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Research Article

Using environmental DNA to extend the window of early detection for dreissenid mussels

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Abstract

Tools that bolster early detection of invasive dreissenid mussels are needed to prevent their spread across western North America. In this study, we assessed if environmental DNA (eDNA) can extend the seasonal window for dreissenid mussel early detection beyond that of plankton tows, which are limited to warmer seasons when mussel larvae are present. We focused eDNA sampling efforts at multiple sites in Tiber Reservoir (Montana) where dreissenid mussel abundance is hypothesized to be low. Samples were collected in June and October 2017, when water temperatures were cooler than thermal optima for dreissenid reproduction, and in July 2017 when water temperatures were warmer and conducive for reproduction. We detected dreissenid mussel DNA in June, July and October even though no dreissenid mussels were observed using non-molecular tools in 2017. A subset of positive and negative eDNA samples was analyzed by an independent lab and results were corroborated. We then estimated the effort needed for 95% probability detection of dreissenid DNA at each site within Tiber Reservoir and found that as many as 27, 14, and 34 samples needed to be collected in June, July and October, respectively. To further validate the utility of eDNA, we also present ancillary eDNA results from other waters in the Flathead Reservation (Montana) where dreissenid mussels have never been detected and from waters with established zebra mussel populations in the upper Mississippi River, which were sampled in the spring when water temperatures were cooler than thermal optima for dreissenid reproduction. All Flathead Reservation samples were negative for dreissenid mussel DNA, while all upper Mississippi River samples were positive. This study adds to a growing body of research that demonstrates eDNA is a highly sensitive tool for dreissenid mussel surveillance in newly invaded waters, including colder seasons when non-molecular tools are likely to be less effective or more challenging to employ.

Key words: detection probability, molecular, Montana, surveillance, Tiber Reservoir

Introduction

Zebra and quagga (*Dreissena polymorpha* Pallas, 1771 and *D. rostriformis* Deshayes, 1838; dreissenids) mussels are prolific aquatic invaders that now occur in most major water basins in North America. Once established, dreissenids can cause significant economic and ecological impacts that result in annual expenditures of 100s of millions of dollars for control and

mitigation efforts (e.g., Prescott et al. 2013). Dreissenids have spread outward from their point of original introduction in the Great Lakes region on the trailers, hulls and in the bilges of recreational and commercial boats (Johnson et al. 2001). No technologies currently exist for eradicating dreissenids in open water so managers of uninvaded waters in western North America have invested heavily in prevention and early detection efforts. Early detection bolsters prevention efforts since rapid-response management strategies can be put in place to contain dreissenids and prevent their spread to other uninvaded waters. In addition, control technologies can be used to keep dreissenids at low abundance and to allow managers time to mitigate potential mussel impacts (Hosler 2011).

Plankton tow sampling is the current standard for early detection of dreissenids in western North America. Using a net, large amounts of water and debris are collected and then concentrated for microscopic examination of the free-swimming larval form (i.e., veligers) of dreissenid mussels. Taxonomic identity of an observed veliger is then confirmed with a DNA test (e.g., PCR). While plankton tows allow for unambiguous results when a veliger is detected, this method requires a breeding population rather than just adults and is limited to the several weeks following a spawning event, when veligers are most likely to be in the water column (Nichols 1996). Quagga and zebra mussels begin spawning when water temperatures are > 10 °C and 12 °C, respectively (McMahon 1996; Mills et al. 1996), so veliger availability in many northern latitude waters is largely limited to warmer months. In the heavily dreissenid-infested Lake Erie, for instance, no veligers were found in the water from October to April (Garton and Haag 1993).

Environmental DNA (eDNA) is a newer technology that may broaden the seasonal window of existing early detection monitoring efforts for dreissenids. Environmental DNA is a molecular approach that can detect DNA diffused from target organisms into a water body from easy to collect water samples. To date, eDNA analysis had demonstrated improved sensitivity and considerable time and cost benefits over traditional survey methods for many target species at low abundance (Rees et al. 2014), including dreissenid mussels (Gingera et al. 2017). In addition, eDNA is not restricted to specific life stages or seasonal windows though detection probabilities do increase with abundance and vary seasonally (de Souza et al. 2016; Wilcox et al. 2016). However, interpreting results is not straightforward since eDNA approaches only detect DNA, regardless of the presence or state (alive *v.* dead) of the target taxa. DNA by itself can enter a waterbody through numerous pathways, including carcasses, slime residue, and predator feces (Merkes et al. 2014). Thus, a positive eDNA detection can occur even though the living target taxa was never present in the sampled waterbody.

