distance from the source and water velocity. Darker colors indicate higher eDNA concentration, and the gray area indicates eDNA concentrations below the limit of detection (LOD; 0.54 copies/mL in 2-L sample or 10.76 copies/μL). (A) Source eDNA concentration of 1.0 copy/mL. (B) Source eDNA concentration of 5.0 copies/mL.

increase the probability of eDNA detection, as observed for *Nodularia nipponensis* (Sugawara et al. 2022).

Our detection of *S. ambigua* eDNA in the wild was lower than expected given that we surveyed at three sites where *S. ambigua* is known to occur. Low detection may be due in part to factors associated with spring sampling as discussed previously. However, the unique habitat use of *S. ambigua* also may contribute to lower eDNA concentrations in the water than for other mussel species. Because *S. ambigua* typically occurs in cavities under large rocks, a large proportion of eDNA produced by individuals may remain in those cavities where it is not readily suspended in the water column or readily detected by conventional sampling.

Table 2. Detection of *Simpsonaias ambigua* environmental DNA at four sites in the Sydenham River, Ontario, Canada, in March 2020. Sites are arranged from upstream to downstream. Amplification is the number of quantitative PCR replicates within each field replicate in which *S. ambigua* eDNA was detected. *Cq* is the quantification cycle. NA = not applicable.

Site	Field Replicate	Amplification	Mean (range) *Cq*
LSC-SYR-33	1	0/6	NA
	2	0/6	NA
	3	0/6	NA
LSC-SYR-44	1	0/6	NA
	2	0/6	NA
	3	1/6	38.49
LSC-SYR-29	1	0/6	NA
	2	1/6	38.69
	3	0/6	NA
LSC-SYR-05	1	3/6	38.34 (37.57–38.85)
	2	2/6	37.96 (37.46–28.39)
	3	0/6	NA

Our estimates of shedding rate for *S. ambigua* were comparable to shedding rate of *Lampsilis siliquoidea* (5.4×10^4–2.4×10^6 copies/h/mussel; Sansom and Sassoubre 2017), but they are higher than shedding rates reported for *N. nipponensis* (0.0066 and 0.33×10^6 copies/h/individual; Sugawara et al. 2022). The similarity between *S. ambigua* and *L. siliquoidea* is surprising because *S. ambigua* is much smaller and eDNA shedding rates tend to increase with biomass (Takahara et al. 2012; Maruyama et al. 2014). However, in wild Brook Trout (*Salvelinus fontinalis*), eDNA shedding rates scaled nonlinearly and allometrically with biomass (Yates et al. 2020a, 2020b). Apart from biomass, shedding rate may be related to behavior and metabolism (Maruyama et al. 2014; Klymus et al. 2015). The juvenile *S. ambigua* used in our study were more active than adult *L. siliquoidea* used in the Sansom and Sassoubre (2017) study (I. Porto-Hannes, unpublished data; B. Sansom, personal communication). *Simpsonaias ambigua* juveniles constantly moved vertically and horizontally within the substrate and sometimes crawled up the tank sides or onto the air stones. This behavior may have resulted in greater shedding rates than expected given their small size. In addition, biomass may be a more important determinant of shedding rate in animals that shed skin or scales, which are proportional to biomass. Soft tissues of mussels are enclosed in a hard shell that does not decay readily (Gutiérrez et al. 2003; Strayer and Malcom 2007); consequently, for these animals, activity and filtering rate may be more important determinants of shedding rate than biomass. Further studies are needed to understand how biomass, habitat conditions, and behavior affect shedding rates within and among freshwater mussel species.

Higher concentrations of eDNA can occur in sediment than in the water column because of settling or direct deposition of feces and pseudofeces (Turner et al. 2015). We observed an unexpected increase in eDNA concentration late in our laboratory experiments that coincided with tilting the tanks to obtain a water sample, which probably resuspended eDNA in the substrate. In the wild, eDNA in sediments will not be detected in water samples unless it is resuspended by high flows or other factors (Jerde et al. 2016; Shogren et al. 2017, 2019). Resuspension of eDNA in sediment is an important factor affecting eDNA detection, especially for benthic organisms such as mussels, and this factor needs further investigation. The detection of *Margaritifera monodonta* eDNA was higher in benthic samples than in water column samples (Lor et al. 2020). However, Currier et al. (2017) found no differences in mussel eDNA detection between surface and subsurface water samples in lotic habitats.

The eDNA decay rate can be influenced by factors such as enzymatic breakdown, microbial grazing, and UV light (Andruszkiewicz et al. 2020). In our study, k values were similar between tap water (from 0.077 $\pm$ 0.024 to 0.144 $\pm$ 0.015/h) and environmental water (0.164 $\pm$ 0.012/h). Our k values also were similar to decay rates reported for *N. nipponensis* (0.074 $\pm$ 0.021/h; Sugawara et al. 2022) and *Cumberlandia monodonta* (0.067/h; K. Klymus, personal communication), but they were an order of magnitude larger than for *L. siliquoidea* (0.0097–0.053/h; Sansom and Sassoubre 2017) and *Actinonaias ligamentina* (0.045/h; K. Klymus, personal communication).

