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SURVEYS FOR RARE AND ENDANGERED MUSSEL SPECIES IN KANSAS: TRADITIONAL METHODS AND ENVIRONMENTAL DNA ANALYSIS

Being

A Thesis Presented to the Graduate Faculty

of the Fort Hays State University in

Partial Fulfillment of the Requirements for

the Degree of Master of Science

by

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The Master of Science Degree

By

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ABSTRACT

Chapter 1.

The Mucket (*Actinonaias ligamentina*) is one of the rarest freshwater mussel species in the state of Kansas. It historically occurred in the Kansas and Osage River systems in the state. The Mucket has been extirpated from most streams and rivers in Kansas where it historically occurred, and few specimens have been documented in recent years. The objective of my project was to determine the conservation status of the Mucket by conducting qualitative surveys in the Osage River system of eastern Kansas. My goals were to collect information that will aid in the recovery of the Mucket and to provide information regarding the mussel communities in these streams. During the summer of 2016, I surveyed 14 sites on the Marais des Cygnes River, 5 sites on Pottawatomie Creek, and 4 sites on the Marmaton River. I collected one live Mucket on Pottawatomie Creek. During the course of the survey, I collected 2,993 live mussels representing 19 species.

Chapter 2.

The Cylindrical Papershell, *Anodontoides ferussacianus,* is listed as a Kansas species in need of conservation (SINC). It only persists in short segments of the Saline and Smoky Hill Rivers. Surveys for the Cylindrical Papershell were conducted in the summers of 2011 and 2015 by Fort Hays State University graduate students with funding from the Kansas Department of Wildlife, Parks and Tourism Chickadee Checkoff Program. I supplemented these surveys by using environmental DNA (eDNA) analysis. Water samples were collected and used to detect the presence of the Cylindrical

Papershell. Species detection using eDNA is an emerging field in aquatic ecology. The primary applications for this sampling technique are early detection of nonnative species and the detection of rare or threatened species. To successfully conserve the Cylindrical Papershell, we must know its current range. Collecting presence and absence data for rare and threatened species is difficult, and is often an expensive and time consuming task. Being able to consistently detect the presence of the Cylindrical Papershell by extracting its DNA from water samples would help determine where to allocate resources to conserve the species. My project refined methods for eDNA analysis and assessed the effectiveness of using eDNA to detect freshwater mussels.

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I thank the Chickadee Checkoff Grant Program of the Kansas Department of Wildlife, Parks and Tourism (KDWPT). I thank Trista Gasper of Fort Hays State University (FHSU) Department of Biological Sciences for her help securing transportation for the project and ordering lab materials. I thank Mark Eberle for all of his help with revisions and edits.

I thank all the landowners for their hospitality in allowing us to conduct mussel surveys on their property. I thank FHSU students Kasandra Brown, Ernesto Flores, Vanessa Salazar, and Ariel Snyder for assisting with field work. This project would not have been possible without them.

"You must refuse to accept the common delusion that a career is an adequate context for a life. The logic of success insinuates that self-enlargement is your only responsibility, and that any job, any career will be satisfying if you succeed in it. But I can tell you, on the authority of much evidence, that a lot of people highly successful by that logic are painfully dissatisfied. I can tell you further that you cannot live in a career, and that satisfaction can come only from your life. To give satisfaction, your life will have to be lived in a family, a neighborhood, a community, an ecosystem, a watershed, a place, meeting your responsibilities to all those things to which you belong." – Wendell Berry

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PREFACE

This thesis is written in the style of the Fort Hays State University Graduate School.

INTRODUCTION

Native freshwater mussels (Bivalvia: Unionidae) are an imperiled taxonomic group of great ecological importance. Seventy-two percent of the 297 recognized taxa in North America are considered endangered, threatened, or of special concern (Williams et al. 1993). The status of freshwater mussels in Kansas is equally concerning. Of 48 species originally documented in the state, 6 are now extirpated, 1 lacks reproductively viable populations, and 38 have reduced ranges or declining populations (Angelo et al. 2009). Kansas mussel populations are noteworthy from the perspective of conservation because 40 species reach the western limits of their distributions in the state (Angelo et al. 2009). These peripheral populations have less gene flow than populations near the center of a species' distribution and are often genetically distinct (Lesica and Allendorf 1995). Recent surveys conducted in Kansas revealed a statewide decline in mussel populations (Angelo et al. 2009). As a result, the Kansas Department of Wildlife, Parks and Tourism (KDWPT) has designated 23 species of mussels as threatened, endangered, or in need of conservation (SINC) (Angelo et al. 2009). The main drivers of freshwater mussel decline are habitat destruction from dams, channel modification, and siltation, and the introduction of invasive mussel species (Williams et al. 1993).

Kansas freshwater mussels are ecologically important because they provide valuable ecosystem functions and are good indicators of aquatic ecosystem health (Williams et al. 1993; Angelo et al. 2009). The ecosystem functions they provide include enhancing water clarity, transferring nutrients from the water column to the substrate, and aerating the sediment layer (Angelo et al. 2009). Freshwater mussels are a food source for

predatory fish and wildlife species, and discarded mussel shells provide attachment sites and shelter for eggs and other aquatic invertebrates (Angelo et al. 2009). Dense mussel beds stabilize stream substrates during high flow events (Angelo et al. 2009). Freshwater mussels also serve as indicators of disturbances in the local environment because they are sensitive to changes in water and sediment quality, physical habitat conditions, and fish community composition (Williams et al. 1993). Abrupt declines in mussel populations can alert biologists to deleterious environmental changes and can assist them in properly diagnosing the underlying issue causing the decline (Angelo et al. 2009).

CHAPTER 1

SURVEY OF FRESHWATER MUSSEL POPULATIONS IN THE UPPER OSAGE RIVER SYSTEM IN KANSAS WITH EMPHASIS ON THE STATUS OF THE MUCKET (*Actinonaias ligamentina*)

INTRODUCTION

The Mucket (*Actinonaias ligamentina*) occurs throughout much of the east-central United States. The distribution of the species extends from Minnesota and portions of southern Canada, east to the Appalachian Mountains, south into Mississippi, and west into eastern Kansas (Figure 1). According to NatureServe (http://www.natureserve.org/), the conservation status of the Mucket is stable over much of its range; however, it is critically imperiled in Kansas, Mississippi, and New York, and it has likely been extirpated in Louisiana. The species inhabits medium to large rivers, usually in riffles less than 1 m in depth with coarse sand and gravel substrates (Parmalee and Bogan 1998; Obermeyer 2002). In Minnesota, the Mucket was reported to be widely distributed but occurring in low abundances (Dawley 1947).

The Mucket is a bradytictic species, meaning they spawn in summer, and fertilized eggs overwinter in the females before being released in spring. Females brood the eggs until June, when they are released as larval mussels called glochidia (Moles and Layzer 2008). The Mucket does not have a mantle flap lure, and Barnhart et al. (2008) suggested it has been secondarily lost. The species now deploys a fragile conglutinate or releases glochidia directly into the water (Barnhart et al. 2008). Glochidia attach to the gills of host fish and develop into juvenile mussels within a few days to two weeks, when

they detach and settle onto the streambed (Parmalee and Bogan 1998; Miller 1999).

Possible host fish in Kansas include Largemouth Bass (*Micropterus salmoides*), Smallmouth Bass (*M. dolomieu*), Bluegill (*Lepomis macrochirus*), Green Sunfish (*L. cyanellus*), Orangespotted Sunfish (*L. humilis*), White Bass (*Morone chrysops*), Black Crappie (*Pomoxis nigromaculatus*), White Crappie (*P. annularis*), Sauger (*Sander canadensis*), and Tadpole Madtom (*Noturus gyrinus*) (Miller 1999; Miller et al. 2008).

The exterior valve of the Mucket is smooth, nondescript, and oval to quadrate in shape. The shell is moderately thick to thick, depending on the age of the individual. The pseudocardinal teeth are large and erect. The exterior of the shell is yellowish brown and occasionally accented with thin, green rays (Miller 1999; Miller et al. 2008). The umbo has a sculpture of many fine concentric lines (McMurray et al. 2012). Maximum shell length is 178 mm (Obermeyer 2002).

The Mucket is one of the rarest freshwater mussel species in Kansas. It historically occurred in the Kansas and Osage river systems in the state, but it has now been extirpated from most streams and rivers where it historically occurred, and few live specimens have been documented in recent years (Obermeyer 2002).

In 1994, the Kansas Department of Health and Environment collected two unweathered valves from Pottawatomie Creek near Lane, Kansas (Obermeyer et al. 2000; Obermeyer 2002). In 1996, Bob Angelo observed a live Mucket and one freshly dead specimen from a gravel riffle in the Marais des Cygnes River, near the confluence of 8 Mile Creek near Ottawa, Kansas (Miller 1997). On 8 August 1997, Karen Couch and Brian Obermeyer collected one live and one freshly dead Mucket in the Marais des

Cygnes River in Miami County, Kansas (T18, R21, Sec. 1). They reported that both specimens appeared to be old (Miller 1998). In 1999, slightly weathered valves were collected from a gravel bar in the Marmaton River approximately 5.6 km west of Fort Scott, Kansas (Obermeyer et al. 2000; Obermeyer 2002). On 21 August 2002, Ed Miller and others reported a live Mucket from the Marais des Cygnes River within Marais des Cygnes National Wildlife Refuge in Linn County, Kansas (38.22368°, -94.62962°) (Couch and Obermeyer 2004). In 2003, Megan Bradburn (2009) reported two Muckets collected during timed searches in the Marais des Cygnes River within the national wildlife refuge. Curtis Wolf quantitatively surveyed 10 sites on the Marais des Cygnes River during the summers of 2003 and 2004. He collected 2,173 mussels but no live Muckets (Wolf and Stark 2008). In 2006, Megan Bradburn (2009) collected two live Muckets in the Marais des Cygnes River within the national wildlife refuge.