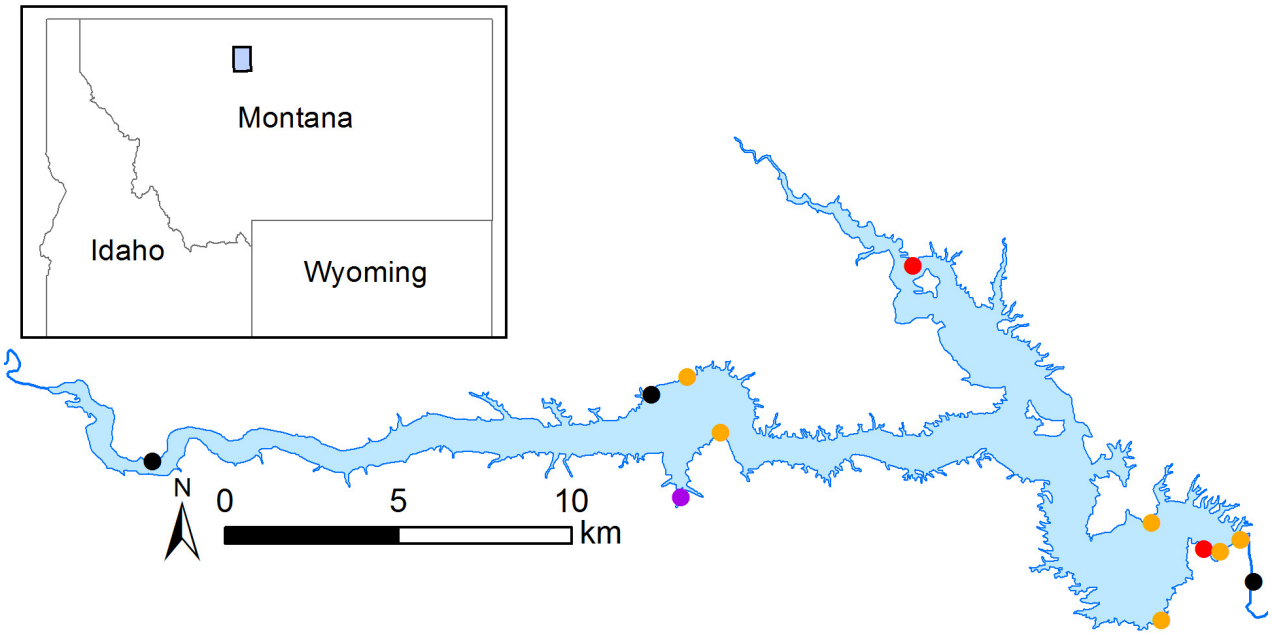

Figure 1. Sites where environmental DNA was used to survey for dreissenid mussel DNA in Tiber Reservoir in northcentral Montana. Sites where dreissenid veligers were detected in 2016 and had ≥ one positive eDNA sample in 2017 are designated in red. Sites where dreissenid veligers were not detected in 2016, but had ≥ one positive eDNA sample in 2017 are designated in orange. The site where dreissenid veligers were detected in 2016, but had no positive eDNA sample in 2017 is designated in purple. Sites lacking any detection in 2016 and 2017 are designated in black.

Table 1. Sites where environmental DNA was used to survey for dreissenid mussel DNA in Tiber Reservoir in northcentral Montana. We report the percent of positive eDNA water samples per month and, for just positive water samples, the proportion of positive PCR replicates for the DRE16S, DPO1, and QMCOI (only for October samples) assays. Please refer to Figure 1 for description of color codes.

		% Positive samples (n = 3)			Proportion positive PCR replicates		
Site ID	**Site**	**Jun.**	**Jul.**	**Oct.**	**Jun.**	**Jul.**	**Oct.**
WC	Willow Creek Arm	0	66	33	–	5/8, 3/8	2/8, 0/4, 1/4
VF	VFW Campground	0	66	0	–	7/8, 8/8	–
BE	Bootlegger East	0	100	–	–	12/12, 11/12	–
ED	East Dam	0	66	0		8/8, 8/8	–
TP	Turner Point	33	33	0	2/8	2/8, 0/4	–
WDA	West Dam	0	66	0	–	5/8, 2/8	–
WDI	West Dike	0	66	0	–	6/8, 7/8	–
MA	Marina	0	33	0	–	4/4, 4/4	–
SB	South Bootlegger	0	0	0	–	–	–
DV	Devon	0	–	–	–	–	–
MR	Marias River	0	–	–	–	–	–
BW	Bootlegger West	0	0	0	–	–	–

Here, we present eDNA results from a Montana Reservoir where quagga mussel veligers were detected with plankton tows in 2016 (Figure 1). At date, no zebra mussel veligers have been detected and no adult dreissenid has been found despite intense survey efforts so dreissenids are likely to be at low abundance. Therefore, this reservoir provides an opportunity to learn about dreissenid early detection efficacy at the early stages of invasion using eDNA approaches. To further validate the utility of eDNA, we also present ancillary eDNA results from other waters in Montana where dreissenid mussels have never been detected and from waters with established zebra mussel populations in the upper Mississippi River. Given

that plankton tow surveillance is most effective when water temperatures are within the reproductive thermal optima, we tested if eDNA broadens the seasonal window for early detection surveillance in the Montana Reservoir by collecting eDNA water samples when water temperatures are cooler than thermal optima for dreissenid reproduction. This study adds to the small body of literature that examines the applicability of eDNA tools when mussel abundance is low and reflective of a new introduction (Gingera et al. 2017; Hosler 2017).

Materials and methods

Study sites

Our focal study occurred at Tiber Reservoir (also known as Lake Elwell) in north central Montana (Figure 1). Tiber Dam, a US Bureau of Reclamation facility, impounds the Marias River and forms Tiber Reservoir. The Marias River is a tributary to the Missouri River. Present operation of the Tiber Dam includes flood control, irrigation, hydropower, municipal and industrial water supply, and recreation. There are nine roadside access points to the reservoir, six of which have concrete boat launches and one of which has a marina. At full pool, total storage capacity of the reservoir is 1,919,169,065 m^3 at an elevation of 920 m, surface area is 51 km^2, mean depth is 15 m, maximum depth is 43 m, and mean hydraulic retention time is 318 days. A 5-km long earthfill dike is adjacent to the dam embankment. The upstream faces of the dam and dike are covered by large boulder-riprap, as are the littoral zones adjacent to the nine access points. Substrate composition of Tiber Reservoir is poorly described. However, it is located within the Telegraph Creek Formation that is composed of gypsiferous, poorly cemented sandstone, and firm shale, so these rock types are likely to be common substrate. Tiber Reservoir has a popular sports fishery for walleye (*Sander vitreus* Mitchill, 1818) and northern pike (*Esox lucius* Linnaeus, 1758), which is primarily accessed by motorized watercraft.