Our predictions from the eDNA transport model should be viewed with at least two caveats. First, this model is one-dimensional: it considers downstream dispersion of eDNA, but not lateral dispersion or settling of eDNA into the substrate. River hydro-geomorphological features have been incorporated in a framework that reconstructs upstream distribution and abundance of a target species across a river network, based on observed eDNA concentration (Carraro et al. 2018). This framework assumes a homogenous distribution of the target species and eDNA production within a river channel. Although the distribution of *S. ambigua* is highly heterogeneous, this framework can be used as a null model. Second, the model is based on decay rates of eDNA present in the water column, but it does not consider settling of eDNA.

Despite the caveats inherent in the eDNA transport model, our model results provide recommendations about optimal sampling designs for eDNA detection. When expected eDNA concentration is low (e.g., 1.0 copy/mL), sampling sites should be spaced at intervals of $<$2.0 rkm if flow velocity is low. Even if flows are higher, sites should be spaced $<$5.0 rkm apart to ensure consistent detection. When expected eDNA is higher (e.g., 5.0 copies/mL), sites can be spaced up to 10 rkm apart at moderate to high flow velocity, and $\sim$5 rkm apart at all but the lowest flow conditions. The potential for seasonal variation in eDNA concentration also should be considered, and, if possible, sites should be resampled in different seasons. It is important to consider whether eDNA is present near the LOD, the ability to consistently detect eDNA, and that nondetection may represent Type II error and should not necessarily be interpreted as evidence that the species is absent (Klymus et al. 2020). Incorporating in a sampling design the effects of source eDNA concentration, flow velocity, seasonality, target species habitat use, and other factors can minimize Type II error. In addition, systematic sampling throughout a watershed can reveal consistent, large-scale patterns that more accurately indicate the distribution of a species.

The eDNA detection of a target species is a cost-effective way to provide information necessary to prioritize sites for more time-consuming conventional sampling. However, for rare and threatened species, such as *S. ambigua*, management decisions should not be made based solely on the detection of eDNA. Although considered part of the standard fisheries and wildlife management toolkit for population detection, assessment, and monitoring (Klymus et al. 2020, and references therein), eDNA methods cannot replace conventional methods and population monitoring but they can complement and augment them.

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APPENDIX

Table 2. *Simpsonaias ambigua* species-specific primer–probe sets designed from mitochondrial DNA sequences from the cytochrome oxidase subunit I gene (COI) and NADH dehydrogenase gene (ND1). Parameters were developed based on the following criteria: (1) DNA fragment size range 80–150 bp, (2) GC content 35–65% for both primer and probe, (3) primer annealing temperature range 58–63°C, (4) probe annealing temperature range 68–73°C, (5) maximum difference in annealing temperature between primer and probe of 10°C, and (6) primer pair specificity. Cross-amplification refers to nontarget species that amplified (see also Table 1). Minimum DNA sequences mismatches with nontarget species refers to the minimum number of nucleotides mismatches between the primer–probe and the nontarget species sequence.

Primer	Gene	Sequence 5′–3′	Primer Length (bp)	Fragment Size (bp)	GC Content	Annealing Temp. (°C)	Cross-Amplification (In Vitro Testing)	Minimum DNA Sequence Mismatches with Nontarget Species
SamCOI_Probe1	COI	TGAGGTCTTCGTTG GTGGAAAGAGGT	26	125	50	62	*Obliquaria reflexa*, *Amblema plicata*, *Truncilla truncata*, *Sagittunio nasutus*, *Potamilus fragilis*, *Lampsilis cardium*, *Alasmidonta undulata*, *Pleurobema sintoxia*	1<
SamCOI_FWD1	COI	ATCGGTGCTCCT GATATGGC	20		55	57		2<
SamCOI_RVS1	COI	ACCGTTCAACCAG TACCCAC	20		55	57		3<
SamCOI_Probe2	COI	CGGTGCTCCTGATATG GCTTTTCCTCG	27	123	56	63	Not tested	2<
SamCOI_FWD2	COI	TGGTAATTGGCT TGTTCCCT	20		45	54		1<
SamCOI_RVS2	COI	TCCACCAACGAA GACCTCAA	20		50	56		2<
SamND_Probe1	ND1	AACCCGCAGCAGA CGCCTTG	20	125	65	63	*Sagittunio nasutus*, *Obliquaria reflexa*	3<
SamND_FWD1	ND1	ACTAGGGCTTAGT GGCATTCC	21		52	57		4<
SamND_RVS1	ND1	AGGGCGAGTATAG TTATTGGGG	22		50	56		4<
SamND_Probe2	ND1	TGGCTACTTTCAAATTC GAAAAGGCCC	27	105	44	70	Not tested	3<
SamND_FWD2	ND1	TGGCTGTAGCATTTT TCACCC	21		48	60		1<
SamND_RVS2	ND1	TGGAATGCCACTA AGCCCTA	20		50	60		3<