In August of 2000, during a period of extreme drought, the Kansas City Power and Light Company at the La Cygnes power plant withdrew water from the Marais des Cygnes River at a rate 35-50 cubic feet per second (cfs); the river was only flowing at 60 cfs (Obermeyer et al. 2001; Bradburn 2009). Additional withdrawals from a downstream water district resulted in flows less than 5 cfs in the river at the Marais des Cygnes National Wildlife Refuge (Obermeyer et al. 2001). Consequently, an estimated 2,000 freshwater mussels were stranded and perished (Bradburn 2009). Bradburn (2009), reported that the Mucket was one of the species stranded during the event, but there was no record of how many Muckets perished.

Critical Habitat for the Mucket (*Actinonaias ligamentina*) in Kansas

(As listed in the recovery plan for freshwater mussels in the Upper Osage River System, Obermeyer 2002)

- Marais des Cygnes River: from the confluence of Hundred and Ten Mile Creek (Osage-Franklin County border) to the Kansas-Missouri border (Linn County).
- Pottawatomie Creek: from the confluence of the South Fork of the Pottawatomie Creek (Anderson County) to the confluence of the Marais des Cygnes River (Miami County).
- Marmaton River: from the confluence of Paint Creek to the City of Fort Scott (Bourbon County).

In the Kansas Comprehensive Wildlife Conservation Plan, the Mucket has been listed as a species of greatest conservation concern, and it was listed as a state endangered species in 1999 (Obermeyer 2002; Wasson et al. 2005).

The objective of this project was to determine the conservation status of the Mucket by 1) conducting qualitative surveys in the Osage River system of eastern Kansas, 2) describing the habitat occupied by the Mucket, and 3) documenting recruitment by using length-frequency distributions. The goal was to collect information that could aid in the recovery of the Mucket in Kansas.

METHODS

Study Area

In Kansas, extant populations of *A. ligamentina* are restricted to the Osage River system—specifically the Marais des Cygnes River, Pottawatomie Creek, and potentially the Marmaton River (Obermeyer 2002). The Osage River system is in the Central Irregular Plains ecoregion of Kansas and Missouri. Historically, the region consisted of prairie habitats with forested riparian areas. A relatively small portion of the grassland habitat has been converted into row-crop agriculture, and the forested riparian areas have been reduced in width (Obermeyer 2002; Bradburn 2009). For example, 171,251 acres (51%) of Franklin County are cropland and 143,099 acres (42%) are pasture and rangeland (Wolf and Stark 2008).

The largest stream in the Osage River system is the Marais des Cygnes River. It is 241 river kilometers long, drains an area of 8,474 km², and is a 6th order stream when it exits the state (Obermeyer 2002; Wolf and Stark 2008). Melvern, Pomona, and Hillsdale reservoirs are federal impoundments that influence the flow of the Marais des Cygnes River. Melvern Lake is the only one located on the mainstem (Wolf and Stark 2008). There are many low-head dams on the Marais des Cygnes River (Figure 3). These impoundments alter the geomorphology, hydrology, water chemistry, and biological communities of lotic environments, and they prevent the upstream dispersal of host fish during times of low flows (Dean et al. 2002). Wolf and Stark (2008) reported that the low-head dam west of Ottawa and the one at Osawatomie each impounded the river approximately 10 km upstream.

In Missouri, 82% of the Marais des Cygnes River has been channelized or impounded. All that remains of the original river channel is a 14.5-km section upstream from Truman Reservoir, Missouri (Obermeyer 2002; Bradburn 2009). Due to the large-scale habitat destruction in Missouri, any effort to conserve the freshwater mussels in the Marais des Cygnes River must occur in Kansas (Obermeyer 2002).

Pottawatomie Creek has a drainage area of 1,400 km². It enters the Marais des Cygnes River near Osawatomie as a 5th order stream (Obermeyer 2002). There is a low-head dam present near the town of Lane, Franklin County.

The Marmaton River has a drainage area of 1,120 km² and exits the state in Bourbon County as a 4th order stream. During periods of low flow, all water in the Marmaton River comes from the outflow of Fort Scott's wastewater treatment facility (Obermeyer 2002).

During the summer of 2016, I surveyed mussels in the Marais des Cygnes River, Pottawatomie Creek, and Marmaton River. Sample locations were selected within stream reaches designated as critical habitat in the recovery plan (Obermeyer 2002). I used Google Earth to identify locations with riffle habitat and mobile version 3.4.1 of ONX Hunt Maps to collect landowner information.

Qualitative Protocol

Qualitative surveys were conducted by using timed searches. Mussels were collected by feeling through the substrate and detecting individuals by hand. All mussels collected were kept in mesh bags until the end of the timed search. All mussels were identified, counted, and measured with calipers to the nearest millimeter. Length, height, and width were measured to use in estimating body size and age distribution within the

mussel communities. After data collection, all live mussels were returned to the stream by placing the anterior end of the valve into the substrate. The live Mucket was tagged with a numbered polyethylene tag attached with Permatex super glue to each valve posterior to the umbo.

After the qualitative survey, sandbars and the banks of the river were searched for dead valves. Species represented by dead valves but absent from the timed searches were recorded and all potential Mucket valves were collected as vouchers. The relative abundance as catch-per-person-hour (catch per unit of effort, CPUE) and species richness were calculated for each site. Simpson's index of diversity was calculated for each stream.

RESULTS

From July 2016 to September 2016, 23 sites were qualitatively surveyed among the three streams (Appendix 1, Figure 2). Fourteen sites were surveyed on the Marais des Cygnes River, 5 sites on Pottawatomie Creek, and 4 sites on the Marmaton River.

Marais des Cygnes River

In the Marais des Cygnes River, 2,149 live mussels were collected among 16 species, with a calculated Simpson's diversity index of 0.83. The total person hours for this river were 48.74 and the mean CPUE was 44.1. No live Mucket was collected, but weathered and relic valves were collected at sites MDC-2, MDC-3, MDC-7, MDC-8, MDC-10, and MDC-13 (Table 1). At site MDC-6, a slightly weathered valve of a Rock Pocketbook (*Arcidens confragosus*) (state threatened) and two weathered valves of a Black Sandshell (*Ligumia recta*) (extirpated in the state) were collected. A live Butterfly (*Ellipsaria lineolata*) (state threatened) was collected in a long gravel bar at site MDC-12. It was 72 mm long, 56 mm high, and 38 mm wide. Four live Fawnsfoot (*Truncilla donaciformis*) (SINC) were collected at 3 sites: MDC-4, MDC-7, and MDC-8.

The Pistolgrip (*Tritogonia verrucosa*), Pimpleback (*Quadrula pustulosa*), Wabash Pigtoe (*Fusconaia flava*), and Threeridge (*Amblema plicata*) were the most frequently observed species and accounted for 26.5%, 19.92%, 17.96%, and 14.6% of all live mussels collected, respectively. The largest mussel bed surveyed was approximately 150 m downstream from a low-head dam at site MDC-2. I observed 792 mussels among 12 species, with a CPUE of 125.1 (Table 1).

The Mucket, Rock Pocketbook, Black Sandshell, Pondmussel (*Ligumia subrostrata*), and Giant Floater (*Pyganodon grandis*) were represented only by dead valves. The nonnative Zebra Mussel (*Dreissena polymorpha*) was encountered at 8 of 14 sites on the Marais des Cygnes River, always in low abundance (Table 1).

Pottawatomie Creek

In Pottawatomie Creek, 226 live mussels among 13 species were collected, with a calculated Simpson's diversity index of 0.75. The total person hours were 16.5 with a mean CPUE of 13.7 (Table 2). The only live Mucket collected during this survey was in Pottawatomie Creek at site P-1, in the thalweg near the end of a long riffle. The substrate was large gravel/cobble and the water depth was less than 30 cm (Figures 4, 5). It was 92.6 mm long, 55.98 mm high, and 30.12 mm wide. I tagged it with number A179 and placed it back in the stream where it was collected (38.48416°, -94.99980°).

Eighty-six percent of the mussels collected in Pottawatomie Creek were from site P-1. A total of 194 live mussels were collected at the site; the second highest total was at site P-4, with 12 live mussels. The most frequently observed species were Threeridge, Pistolgrip, and Mapleleaf (*Quadrula quadrula*), which represented 39.8%, 27.4%, and 11.0% of all live mussels collected, respectively. The Plain Pocketbook (*Lampsilis cardium*) and Spike (*Elliptio dilatata*) were represented only by dead valves. The Zebra Mussel was not observed on Pottawatomie Creek (Table 2).

Marmaton River

In the Marmaton River, 4 sites were surveyed and 558 live mussels were collected. A species richness of 13 and a Simpson's diversity index of 0.75 were recorded. The total person hours were 8.5 and the mean CPUE was 65.6 mussels. No live

Mucket was collected (Table 3). Sixteen Spikes (SINC) were collected at site M-3 and another 16 at site M-4. They ranged in length from 46-135 mm, indicating healthy recruitment. At site M-4, a live Plain Pocketbook was collected. It was 79 mm long, 59 mm high, and 39 mm wide. Six Creepers (*Strophitus undulatus*) (SINC), were observed at site M-3. They ranged in length from 67 to 91 mm.