In fall 2016, the US Bureau of Reclamation and Montana Fish Wildlife & Parks (MFWP) detected dreissenid veligers using cross-polarized light microscopy in multiple plankton samples collected in mid-July and mid-August from three sites in Tiber Reservoir (Figure 1, Table 1). One of these suspected veligers was PCR-confirmed as a quagga mussel by the U.S. Bureau of Reclamation lab in Denver, CO following methods described in Carmon et al. (2014). Upon learning of this detection, Montana agencies resampled the reservoir in October and November 2016 with multiple surveillance tools including plankton tows, visual shoreline and structure surveys, dive teams, and scientific research canines. No mussels were detected, however scientific research canines did alert at two sites (South Bootlegger and Turner's Point; Figure 1). Thus, Tiber Reservoir represents a great site for testing the efficacy of eDNA to detect a low abundance organism.

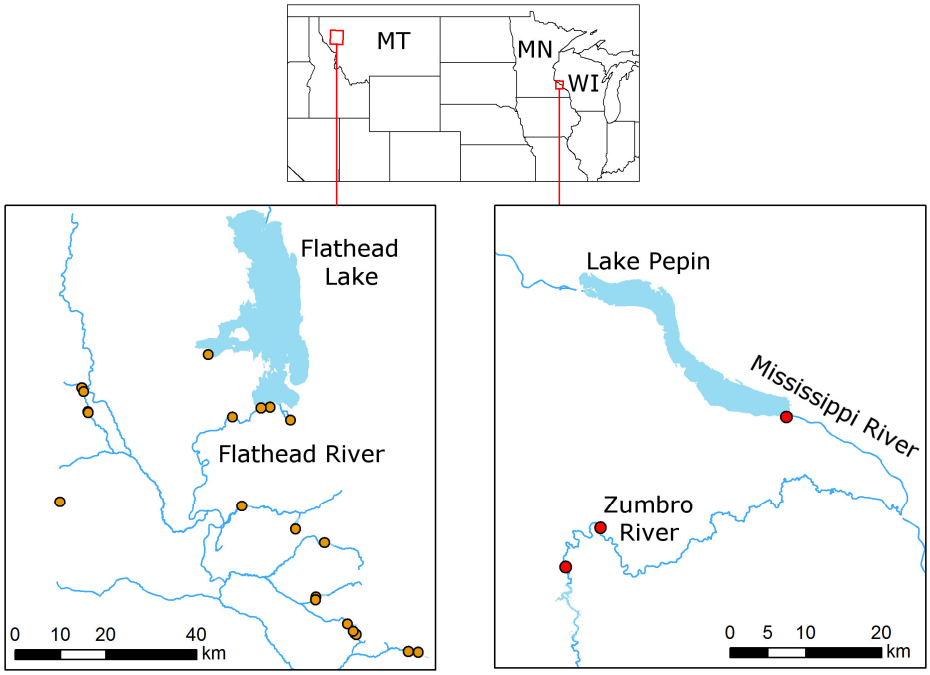

Figure 2. eDNA sampling sites on waters within or near the Flathead Reservation, Montana (MT) and along the Minnesota (MI)-Wisconsin (WI) border. Water samples scored as positive are indicated by filled red circles, while water samples scored as negative are indicated by filled orange circles.

Additional eDNA water samples were also collected from 14 water bodies on the Confederated Salish and Kootenai Tribes' (CSKT) Flathead Reservation in western Montana in August, September, and October 2017 and from three water bodies near the Minnesota-Wisconsin border in March 2018 shortly after ice-out (Figure 2, Supplementary material Table S1). The Flathead Reservation water bodies are headwaters of the Columbia River Basin, where no dreissenid mussels have been documented despite intensive monitoring, so serve as a potential means of testing for eDNA false-positives from non-target taxa. It is important to note that the Flathead Reservation waters and Tiber Reservoir are in different river basins so have different species assemblages. The Minnesota-Wisconsin water bodies have had established populations of zebra mussels since as early as the 1990s, so serve as positive field controls.

Sampling

Tiber Reservoir

Montana Fish Wildlife & Parks (MFWP) collected eDNA water samples at up to 12 sites in Tiber Reservoir in June, July, and October 2017 (Figure 1; Table S1). All sites were at recreation access points (i.e., highest use sites), and three of these sites (South Bootlegger, Willow Creek, VFW Campground) were collocated with 2016 plankton tow positive detections. In June and July, all eDNA samples were collected near-shore from a boat by MFWP that had only been used at Tiber Reservoir. Samples were collected from the

Table 2. For eDNA sampling sites on waters within or near the Flathead Reservation, Montana and along the Minnesota-Wisconsin border, the percent of positive eDNA water samples per month and, for just positive water samples, the proportion of positive PCR replicates for the DRE16S, and DPO1assays. The asterisk (*) indicates a sampling occasion when n = 6.