The most frequently observed species in the Marmaton River were Threeridge, Mapleleaf, Wabash Pigtoe, and Pistolgrip, which represented 42%, 19.8%, 11.3%, and 9.3%, respectively, of all mussels collected from the Marmaton River (Table 3). The Giant Floater, Yellow Sandshell (*Lampsilis teres*), Pondmussel, and Round Pigtoe (*Pleurobema sintoxia*) were only represented by dead valves.

I planned to conduct a quantitative survey and to quantify the habitat at site P-1, where the Mucket was located; however, unseasonable September rains prevented us from surveying there and on the Marais des Cygnes River at the Marais des Cygnes National Wildlife Refuge (Figures 6-9).

DISCUSSION

The Mucket that was collected in Pottawatomie Creek (length 92.6 mm) was substantially shorter than the maximum shell length of 178 mm. The periostracum was unweathered and had broad bands of thin green rays, as expected in younger individuals perhaps 5-15 years old.

There were not enough Muckets collected to determine habitat associations; however, the individual collected occurred in habitat similar to the presumed habitat preference described in the Kansas Recovery Plan (Obermeyer 2002). The mussel was collected at the downstream end of a long, shallow riffle in a substrate of large gravel and cobble (Figures 4, 5).

The survey indicated that the Mucket persists in the state. Dawley (1947) noted that in Minnesota, the species was widely distributed but occurred in low abundances. The rarity of the Mucket warrants the current conservation status of state endangered, and work must continue to conserve the species in Kansas.

Wolf and Stark (2008) noted that stream bank erosion and highly incised banks (>4 m) were prevalent in the Marais des Cygnes River. This was consistent with what I observed at most sites and it was especially severe at site MDC-6 (Figure 10). The right bank of the river lacked a riparian buffer and crops were planted near the edge of a 10-m, incised bank. I recorded a species richness of 7 at the site and 9 more species were represented only by dead individuals, including the Rock Pocketbook (state threatened) and Black Sandshell (state extirpated). This would be an ideal location for a streambank restoration project that would potentially benefit the mussel community.

Wolf and Stark (2008) reported infrequent occurrence of Yellow Sandshell (SINC) and observed no evidence of recruitment in the Marais des Cygnes River. During the 2016 survey 3 individuals were collected in the Marais des Cygnes River, all at site MDC-11. They seemed to be from different age classes, indicating successful reproduction. Their lengths were 123, 88, and 32 mm.

CONCLUSION

Because of widespread habitat destruction in the Marais des Cygnes River in Missouri, the only opportunity to conserve the freshwater mussels inhabiting this river is in Kansas (Obermeyer 2002). It is evident that the distribution of the Mucket has declined in the state of Kansas (Angelo et al. 2009). The results of this survey were consistent with earlier surveys and reports stating that the Mucket is one of the rarest mussels in Kansas and that it occurs at extremely low abundances (Miller et al. 2008). The current conservation status of state endangered is warranted for this species.

To successfully conserve the Mucket we must first identify the sections of river it inhabits. Because of the difficulties associated with collecting rare and endangered species, it would be beneficial to use additional survey techniques, such as environmental DNA (eDNA) analysis, in combination with traditional qualitative surveys. Environmental DNA analysis is a relatively new technique of species detection that uses species specific primers to amplify short segments of mitochondrial DNA collected from water samples (Ficetola et al. 2008). One of the primary applications for the technique is to survey rare and endangered species (Rees et al. 2014). Being able to consistently detect the presence of the Mucket by extracting its DNA from water samples would help us determine where to allocate time, energy, and resources to better conserve the species.

The major cause for the nationwide decline of freshwater mussels is habitat alteration from widespread impoundment of the nation's rivers (Williams et al. 1993). There are an estimated 2 million dams in the United States and 5,700 in Kansas. Kansas is second in the nation behind Texas for states with the most dams (Dean et al. 2002).

Impoundments alter the geomorphology, hydrology, water chemistry, and biological communities of lotic environments (Juracek 1999; Dean et al. 2002). Dams alter the seasonality and temperature of the stream flow, and they often reduce the ability of a stream to move its bed load, causing unnatural deposition and alteration of the river channel (Juracek 1999; Vaughn and Taylor 1999). They also alter the transport of suspended organic matter, which is the main source of food for freshwater mussels (Vaughn and Taylor 1999).

Dams artificially restrict freshwater mussel distributions by preventing the upstream dispersal of glochidia attached to the gills of host fish (Dean et al. 2002). Williams et al. (1993) suggested the most detrimental effect of dams to mussel populations is the disruption of the reproductive cycle caused by the elimination of the host species. Angelo et al. (2009) stated that dams and other barriers to fish migration hinder the reestablishment of mussel colonies after periods of prolonged drought. Wolf and Stark (2008) documented that low-head dams throughout the Marais des Cygnes basin impounded large stretches of the river. Removal of low-head dams could restore the natural river morphology and improve the dispersal and reestablishment of freshwater fishes and mussels, including the Mucket. Enhancing riparian buffers and streambank stabilization would reduce the stream bank erosion and siltation documented by Wolf and Stark (2008).

Even with habitat improvements, current Mucket populations might be too low to recover on their own. The populations might need to be supplemented through artificial propagation. With the apparently low abundance of the Mucket, it will be difficult, if not impossible, to collect individuals for broodstock within the state.

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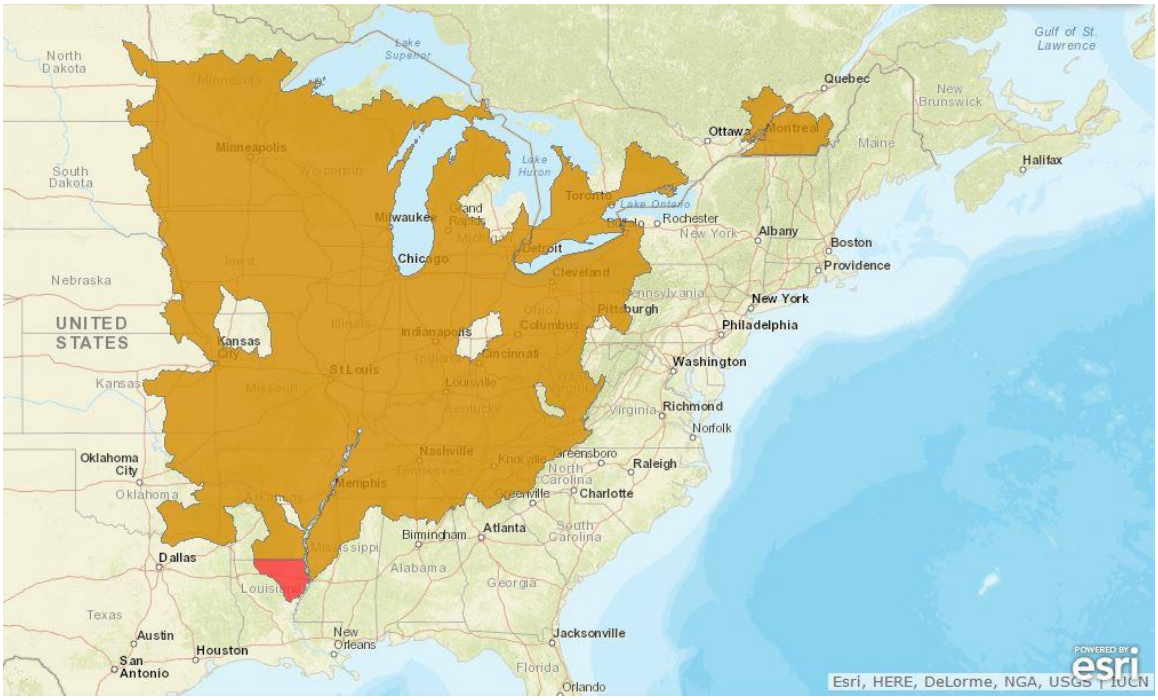
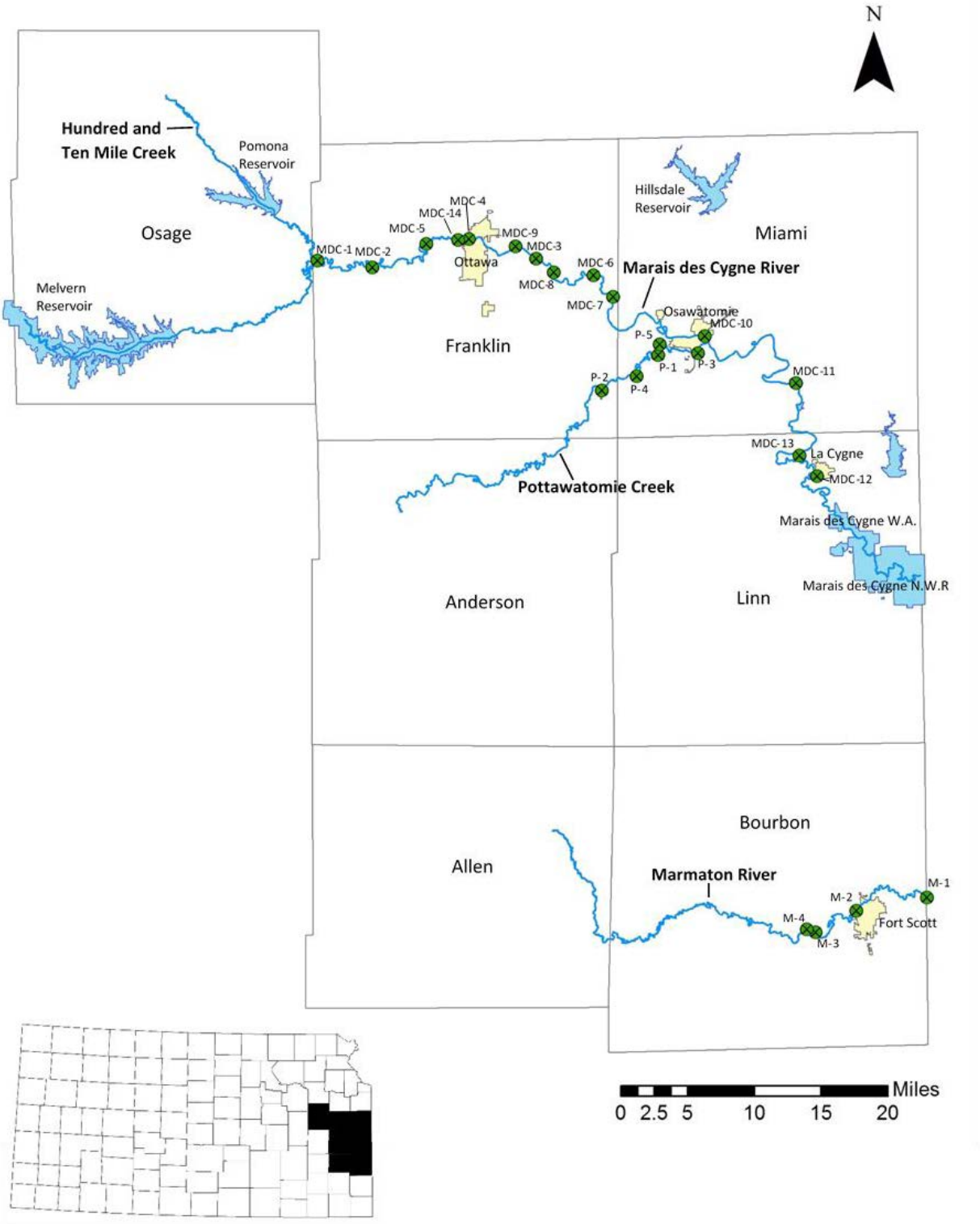