		% Positive samples (n = 3)		Proportion positive PCR replicates
Region	Site	Aug., Sep., Oct.	Mar.	
Flathead Reservation	Black	0, –, 0	–	–
	Crow	0, –, –	–	–
	Flathead Lake, Big Arm	0, –, 0	–	–
	Flathead Lake, Bourchard	0, –, 0	–	–
	Flathead Lake, Polson	0, –, 0	–	–
	Flathead River gage	0, –, 0	–	–
	Kicking Horse	0, –, –	–	–
	Lower Jocko	0, –, 0	–	–
	Lower Lone Pine	0, 0, –	–	–
	Lower Lone Pine gage	0, –, –	–	–
	McDonald	0, 0, 0	–	–
	Mission	0, 0, 0	–	–
	Mission gage	0, 0, 0	–	–
	Rainbow	0, 0, –	–	–
	St. Mary's	0, –, 0	–	–
	Turtle	0, –, 0	–	–
	Twin Lakes lower	0, 0, 0	–	–
	Twin Lakes upper	0, 0, 0	–	–
	Twin Lakes gage	0, 0, 0	–	–
	Upper Lone Pine	0, 0, –	–	–
	Upper Lone Pine gage	0, –, –	–	–
Minnesota-Wisconsin	Lake Pepin	–	100	12/12, 12/12
	Zumbro River	–	100	12/12, 12/12
	Zumbro River gage	–	100*	24/24, 24/24

stern while the boat was driven in reverse to minimize DNA contamination from the boat hull. In October, samples were collected by wading from shore since winds were too strong to safely navigate a boat. Surface water temperatures were 14–17 °C, 21–24 °C, and 9–11 °C when June, July, and October samples were collected, respectively.

At each site, three 3.79 L water eDNA samples were collected from the subsurface (~ 20 cm depth) in sterile plastic jugs (ULINE model no. S-16912). These jugs had not been used previously and each jug and cap were rinsed three times with water from the sample site immediately prior to collection. Filled jugs were placed on ice inside coolers and transported to an indoor space where samples were immediately filtered. Field blanks of deionized water were collected at each site and one travel blank of deionized water was placed in each cooler. Water samples were filtered through a 47 mm, 1.2 µm Whatman® glass-fiber filter (GE Healthcare) attached to a vacuum manifold using a peristaltic pump (Geotech Environmental Equipment Inc.). Filters were placed in individual sterile Whirl-Pak® bags (Nasco Corporation) filled with silica desiccant and shipped to the USGS Upper Midwest Environmental Science Center (UMESC) for analysis. Upon their receipt, samples were frozen at −20° C until DNA extraction.

MFWP also used plankton tow sampling (n = 131), artificial substrate sampling (n = 29), underwater inspections using divers and snorkelers (n = 2), shoreline surveys (n = 31), and scientific research canine surveys (n = 7) to monitor for dreissenids in 2017. Of the 131 plankton tow samples, three samples were collected in parallel with eDNA samples at each of the 10 sites in June and July. Plankton tow samples were transported to MFWP's Montana Aquatic Invasive Species Laboratory in Helena, MT, where trained technicians used cross polarized light microscopy to analyze samples for dreissenid veligers. Sampling methods for these non-molecular techniques are described in MFWP's 2017 annual AIS Monitoring Report (Schmidt and McLane 2018).

CSKT Flathead Reservation

Water samples were collected by a CSKT and USGS biologists using identical field methods as Tiber Reservoir. Three replicate water samples were collected at each site at all sites in August (n = 21) and at a subset of sites in September (n = 9) and October 2017 (n = 14; Figure 2, Table S1). For several of these waters, samples were also collected at a USGS or Flathead Reservation streamgage immediately downstream of the waterbody. Filtration and preservation methods were identical to those used in Tiber Reservoir.

Minnesota-Wisconsin border

In March 2018, USGS biologists collected water samples from two sites on the Zumbro River downstream of Lake Zumbro, Minnesota where zebra mussels were first found in 2000 (Figure 2, Table S1). One site was 3.3 km downstream, where three, 2 L water samples were collected from the river's bank, and the other site was 20 km downstream, where six, 2 L water samples were collected from the river's bank. USGS biologists also collected three, 2 L water samples from Lake Pepin, a large reservoir on the Mississippi River on the Minnesota-Wisconsin border where zebra mussels became established in the 1990s (Figure 2). At all sites, sampling occurred shortly after ice-out so water temps were approximately 4 °C and veligers were unlikely to be present. Filtration and preservation methods were identical to those used in Tiber Reservoir.

Molecular analyses

DNA was extracted from frozen filter samples using the Investigator Lyse & Spin Basket Kit (Qiagen) in concert with the gMAX Mini Genomic DNA Sample Kit (IBI Scientific) and eluted in 100 µl buffer. A laboratory negative control sample was prepared by processing 100 µl molecular grade water concurrently with the test samples. Purified DNA was used as template in up to three separate quantitative PCR (qPCR) reactions. First,

Table 3. Summary of the eDNA markers and their reaction profiles developed in this study for zebra mussel and quagga mussel using qPCR and the 16s rRNA and cytochrome oxidase *c* subunit 1 (COI) mitochondrial genes.