Figure 1. Distribution map of *Actinonaias ligamentina* from iucnredlist.org. (http://maps.iucnredlist.org/map.html?id=189504).

Figure 2. Mucket survey locations in the Marais des Cygnes River, Pottawatomie Creek, and Marmaton River in Franklin, Linn, and Bourbon counties, Kansas in 2016.



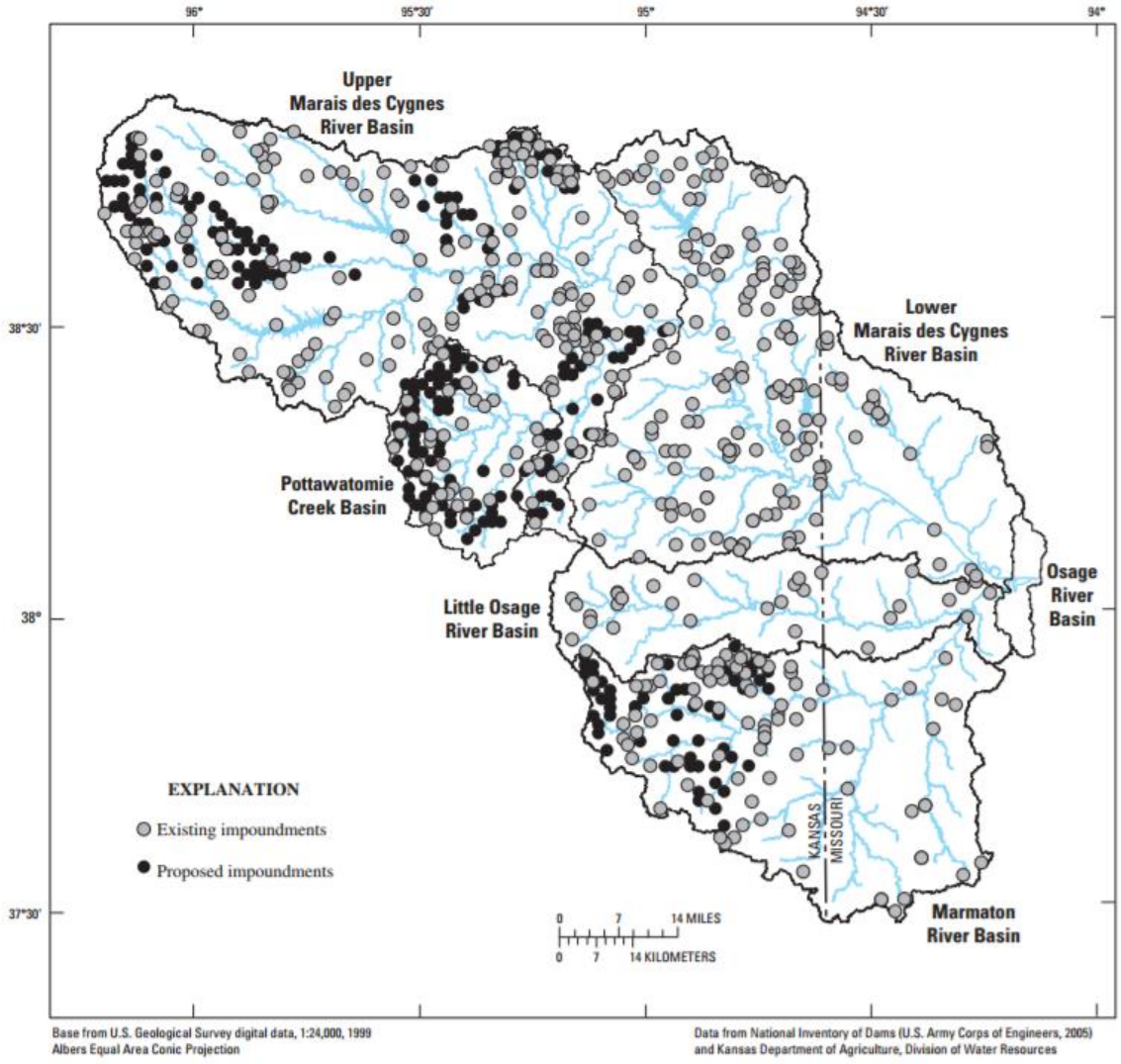


Figure 3. Existing and proposed impoundments of the upper Osage River basin (Heimann et al. 2007)

Figure 4. Substrate at location where the live *Actinonaias ligamentina* was collected at site P-1 in Pottawatomie Creek, Kansas in 2016.

Figure 5. View of the riffle at site P-1 in Pottawatomie Creek, Kansas, where the live *Actinonaias ligamentina* was collected in 2016. The arrow indicates the direction of stream flow.

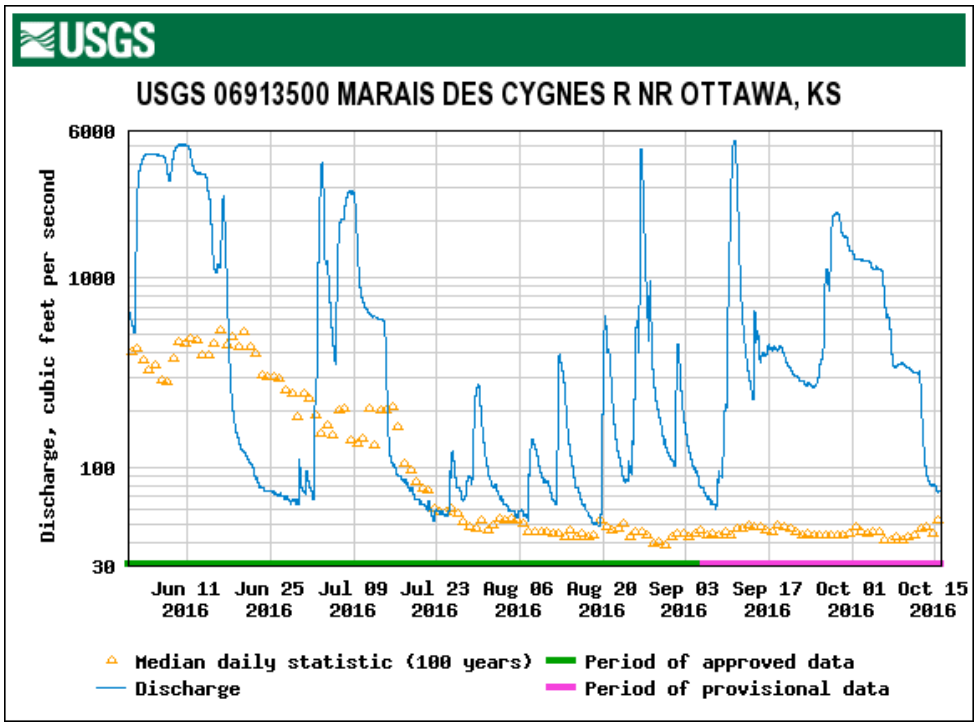


Figure 6. Hydrograph for the Marais des Cygnes River near Ottawa, Kansas during survey period in 2016.