Target gene	Marker name	Amplicon size (bp)	Marker	Sequence (5' – 3')
16s rRNA	DRE16s	139	Forward	TGGGGCAGTAAGAAGAAAAAAATAA
			Reverse	CATCGAGGTCGCAAACCG
			Probe	6FAM-CCGTAGGGATAACAGC-MGBNFQ
			Profile	95°C 30sec/40 cycles of 95°C 5sec-60°C 15sec-72°C 10sec
COI	DRE2	116	Forward	TGGGCACGGGTTTTAGTGTT
			Reverse	CAAGCCCATGAGTGGTGACA
			Probe	6FAM-CGTCCTTGGTG-MGBNFQ
			Profile	95°C 2min/55 cycles of 95°C 1min-55°C 1min-72°C 30sec
COI	DREQM	104	Forward	CTCTTCATATCGGTGGAGCTTC
			Reverse	CAAAGGCACCCGATAAAACTG
			Probe	CCCGGCACGTATATTTCCTCATGTT
			Profile	95°C 30sec/40 cycles of 95°C 5sec-61°C 15sec-72°C 10sec

we screened purified DNA using the Dreissenid 16S rRNA-specific marker set ("DRE16S", Gingera et al. 2017), which is genus-specific so amplifies both quagga and zebra mussel DNA (Table 3). Inhibition was present in the majority of June samples from Tiber Reservoir. Consequently, we modified the DRE16S assay presented in Gingera et al. 2017 to include a mastermix (Quantabio PerfeCTa qPCR ToughMix) robust to inhibition and changed the thermal cycling protocol to be within the mastermix manufacture guidelines. The annealing temperature of 60 °C is the same as the original assay. This modified assay was tested on a panel of off-target species and maintained previous levels of sensitivity and specificity. Each qPCR reaction contained: 2X Quantabio PerfeCTa qPCR ToughMix, 500 nM forward and reverse primers, 250 nM probe, 2 µl eDNA sample template, and molecular grade water to a final volume of 25 µl. Marker sequences and thermal cycling profiles are described in Table 3. Each sample was tested in quadruplicate reactions.

For any samples that amplified with DRE16S, we then tested DNA with a zebra mussel COI-specific marker set ("DRE2" primers and "DPO1" probe, developed at USGS and described in Amberg et al. 2019), and for only October samples, a quagga mussel COI-specific marker set ("QMCOI" primer" and "DPO1" probe, developed at USGS; Table 3). The quagga mussel assay was developed and validated in fall 2017 by USGS UMESC. Sequences were designed using the PrimerQuest® program (IDT, Coralville, USA; http://www.idtdna.com/Scitools). Specificity was confirmed by Basic Local Alignment Search Tool (BLAST) search and alignment to a collection of 20 aligned quagga mussel and zebra mussel COI sequences using Geneious® 10.2.3 software (http://www.geneious.com). Optimal conditions were defined by testing a range of annealing temperatures (55–61 °C) against genomic DNA from three quagga and three zebra mussel specimens as well as a panel of genomic DNA from 15 off-target species (Table S2). We were not able to test June and July samples with this assay because DNA extract from these samples had been shared with partners and fully consumed.

Each DRE2 qPCR reaction and each QMCOI qPCR reaction contained: 2X Quantabio PerfeCTa qPCR ToughMix, 500 nM forward and reverse primers, 250 nM probe, 2 µl eDNA sample template, and molecular grade water to a final volume of 25 µl. Each sample was tested in quadruplicate reactions. Marker sequences and thermal cycling profiles are described in Table 3. Each sample was tested in quadruplicate reactions.

For all three assays, the limit of quantitation (LOQ) was 10 copies per reaction and the limit of detection (LOD) was 1 copy per reaction. Standard curves were prepared from gBlocks® synthetic DNA positive control material in a 10-fold dilution series ranging from 10,000 copies/µl to 10 copies/µl. Each sample was tested in quadruplicate reactions. Triplicate reactions spiked with 100 copies of gBlocks® positive control DNA were run in parallel to measure qPCR inhibition. A qPCR no template control (NTC) reaction was also set up for each sample on each plate (total = 10 NTC reactions per 96 well plate). For all plates, the qPCR efficiency was between 90–110% and the R^2 values were ≥ 0.98. Inhibited samples were first cleaned up with post-extraction spin-column purification according to the manufacturer's instructions (OneStep™ PCR Inhibitor Removal Kit, Zymo Research, Irvine, CA) and then rerun in quadruplicate.

We used a conservative approach to score samples as positive for dreissenid mussel DNA. First, any sample with a technical replicate that had a level of amplification greater than background was further evaluated in octet reactions to verify amplification using the original marker. If any of these replicates amplified, their product was then sequence verified and rerun using a second marker in a different region of the organism's mitochondrial genome to verify presence of the mitochondrial DNA. The second marker was analyzed in quadruplicate and if amplification was observed, it too was sequenced for verification. Only when two markers detected the presence of dreissenid DNA and the amplified product was verified as a dreissenid mussel was that sample scored as a positive for dreissenid DNA. All sequencing was done at the USFWS Whitney Genetics Lab on an Applied BioSystems 3500 Genetic Analyzer.

Finally, purified DNA from a subset of positive and negative samples from the June and July sampling events were sent to Pisces Molecular (Boulder, CO) for molecular analysis. Submitted samples included two samples from a Minnesota lake with documented zebra mussels, two samples from a Minnesota lake with no documented zebra mussels, eight samples from Tiber Reservoir in July that amplified for zebra mussels, and two samples from Tiber Reservoir in July that did not amplify for dreissenids. Pisces Molecular used a proprietary qPCR assay in a multiplex reaction that targets the ITS1 region with a single primer, inclusive of both zebra and quagga mussels, and two individual species-specific hydrolysis probes with different fluorescent dyes. They tested samples in triplicate

reactions; samples with 2 out of 3 replicates positive were scored as positive. Samples with only 1 out of 3 replicates positive were repeated in a second qPCR run, again in triplicate replicates. If any of these second qPCR replicates were positive, then the sample was scored as positive.