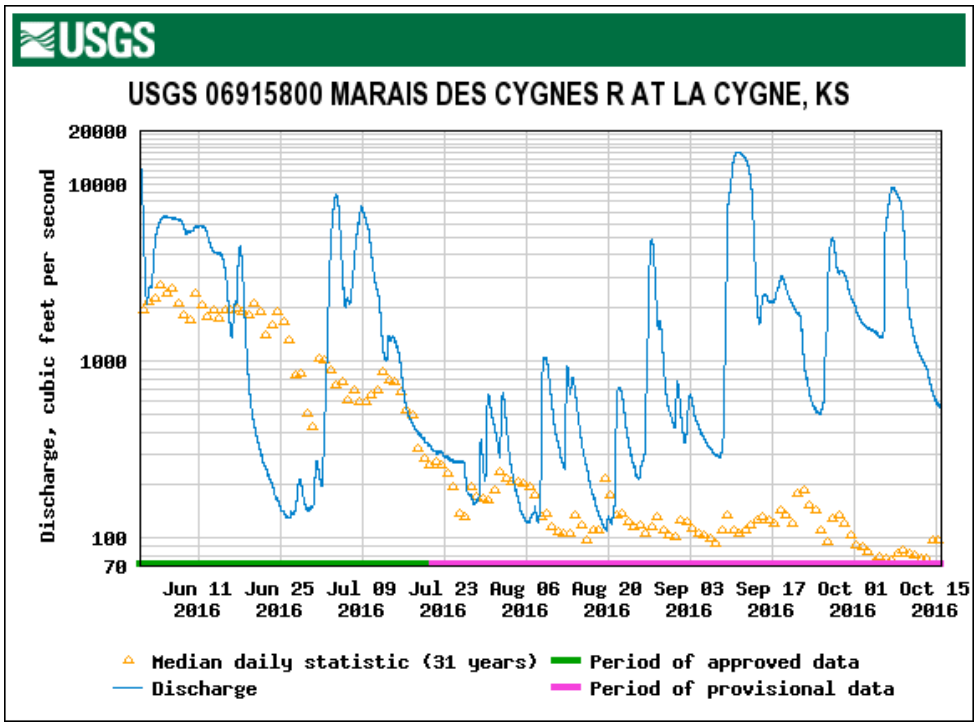


Figure 7. Hydrograph for the Marais des Cygnes River near La Cygne, Kansas during survey period in 2016.

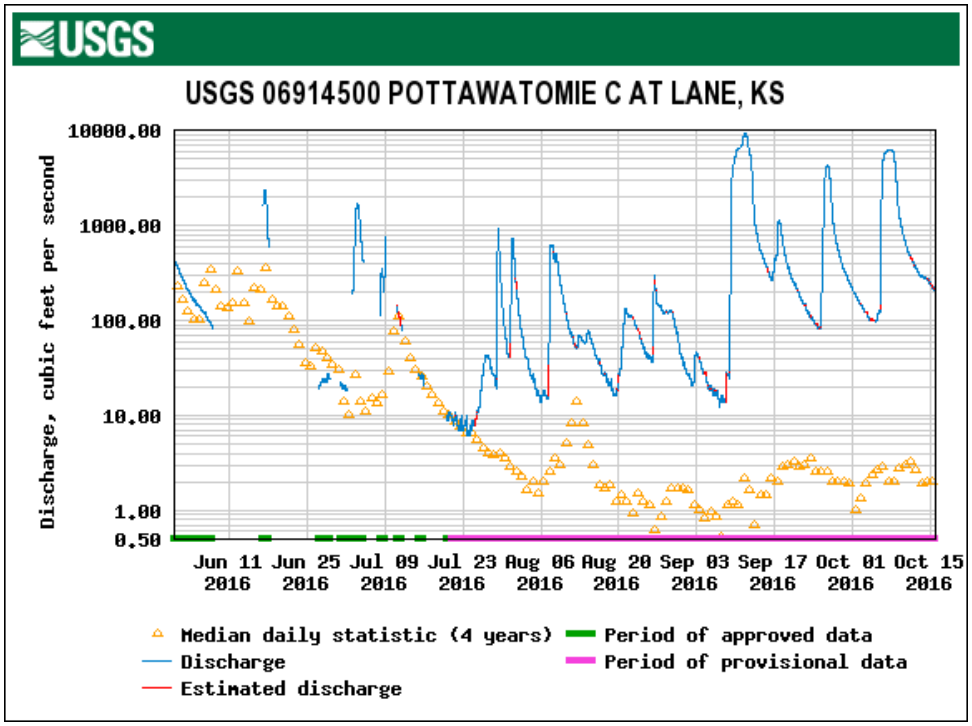


Figure 8. Hydrograph for Pottawatomie Creek at Lane, Kansas during survey period in 2016.

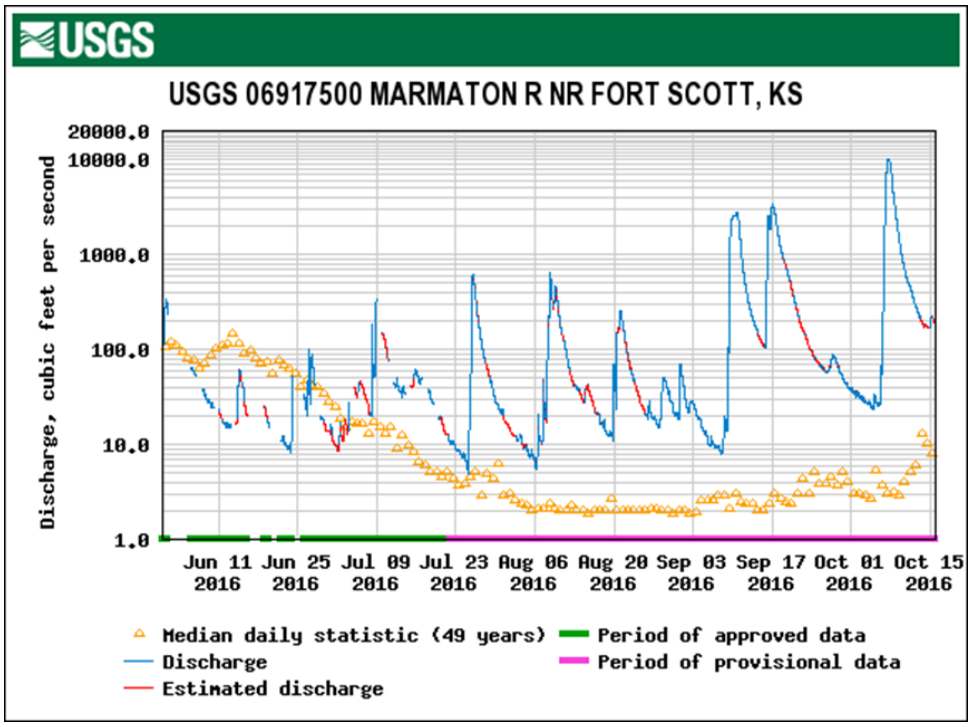


Figure 9. Hydrograph for the Marmaton River near Fort Scott, Kansas during survey period in 2016.

Figure 10. A view of stream bank erosion in the background of site MDC-6 on the Marais des Cygnes River, Kansas. The arrow indicates the direction of stream flow.

Species	MDC-1	MDC-2	MDC-3	MDC-4	MDC-5	MDC-6	MDC-7	MDC-8	MDC-9	MDC-10	MDC-11	MDC-12	MDC-13	MDC-14	Total
Actinonaias ligamentina	-	W	R	-	-	-	R	W	-	W,R	-	-	W	-	0
Arcidens confragosus	-	-	-	-	-	W	-	-	-	-	-	-	-	-	0
Amblema plicata	W	187	-	31	21	11	7	8	5	7	3	21	12	2	315
Elliptio dilatata	-	4	2	-	W	W	13	8	W	W	-	W	W	-	27
Ellipsaria lineolata	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1
Fusconaia flava	8	77	28	16	-	26	74	98	6	1	-	27	20	5	386
Lampsilis teres	-	-	-	-	-	-	-	-	-	-	3	-	-	-	3
Lasmigona complanata	2	2	-	2	2	1	-	-	1	-	1	-	3	W	14
Leptodea fragilis	W	7	W	6	1	2	2	5	3	W	W	4	W	3	33
Ligumia recta	-	-	-	-	-	W	-	-	-	-	-	-	-	-	0
Ligumia subrostrata	W	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Megalonaias nervosa	-	-	W	-	-	W	1	-	-	-	-	1	6	-	8
Obliquaria reflexa	3	39	32	22	13	W	3	10	4	4	-	3	2	14	149
Potamilus alatus	-	4	4	5	2	W	1	-	4	3	3	6	-	-	32
Pyganodon grandis	-	-	-	W	-	-	-	-	-	-	-	-	-	-	0
Quadrula pustulosa	28	92	51	114	14	10	31	51	6	4	3	9	13	2	428
Quadrula quadrula	2	49	10	34	19	8	1	8	3	2	-	-	-	1	137
Strophitus undulatus	-	5	W	-	-	W	1	2	2	-	-	-	-	-	10
Tritogonia verrucosa	19	313	20	45	40	1	10	55	12	9	2	7	28	9	570
Truncilla donaciformis	-	-	-	1	-	W	1	2	-	-	-	-	-	-	4
Truncilla truncata	1	13	-	6	2	W	3	-	2	W	-	1	1	3	32
Total	63	792	147	282	114	59	148	247	48	30	15	80	85	39	**2,149**
Species Richness	7	12	7	11	9	7	13	10	11	7	6	10	8	8	**16**
Total Person Hours	4.66	6.33	4	2.67	3.33	3.75	3	3	3	2	2	4	4	3	**48.74**
CPUE	13.5	125.1	36.8	105.6	34.2	15.7	49.3	82.3	16	15	7.5	20	21.3	13	**44.09**
Diversity	**0.83**														

Table 1. Qualitative mussel survey results from each site in the Marais des Cygnes River, Kansas in 2016, including other observed dead valves (W= weathered, R= relic). CPUE (catch-per-person-hour) calculated for each site and a total CPUE for all live freshwater mussels collected in the Marais des Cygnes River. Diversity was calculated as Simpson's index of diversity.