Statistical analyses

To provide insight on the effort needed for high probability detection of dreissenid DNA, we used the eDNAoccupancy R package to model probabilities of eDNA detection in Tiber Reservoir (Dorazio and Erickson 2017). This package fits Bayesian, multi-scale occupancy models. Our data included three, nested levels of sampling: primary sample units within Tiber Reservoir (i.e., a site), secondary sample units (i.e., a water sample) collected from each primary unit, and subsamples (i.e., PCR technical replicates) of each secondary sample unit. The focus of our analysis was to estimate θ_i (conditional probability of occurrence of dreissenid eDNA in each sample of location *i,* given that dreissenid eDNA was present at that location) for each sampling event based only on DRE16S assay results, since all water samples were tested using this assay. Probability of occurrence of dreissenid eDNA among all surveyed locations (ψ) and the conditional probability of detection of dreissenid eDNA in each qPCR replicate of an eDNA sample given that dreissenid eDNA was present in the sample (p) were modeled as constant. Models for each sampling event (June, July, and October) were run separately. Model estimates and standard errors were computed using a Markov chain containing 11,000 iterations (1000 burn-in).

Following Hunter et al. (2015), we then used the derived estimates of θ_i to make inferences about $\theta^* = 1 - (1 - \bar{\theta})^n$, which denotes the cumulative probability of occurrence of dreissenid eDNA (i.e., samples that were scored positive for either quagga or zebra mussel DNA) in n samples taken from a location that contained dreissenid eDNA. We computed θ^* for a sequence of samples sizes (n = 1, 2, ….) using an estimate of $\bar{\theta} = (\sum_{i=1}^{m} z_i \theta_i) / \sum_{i=1}^{m} z_i)$, the average conditional probability of occurrence of dreissenid eDNA in a single sample at location i averaged over all survey locations where dreissenid eDNA was actually present.

Results

Tiber Reservoir

Dreissenid mussel DNA was detected in June, July, and October samples in the eastern portion of Tiber Reservoir (Figure 1). One water sample was scored as positive in June for quagga mussel DNA and in October for quagga and zebra mussel DNA, while 15 water samples were scored as positive in July for quagga and zebra mussel DNA (Table 1). No dreissenids were documented with non-molecular survey tools in 2017,

including plankton tow samples that were collocated with eDNA samples in June and July.

Positive DNA samples occurred at multiple sites across time. The positive June sample was from Turner Point, near the southcentral shore, while the positive October sample was from Willow Creek Arm, near the northeastern shore. In July, dreissenid DNA was detected at eight of 10 sampled sites throughout the eastern half of Tiber Reservoir (Table 1). We detected dreissenid DNA in 2017 at two of the three sites (Willow Creek Arm and VFW Campground) where dreissenid veligers were detected in 2016 and at one of the two sites (Turner Point) where scientific research canines alerted in 2016 (Table 1).

In general, positive June and October DNA samples had fewer PCR replicates amplify than positive July samples (Table 1). The single positive sample in June had only one PCR replicate that amplified in the first round of quadruplicate reactions with the DRE16S marker, but amplification also occurred in additional rounds of octet reactions using this marker. Amplicons were sequence verified as quagga mussel DNA. In July, 12 of the 15 positive samples had greater than two PCR replicates amplify. Importantly, eight of these samples amplified in DRE16S and in DRE2 reactions. Amplicons were sequenced, and DNA was verified as quagga mussel and zebra mussel. The single positive October sample had two PCR replicates that amplified in the first round with the DRE16S marker, but amplification also occurred in additional rounds of quadruplicate reactions using this marker and the QMCOI marker. Amplicons were sequenced and verified as quagga mussel and zebra mussel DNA.

One field control in July had one technical replicate that amplified with the DRE16S marker though the estimated copy number was much lower (11 copies per reaction) than the mean for positive field samples (952 copies per reaction). A rerun of this positive field control in octet was negative for all reactions. All other field, lab and extraction controls did not amplify dreissenid DNA so were scored as negative.

Pisces Molecular corroborated all USGS positive results, including the July field control. In addition, Pisces Molecular scored a Tiber sample as positive (2 of 6 replicates amplified) for zebra mussel DNA that USGS had scored as negative.

Estimates of median θ_i (± 95% CI) for the single sites in June and October where DNA was detected was 0.58(0.11–0.98) and 0.47(0.09–0.94), respectively. Median θ_i in July at the nine sites where DNA was detected ranged from 0.39(0.07–0.80)–0.84(0.39–0.99). The 95% credibility intervals of the cumulative probability of eDNA occurrence (θ^*) in at least one sample was 4–27 samples in June, 4–14 samples in July, and 5–34 samples in October (Figure 3).