Species	P-1	P-2	P-3	P-4	P-5	Total
Actinonaias ligamentina	1	-	-	-	-	1
Amblema plicata	84	4	W	1	1	90
Elliptio dilatata	-	-	W	-	-	0
Fusconaia flava	-	-	1	1	-	2
Lampsilis cardium	-	-	R	-	-	0
Lampsilis teres	2	-	W	W	-	2
Lasmigona complanata	5	-	1	-	W	6
Leptodea fragilis	1	-	W	1	-	2
Obliquaria reflexa	8	-	-	-	2	10
Potamilus alatus	-	2	-	W	1	3
Quadrula pustulosa	14	-	-	2	1	17
Quadrula quadrula	25	-	-	W	-	25
Strophitus undulatus	1	-	-	-	-	1
Tritogonia verrucosa	48	4	-	7	3	62
Truncilla truncata	5	-	-	W	-	5
Total	194	10	2	12	8	**226**
Species Richness	11	3	2	5	5	**13**
Total Person Hours	8	2	2	3	1.5	**16.5**
CPUE	24.25	5	1	3.7	5.3	**13.7**
Diversity	**0.75**					

Table 2. Qualitative mussel survey results from each site in Pottawatomie Creek, Kansas in 2016, including other observed dead valves (W= weathered, R= relic). CPUE (catch-per-person-hour) calculated for each site and a total CPUE calculated for all live freshwater mussels collected in Pottawatomie Creek. Diversity was calculated as Simpson's index of diversity.

Species	M-1	M-2	M-3	M-4	Total
Amblema plicata	13	R	104	118	235
Elliptio dilatata	-	-	16	16	32
Fusconaia flava	2	R	41	20	63
Lampsilis cardium	-	R	-	1	1
Lampsilis teres	W	-	W	-	0
Lasmigona complanata	5	-	1	-	6
Leptodea fragilis	1	W	5	-	6
Ligumia subrostrata	-	-	W	-	0
Pleurobema sintoxia	R	-	-	-	0
Potamilus alatus	1	-	-	W	1
Potamilus ohiensis	-	-	2	-	2
Pyganodon grandis	W	R	W	-	0
Quadrula pustulosa	12	-	19	9	40
Quadrula quadrula	84	R	22	5	111
Strophitus undulatus	-	-	6	1	7
Tritogonia verrucosa	44	-	7	2	53
Truncilla truncata	-	-	W	1	1
Total	162	0	223	173	**558**
Species Richness	8	0	10	9	**13**
Total Person Hours	3	1	2.5	2	**8.5**
CPUE	54	0	89.2	86.5	**65.6**
Diversity	**0.75**				

Table 3. Qualitative mussel survey results from each site in the Marmaton River, Kansas in 2016, including other observed dead valves (W= weathered, R= relic). CPUE (catch-per-person-hour) calculated for each site and a total CPUE calculated for all of the live freshwater mussels collected in the Marmaton River. Diversity was calculated as Simpson's index of diversity.

Appendix 1. Qualitative survey locations for *Actinonaias ligamentina* on the Marais des Cygnes River (MDC), Potawatomie Creek (PC), and Marmaton River (M) of the Osage River system during the summer of 2016. Discharge data were obtained from the US Geological Survey. Latitude and longitude were collected with a Garmin RINO 650t. Effort was calculated by multiplying the time spent searching by the number of people.

Site	Latitude	Longitude	Date	Effort (Person hours)	Daily Mean Discharge (cfs)	USGS Gaging Station ID
MDC-1	38.59594	-95.50011	8/3/2016	4.7	61	Pomona 06913000
MDC-2	38.587829	-95.419036	8/3/2016	6.3	61	Pomona 0691300
MDC-3	38.59638	-95.17987	8/3/2016	4	58	Ottawa 06913500
MDC-4	38.61991	-95.27675	8/4/2016	2.8	59	Ottawa 06913500
MDC-5	38.61467	-95.33979	8/4/2016	3.3	59	Pomona 06913000
MDC-6	38.57683	-95.09377	8/9/2016	3.8	94	Ottawa 06913500
MDC-7	38.55116	-95.06535	8/9/2016	3	94	Ottawa 06913500
MDC-8	38.58057	-95.15199	8/9/2016	3	94	Ottawa 06913500
MDC-9	38.61077	-95.20797	8/10/2016	3	83	Ottawa 06913500
MDC-10	38.50505	-94.93083	8/18/2016	2	136	La Cygne 06914500
MDC-11	38.44891	-94.79730	8/18/2016	2	136	La Cygne 06914500
MDC-12	38.34086	-94.76861	8/18/2016	4	136	La Cygne 06914500
MDC-13	38.36516	-94.79405	8/19/2016	4	116	La Cygne 06914500
MDC-14	38.618972	-95.292416	9/7/2016	3	97	Ottawa 06913500
P-1	38.48327	-94.99980	8/17/2016	8	26	Lane 06914500
P-2	38.44303	-95.08308	8/17/2016	2	26	Lane 06914500
P-3	38.48533	-94.941027	8/18/2016	2	21	Lane 06914500
P-4	38.45944	-95.032055	9/3/2016	3	39	Lane 06914500
P-5	38.495638	-94.99727	9/3/2016	1.5	39	Lane 06914500
M-1	37.85143	-94.61681	7/18/2016	3	32	Fort Scott 06917500
M-2	37.83765	-94.71984	7/19/2016	1	22	Fort Scott 06917500
M-3	37.81333	-94.77991	7/19/2016	2.5	22	Fort Scott 06917500
M-4	37.81673	-94.79261	7/19/2016	2	22	Fort Scott 06917500

CHAPTER 2

USING ENVIRONMENTAL DNA TO DETECT THE PRESENCE OF THE CYLINDRICAL PAPERSHELL MUSSEL (*Anodontoides ferussacianus*) IN THE SALINE AND SMOKY HILL RIVERS IN KANSAS

Analysis of environmental DNA (eDNA) from water samples is a new technique of species detection that is rapidly being developed and deployed in ecological assessments in aquatic systems (Rees et al. 2014). Environmental DNA in this context is DNA that is suspended in water and has originated from feces, saliva, urine, gametes, or skin cells of organisms living in or visiting an aquatic environment (Rees et al. 2014). Diffusion of eDNA within the water body allows us to detect individuals at locations throughout the environment, not just at locations near the point of origin. Over time, eDNA is fragmented and destroyed by ultraviolet light and microbial activity; thus, positive species detections signify the species is or recently was present in the aquatic environment at the time of water sample collection (Rees et al. 2014). Environmental DNA analysis minimizes stress to the organism and has the potential for higher detection rates than traditional survey methods, especially for species occurring at low densities (Pilliod et al. 2013; Janosik and Johnston 2015). Accurately determining a species presence and distribution is a vital step towards successfully managing or conserving a species (Laramie et al. 2015). The challenge of surveying rare and endangered species is the amount of time, effort, and finances required to detect populations present at low densities (Goldberg et al. 2011; Rees et al. 2014).

Environmental DNA analysis detects species presence by using specific primers to amplify short segments of mitochondrial DNA collected from water samples (Ficetola et al. 2008). The primary applications for eDNA analysis are surveying rare and endangered species and early detection of invasive species (Pilliod et al. 2013).

The natural history characteristics and conservation status of many native freshwater mussels suggests that eDNA analysis could provide helpful insights in the development of conservation plans. As such, I chose to investigate the potential of this technique by using the Cylindrical Papershell (*Anodontoides ferussacianus*), a species in low abundance in western Kansas. The Cylindrical Papershell has a wide distribution in the north-central United States and southern Canada (Sowards et al. 2016). However, in Kansas, the range of the species has been dramatically reduced. The species was historically collected in the Kansas, Missouri, Republican, Smoky Hill-Saline, and Solomon River drainages (Murray and Leonard 1962). It is currently restricted to short reaches in the Saline and Smoky Hill rivers. The relative abundance and density of the Cylindrical Papershell have also declined. In 1983, it was the most abundant species at several sites in the Smoky Hill River (Hoke 1997). In 2011, the relative abundance and density were low in both rivers and no aggregations of the mussel were documented (Sowards et al. 2016). These declines prompted Sowards et al. (2016) to recommend that the Kansas Department of Wildlife, Parks and Tourism (KDWPT) downgrade the status of the Cylindrical Papershell from SINC to endangered. Due to the persistence and increased severity of drought conditions, KDWPT funded an additional survey for the Cylindrical Papershell during the summer of 2015.

During the summer of 2015, Andrew Karlin and I used timed, tactile searches in all wadeable habitats to survey 21 sites on the Smoky Hill River and 19 sites on the Saline River (Figure 1 and Table 3) (Karlin et al. 2017). Quantitative surveys were conducted post hoc at sites SR-08 and SR-16 on the Saline River and at sites SH-11, SH-12, SH-17, SH-21, and SH-22 on the Smoky Hill River (Figure 1) (Karlin et al. 2017). Four live individual Cylindrical Papershell were collected in the Saline River at site SR-16 (Figure 1) (Karlin et al. 2017). In the Smoky Hill River, 4 Cylindrical Papershell were collected at site SH-21, 3 at site SH-22, 2 at site SH-17, and 1 at site SH-19 (Table 3) (Karlin et al. 2017). During the quantitative surveys, 3 live Cylindrical Papershell were collected at site SR-16 and 2 live Cylindrical Papershell were collected at site SR-22, one of which was a recapture from the qualitative survey (Karlin et al. 2017).

The objective of this project was to evaluate the effectiveness of eDNA analysis to detect the presence of freshwater mussels, refine methods for eDNA species detection, and to determine the applicability of the method for surveying rare and endangered populations of freshwater mussels.