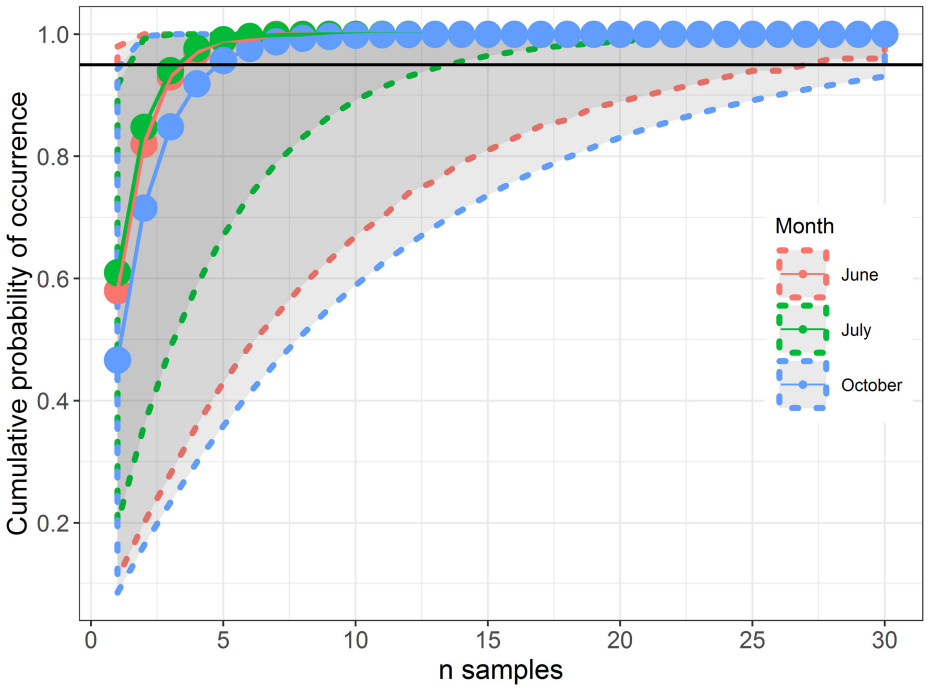

Figure 3. Cumulative probability of occurrence (colored, solid lines) of dreissenid eDNA in *n* samples from Tiber Reservoir, MT in June, July and October 2017. Gray bands outlined with colored, dotted lines display 95% confidence interval for each month. Black, solid line is for reference and indicates a cumulative probability of occurrence = 0.95.

Other sites

All water samples collected from CSKT's Flathead Reservation were negative for dreissenid mussel DNA (Figure 2, Table 2). All water samples collected from Minnesota-Wisconsin sites were positive for dreissenid mussel eDNA and all PCR replicates amplified for the DRE16S and DPO1 markers (Figure 2, Table 2). These results verify that our field and analytical methods do detect dreissenid mussel DNA when mussels are present and that eDNA can detect dreissenid DNA in seasons when veligers are not present.

Discussion

We found that eDNA extended the seasonal window for dreissenid mussel surveillance in northern-latitude waters beyond that of plankton tow samples. In Tiber Reservoir, we detected dreissenid mussel DNA in July, when water temperatures were conducive to dreissenid spawning, but also in June and October, when water temperatures were at or below the thermal minima for dreissenid spawning. Positive June, July, and October eDNA results amplified at multiple markers associated with different regions of the mitochondrial genome and were sequence-verified. In contrast to our eDNA results, plankton tow sampling and other non-molecular sampling techniques did not detect dreissenid mussels in Tiber Reservoir in 2017, underscoring that dreissenid mussels are likely to be at low abundance. We also detected dreissenid mussel DNA in Minnesota-

Wisconsin sites in March, days after ice-out when water temperatures were below the thermal minimum for spawning. This study adds to a growing body of research that demonstrates eDNA is a highly sensitive tool for dreissenid mussel surveillance in newly invaded waters and in seasons when veligers are likely to be rare.

Positive eDNA results can be difficult to corroborate with non-molecular methods since eDNA is often a more sensitive technique (Darling and Mahon 2011; Hosler 2017). When these two methods result in conflicting answers, such as in this study at Tiber Reservoir, decision-making is challenging since eDNA approaches only detect DNA, regardless of the presence or state of the target taxa. Thus, we cannot eliminate the possibility that the eDNA origin in Tiber Reservoir was from a failed introduction, from external sources (e.g., contaminated boat hulls), or from field contamination, rather than fresh DNA from mussel colonization. However, multiple lines of evidence discount, but do not eliminate, support for these alternative explanations. First, we documented multiple positive eDNA detections of both zebra and quagga mussel DNA across time and space. This fluctuating pattern in time and space is contrary to the general trend of eDNA exponential decay after the DNA source has been removed, although exceptions to this trend have been noted (Barnes et al. 2014). The observed temporal pattern, especially the peak in the number of positive detections in July, is consistent with expectations based on target species biology (i.e., spawning patterns and the likelihood of mussels releasing DNA into the water) and frequency of summer watercraft use in Montana that peaks in July (Biggs et al. 2017). Second, dreissenid DNA from 13 samples was amplified using multiple markers associated with different regions of the mitochondrial genome. The use of multiple markers provides redundancy against the stochastic process of DNA degradation (Farrington et al. 2015). Moreover, multiple samples each positive at multiple markers suggests detected DNA has undergone minimal degradation and likely to be from a fresh source. Third, a subset of positive detections was confirmed by an independent lab using different markers, indicating that results were reproducible (Darling and Mahon 2011). Fourth, the source of DNA from the positive field control was likely Tiber Reservoir since field sampling gear had never been used at any other waterbody, and the closest known population of dreissenids is > 900 km away. No other field or laboratory controls were positive. Finally, samples from Minnesota-Wisconsin and Flathead Reservation sites amplified as expected, indicating that our assays detected dreissenid mussels when present and did not amplify non-target DNA.