METHODS

Study Area

I collected water samples from the Saline and Smoky Hill rivers in Ellis and Russell counties, Kansas. In these counties, the rivers are shallow, low-flow streams with irregular high-flow events (Sowards et al. 2016). Both of these rivers flow in an easterly direction, and join near Salina, Kansas, flowing into the Kansas River near Junction City, Kansas. West of Ellis County, the flow of these rivers is typically intermittent or ephemeral. Cedar Bluff Reservoir impounds the Smoky Hill River in eastern Trego County, and Kanapolis Reservoir impounds the Smoky Hill River downstream in Ellsworth County. The Saline River is impounded by Wilson Reservoir in Russell County. Both rivers have several low-head dams (Sowards et al. 2016).

Experimental Design

I targeted eDNA for the Mapleleaf (*Quadrula quadrula*), a common species, and the Cylindrical Papershell, a rare species of freshwater mussel. The Mapleleaf was the most common species collected during the traditional survey; the Cylindrical Papershell was a rare species of conservation concern and the focus of our traditional survey (Table 3). Targeting species occurring at different densities provided the opportunity to estimate the sensitivity of the analysis.

I collected three 2-L samples of water at each site. Significant reductions in species detection have been shown to occur when only 1 or 2 water samples are collected per site (Rees et al. 2014). A fourth, empty 2-L bottle was carried into the field as a control. In the laboratory the control bottle was filled with deionized water and filtered

before the environmental samples. Amplification of DNA in the control sample would alert us of cross contamination of DNA. The bottles and funnel were autoclaved between each sampling event, and countertops, forceps, and other tools used were washed in a 50% bleach solution between analyses of sites.

Field Sampling/ Filtration

In the fall of 2015, I collected water samples from 17 sites on the Smoky Hill River and 8 Sites on the Saline River. In the spring of 2017, I collected additional water samples from sites SH-1, SH-22, SH-21 and SH-19. From the bank of the river, reaching with a gloved hand, I used 2-L, wide-mouth, polypropylene, Nalgene bottles to collect 3 water samples at each site. The 3 samples were collected approximately 50 m apart, starting at the downstream end of the site. They were transported on ice to the laboratory and stored at 4°C for a maximum of 6 hours. I used a 350-ml, polysulfone analytical funnel seated on top of a 4-L glass vacuum filter flask connected by hosing to a filter pump aspirator attached to a faucet. The aspirator created a vacuum suction when the water was turned on. I filtered each water sample through a 47-mm diameter glass microfiber filter with a pore size of 1.5µm (Sterlitech Corp., Kent, WA) as recommended by Eichmiller et al. (2016). Multiple filters were used per sample due to clogging. The filters were placed in a 50-mL vial of 100% ethanol and stored in a freezer until the DNA was extracted.

eDNA Extraction

I tested 4 eDNA extraction techniques. We used the FastDNA Spin Kit (MP Biomedicals, Santa Ana, CA) as recommended by Eichmiller et al. (2016). I used the Qiagen Dneasy Blood and Tissue kit, and used it in combination with a Qiagen

Qiashredder (Qiagen Company, Hilden, Germany) as described in Goldberg et al. (2011). I also vortexed and boiled the filters in 1.5 mL of Tris-EDTA (TE) buffer for 10 minutes as described by Vasuki et al. (2001). The FastDNA Spin Kit was used to extract eDNA from sample SR16-2 collected from the Saline River.

A second extraction of eDNA from sample SR16-2 was attempted by using the Qiagen Dneasy Blood and Tissue kit. I removed 2 filters from the sample, cut them in half, and left them out in an uncovered petri dish to dry overnight. The following morning the filters were cut into small pieces and individually placed into a 1.5-mL Eppendorf tube. A 600 µL solution of ATL buffer and Proteinase K was added to each tube, and the tubes were placed in a 56°C water bath and incubated for 48 hours. After incubation, I used the Qiagen protocol for the rest of the extraction. A NanoDrop 2000c UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to determine the concentration and quality of the DNA.

I used the eDNA extraction method described by Goldberg et al. (2011) for a site on the Smoky Hill River where we collected 234 Mapleleaf during the traditional survey. The filters of sample SH25-1 were cut in half with a sterile razorblade; one half was placed back into the ethanol and freezer for reserve, and the other half was set out to dry overnight. The following morning the ½ filter was cut into 4 smaller pieces with a sterile razorblade. The 2 pieces were placed in separate 2-mL tubes and 180 µL of solution ATL was added to each. The filters were gently homogenized by using a handheld micro pestle. I added 20 µL of proteinase K to each tube and placed them in a 56°C water bath to incubate for 48 hours. The contents of the tubes were then transferred to the Qiagen Qiashredder tubes and centrifuged at 17,000g for 2 minutes. The supernatant was then

transferred into a regular 2-mL tube. The rest of the DNA isolation followed the Qiagen Dneasy Blood and Tissue extraction kit protocol until the final elution step, where I added 15 µL of water instead of AE buffer. After the elution step, the supernatant from all of the tubes of the same sample were combined. This technique was used for all environmental samples collected in 2015 (Appendix 1).

In an attempt to save time, the 2017 samples were extracted by using the TE boil method. The filters were cut in half with sterile scissors; half of the filters were placed in ethanol and stored in the freezer for reserve, and the other half were placed in a 15-mL conical polypropylene centrifuge tube with 1500 µL of Tris-EdTA solution. The samples were vortexed, boiled for 10 minutes, and vortexed again. The liquid was transferred to a 1.5-mL tube and centrifuged for 3 minutes at 13,000 g. The supernatant was transferred to a new tube. Samples 1, 2, and 3 were pooled taking 100 µL of each, adding it to a clean tube, and vortexing the sample. The NanoDrop results of the 2017 pooled samples are listed in Appendix 1.

Primer Design

Standard PCR Primer Design

I designed species specific molecular markers for the Cylindrical Papershell and Mapleleaf. Sequence information for both species was available in a public data base (Genbank, www.ncbi.nlm.nih.gov). I used Clustal Omega software (www.ebi.ac.uk/Tools/msa/clustalo/) to organize the FASTA sequences of all mussel species collected during the 2015 survey. Forward and reverse primers were developed in regions of the sequence that did not correspond to the sequences of the other species present in the survey area. I used BLAST (Basic Local Alignment Search Tool;

GenBank, www.ncbi.nlm.nih.gov) to test the specificity of the primers. The forward and reverse primer sequences I designed are recorded in Table 1.

Table 1. Primer sequences for species specific amplification of mitochondrial DNA. (Standard PCR)

Species	Primer Name	Primer Sequence
Cylindrical Papershell (*Anodontoides ferussacianus)*	CP NADHF6	5'- GTC ACG TAC CTC CTA ATT TT
	CP NADHR513	5'- GCA TAA TTG CTC AAG TAG ATA T
Mapleleaf (*Quadrula quadrula)*	QuadrulaF212	5'- AAT CAT ACT TAT CAT GGC ACT C
	QuadrulaR497	5'- TAG ACA GGG CGA TAG TTG GTA TA

The specificities of the primers were tested by using known tissue samples taken directly from an individual of each species that was collected during the field survey with the exception of the Pondmussel (*Ligumia subrostrata*); only one individual was collected at site SH21 during the quantitative survey (Karlin et al. 2017). I used the non-lethal DNA isolation technique described by Spicer et al. (2007) to extract tissue from the mussels. The tissue was placed in a tube of 100% ethanol, transported on ice, and stored at -20 degrees Celsius. I used the Qiagen Dneasy Blood and Tissue kit to isolate DNA from the mussel samples. I deviated from the Qiagen protocol for the final elution step; I added 50 µL of water into the column instead of 200 µL of AE buffer. I analyzed the quality and concentration of the DNA using a NanoDrop 2000c UV-Vis spectrophotometer. The Cylindrical Papershell and Mapleleaf primers were used to run a standard PCR using the thermal cycle program MCRE 52L (Table 4). Gel electrophoresis was conducted at 120 volts for 30 minutes to determine if the PCR successfully amplified the short segment of DNA the primers were designed to detect.

The genes in the PCR product of the Cylindrical Papershell and Mapleleaf were cloned using *E. coli* transformation. The plasmid was isolated from the *E. coli* and then

digested by using EcRI digestion protocol. I used the results from gel electrophoresis and the NanoDrop to determine which plasmids to send for sequencing. This step verified the primer was identifying the target species.

Real-Time PCR Primer Design

The DNA sequenced from the cloned plasmids was used to develop the species specific primers for the Real Time-PCR assay. Clustal Omega was used to align the FASTA sequences of the Cylindrical Papershell and Mapleleaf to the Genbank sequences from the other mussel species collected during the traditional surveys. I attempted to locate regions in the Cylindrical Papershell and Mapleleaf sequences that differed in nucleotides from the other mussel species. BLAST was used to test the specificity of the primers. The forward and reverse primers used in the RT-PCR assay are recorded in Table 2.

Table 2. Primer sequences for species specific amplification of mitochondrial DNA. (RT-PCR).

Species	**Primer Name**	**Primer Sequence**
Cylindrical Papershell (*Anodontoides ferussacianus)*	CPF419-441	5'- TCC CAG TTT ATT AGG GCC TTT C
	CPR502-525	5'- CCT TGT CAC GTA CCT CCT AAT TT
Mapleleaf (*Quadrula quadrula)*	MLF9-31	5'- GAC AGG GCG ATA GTT GGT ATA G
	MLR125-144	5'- CCG AGC TAT GGC TCA AAC A

To estimate the sensitivity, and efficiency of the assay, I used different concentrations of known Cylindrical Papershell and Mapleleaf DNA (10 ng/µL - 0.1 pg/µL). In addition, different concentrations of primer were used in the master mix solution (0.1µL, 0.5 µL, and 1.0 µL) to determine the amount of primer required in the eDNA assays. The specificity of the primers were tested by running a RT-PCR assay

with DNA isolated from tissues removed from individual freshwater mussels of each species collected during the field survey with the exception of the Pondmussel.