Our use of a multi-phase approach (i.e., targeting multiple locations on the mitochondrial genome, sequence confirmation, independent lab verification) for reporting the presence of dreissenid DNA in samples provided important insight on steps of the eDNA workflow that warrant

further consideration. Using this workflow, we documented an October sample that amplified for dreissenid mussel DNA (DRE16S) and quagga mussel DNA (QMCOI) but not zebra mussel DNA (DRE2). However, sequences from these positive DRE16S and QMCOI amplicons had a 100% match with both quagga and zebra mussels. We also documented an additional sample that was scored as positive for zebra mussel DNA by the external lab (Pisces Molecular) that USGS had scored as negative. These results indicate that the sensitivity of the zebra mussel marker used by the USGS could be improved to minimize potential for false-negatives. Additionally, these results underscore the limitations of using sequencing to differentiate sister taxa. eDNA markers are typically short (e.g., 100–200 bp), and mismatches are focused on the 3' end rather than the center (Wilcox et al. 2013). Thus, the inability to sequence 20–40 bp at the ends can have can have a significant impact on differentiating sister taxa. Given these constraints, the most supported interpretation of our results is water samples positive for dreissenid mussel DNA rather than zebra or quagga mussel DNA.

Dreissenid mussel eDNA detections in Tiber Reservoir did vary by season, but comparable variation also occurred in eDNA sampling by Gingera et al. (2017) and is in line with dreissenid natural history and seasonal hydrography. Gingera et al. (2017) sampled sites with documented zebra mussels in Lake Winnipeg (Manitoba, Canada) and found one of four sites positive for zebra mussel DNA in May and four of four sites positive in October. The lower detection rate in May was hypothesized to be due to lower abundance of zebra mussels due to winter die-off, when reduced water levels and freezing temperatures in winter can result in the death of zebra mussels. Zebra mussels are likely more vulnerable to winter mortality than quagga mussels since zebra mussels most often occur in the littoral zone (Dermott and Munawar 1993). Lower detections rates could also be due to dilution since Lake Winnipeg water volume is at near-peak in May (Gingera et al. 2017). The seasonal variability in eDNA detections in Tiber Reservoir, where we detected quagga mussel DNA all months tested, but zebra mussel DNA only in July and October and where DNA detection probability was very low in June, are in line with Gingera et al.'s (2017) hypotheses. Tiber Reservoir is drawn down each winter prior to spring-runoff, so littoral substrate is exposed to freezing temperatures. Pertinent to this study, elevation at the dam forebay was reduced by ~ 2 m by December 2016 relative to summer levels, but then increased by ~ 3 m to a peak water volume by June 2017 (www.usbr.gov; Station ID: LER). In 2017 summer, water levels were rapidly drawn down such that October water levels were ~ 3 m lower than July. Understanding how fluctuating water levels affect eDNA detection probability and dreissenid mussel colonization is an important next step for mussel monitoring and control.

A unique contribution of this work is that we used multi-scale occupancy modeling to provide estimates of the eDNA sampling intensity

required to detect dreissenid DNA in Tiber Reservoir, a relatively large waterbody where dreissenid mussels are new invaders and, if present in 2017, likely rare. Conditional that DNA is present at a site, we estimated that as many as 34 samples are required to detect DNA with very high confidence in June and October, while up to 14 samples are required in July (Figure 3). Assuming that live dreissenid mussels were present, the projected effort required for 95% detection confidence with eDNA seems much lower than that expected for traditional sampling, given the failure of the latter to detect targets in 2017. While the results of these analyses are not transferable to other waters, our results do provide an initial baseline for managers to reference and, in general, suggest that sampling efficiencies are greatest in the mid-summer but that considerable eDNA sampling intensity is still required. More research on the covariates associated with DNA detection probabilities is needed to provide meaningful guidelines for eDNA surveillance within and across waterbodies. For managers challenged with surveillance of large waterbodies and with limited budgets, this research is urgently needed since optimal allocation of effort is critical.

Evidence is now growing that eDNA detections often precede observations of dreissenid mussel colonization. Gingera et al. (2017) had positive eDNA samples from the Red River upstream of Lake Winnipeg, where mussels were documented the following year. Similarly, Hosler et al. (2017) documented multiple examples in the West where water samples were positive for eDNA prior to positive plankton tow samples. Nevertheless, there also exist multiple studies where positive eDNA detections where never corroborated with observation, such as Dunker et al. (2016) where eDNA was used to monitor for northern pike after a piscicide eradication effort. Fear of false-positives has stymied the use of eDNA as a decision-making tool for politically contentious invasive species. Rightfully so, managers do not want to mount costly and unnecessary control efforts for invasive species that are not present. However, false-negatives are likely to be more costly than false-positives (Leung et al. 2002). Decision-support tools that incorporate the socioeconomic and ecological costs of invasive species relative to the costs of potential management actions are needed to provide guidance on how to best incorporate eDNA results into decision making.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Geospatial locations for all sites where eDNA water samples were collected.
Table S2. Off-target species whose genomic DNA was used to conduct *in vitro* validation of the QMCOI marker.

This material is available as part of online article from:
http://www.reabic.net/journals/mbi/2019/Supplements/MBI_2019_Sepulveda_etal_SupplementaryMaterial.xlsx