Standard PCR

In the standard PCR assays, I used thermal cycle program MCRE 52-L (Table 4) and the master mix listed in (Table 5); 5µL of eDNA was used per well. Gel electrophoresis was used to analyze the PCR product and was conducted at 120 volts for 30 minutes. The eDNA amplification was tested by using the Cylindrical Papershell primer and Mapleleaf primer.

Real-Time PCR

I used thermal cycle program eDNA_Sybr_50Cycle (Figure 2) and the intercalating fluorescent dye SYBR Green in the Real-Time PCR assays. The master mix is listed in Table 7. I used the Cylindrical Papershell primer to run multiple assays to detect the presence of eDNA from the water samples.

RESULTS

Standard PCR Primer Design

The NanoDrop results of the DNA collected from the mussel species to test primer specificity are listed in Table 6. These muscle tissues were used to test the specificity of the standard PCR primers and the RT-PCR primers. Figure 3 is the gel electrophoresis of the PCR product that tested the specificity of the Cylindrical Papershell primer. Only the Cylindrical Papershell was amplified, indicating the primer was specific. I ran BLAST for the cloned and sequenced PCR product. The PCR product was 99% identical to the Cylindrical Papershell.

The Mapleleaf primer (Figure 4) was not species specific. Both the Cylindrical Papershell and Pink Papershell had PCR amplification with a similar size, indicating that the primer had also amplified DNA of those species. I reran the PCR using the thermal cycle program MCRE55L, which had a higher annealing temperature at 55°C. The results were similar to the first PCR, but included slight banding on the Lilliput (*Toxolasma parvus*) and Pink Papershell (Figure 5). This indicated that the primer was not specific to the Mapleleaf.

Real-Time PCR Primer Design

The assay using different concentrations of Cylindrical Papershell DNA and primers successfully amplified DNA at all concentrations down to 0.1 pg/µL, and it amplified DNA with 0.5 µL of primer per well (Figure 6). The standard curve for the assay is shown in Figure 7. The assay testing the specificity of the Cylindrical Papershell

primer only amplified the Cylindrical Papershell DNA, indicating the primer was specific (Figure 8).

The assay using different concentrations of Mapleleaf DNA and primers successfully amplified DNA at all concentrations down to 0.1 pg/µL, and it amplified DNA with 0.5 µL of primers per well (Figure 9). The standard curve for the assay is shown in Figure 10. The assay testing the Mapleleaf primer specificity amplified DNA of the Mapleleaf, Fragile Papershell, White Heelsplitter, and Paper Pondshell (*Utterbackia imbecillis*) at high concentrations (10 ng/µl). The Mapleleaf primer was not species specific (Figure 11).

eDNA Standard PCR Results

For SR16-2, the PCR amplified the known Cylindrical Papershell DNA but the eDNA was not detected (Figure 12). In the second attempt of SR16-2, I used both the Cylindrical Papershell and Mapleleaf primers for the PCR. The gel electrophoresis of the PCR products resulted in bright bands for both known positives and no banding on the gel for the environmental sample.

The NanoDrop for SH25-1-1 had a concentration of 188.9 ng/µL and a 260/280 ratio of 1.85. The NanoDrop for SH25-1-3 had a concentration of 47.6 ng/µL and a 260/280 ratio of 1.74. The PCRs using the Cylindrical Papershell and Mapleleaf primers are shown in Figure 13. There is a bright band for both of the known positives and no banding for the eDNA. There also seems to be some contamination in the Mapleleaf primer.

Results of the DNA degradation test for SH25-1 are shown in Figure 14. There was good banding for both SH25-1-1 and SH25-1-3, indicating the DNA was not degraded.

eDNA Real-Time PCR Results

The assay attempting to detect Cylindrical Papershell eDNA used known Cylindrical Papershell DNA at concentrations ranging from 10 ng/µL to 0.01 pgµL. Environmental samples SH17 (C,1,2,3), SH22 (C,1,2,3), SH19 (C,1,2,3), and SR16 (C,1,2,3) were used. The known Cylindrical Papershell DNA samples were amplified at all concentrations down to 0.01 pg. Only the controls were positive for the eDNA samples. The controls of SH-17, SH19, and SR16 were all positive, indicating that the eDNA samples were contaminated (Figure 15). The suspicion was that either the eDNA concentrations were too high and suppressed the reaction or the eDNA samples were inhibited by inorganic material.

I diluted the eDNA reactions to 5 ng/µL- 5pg/µL and spiked several of the eDNA samples with 2 pg/µL of Cylindrical Papershell DNA to test for inhibition. The assay amplified a well of SH22 eDNA at concentrations of 500 pg/µL, 5 pg/µL, and 5 pg/µL, and all of the spiked samples were amplified, indicating the eDNA samples did not inhibited the assay (Figure 16).

I pooled eDNA samples 1, 2, and 3 from sites SH17, SH19, SH22, and SR-16. These samples were diluted to 5 ng/µL, 500 pg/µL, and 50 pg/µL. The assay amplified eDNA from all 4 sites (Figure 17).

Due to contamination of the environmental samples and the apparent ability of this technique to amplify low concentrations of freshwater mussel DNA, I collected

additional water samples with a different technique to sterilize the sampling equipment; however, I did not have time to use the current eDNA extraction technique with the Qiagen Dneasy Blood and Tissue Kit in combination with the Qiashredder. Accordingly, I decided to test the boil in TE buffer technique for eDNA extraction and used the reserve filters of SH21-1 and SH21-2 to test the effectiveness of the technique. SH21-1 had a NanoDrop concentration of 38.6 ng/µl and SH21-2 had a NanoDrop concentration of 39.4 ng/µL. I diluted SH21-1 and SH21-2 to 3.8 ng/µL and 3.9 ng/µL, 380 pg/µL and 390 pg/µL and 38 pg/µL and 39 pg/µL, respectively. The assay amplified DNA in sample SH21-2, indicating the new extraction technique worked (Figure 18).

I collected additional water samples at sites SH1, SH22, SH21, and SH19 and used the same sampling and filtration technique but sterilized the equipment with a 50% bleach solution instead of autoclaving. The NanoDrop results are listed in appendix 1. I pooled samples 1, 2, and 3 for each site and diluted them to 30 ng/µL and 3ng/µL. I conducted the assay by using straight eDNA and the dilutions from each sight. None of the eDNA samples were positive in the assay (Figure 19).

DISCUSSION

The initial results of the Real-Time PCR assay indicated that the water samples were contaminated with DNA. According to Goldberg et al. (2016), standard autoclaving of sampling and filtering equipment is inadequate for destroying nucleic acids. They claimed a treatment of 50% commercial bleach is the minimum to remove extraneous DNA and PCR products (Goldberg et al. 2016). If the contamination came from the bottles, funnel, or both, which were autoclaved between sites, then Cylindrical Papershell eDNA was detected but because of the contamination, I am unable to determine the sites where eDNA was present. However, I was not able to determine the source of contamination and whether the samples were contaminated with eDNA.

The preliminary test of the TE boil DNA extraction technique used for sample SH21 apparently was successful; however, I was unable to detect Cylindrical Papershell DNA when I conducted the assay with the 2017 water samples. As such, I cannot be certain if the extraction technique worked for SH21 or if the assay was positive due to contamination of the sample. The TE boil technique has the potential to dramatically decrease the time required to extract the eDNA and reduce the cost of the analysis. The technique was unsuccessful during the analysis, but more work needs to be done before it is discredited as an ineffective extraction method.

The next step to take in this study would be to extract the eDNA in the 2017 water samples by using the Qiashredder/ Dneasy Blood and Tissue Kit technique and running a Real-Time PCR analysis. The eDNA assays suggested that Real-Time PCR using SYBR

Green is sensitive enough to amplify minute concentrations of DNA and has the potential to detect eDNA of rare and endangered aquatic species.

Several environmental variables differed from the collection of water samples in the fall of 2015 and in the spring of 2017. In 2015, I collected water samples from October to November; in 2017 the water samples were collected in early March. This difference in seasons likely affected the water temperature, although it was not recorded. At lower temperatures, the metabolism of freshwater mussels could decrease filter feeding rates and decrease the amount of DNA sloughed into the environment. The 2017 samples were collected 18 months after field surveys. The Cylindrical Papershell populations might have declined in that time, resulting in decline of eDNA. These are hypothetical questions I am unable to answer due to limitations of my data.

The main difference between the 2015 water collection and the 2017 collection was the increase in water volume and flow. When I collected water samples in the fall of 2015, there was no visible flow in either the Saline or Smoky Hill rivers (Figures 20 and 21). The rivers were reduced to isolated pools functioning as lentic environments. A positive detection of eDNA in these non-flowing environments would provide us certainty that the species were present at the location where the sample was collected. In the spring of 2017, when I collected the water samples, there was flow of 3 cfs (Figure 22). A positive detection of eDNA in a lotic environment would affect the distance between the collection site and the eDNA source. Deiner and Altermatt (2014) detected eDNA of a lake-dwelling invertebrate (*Daphnia longispina*) 12.3 km downstream from the lake. This indicates that invertebrate eDNA can persist over relatively large distances. Deiner et al. (2016) described rivers as conveyor belts of biodiversity information.

Other variables to consider when designing an eDNA survey are turbidity, stream flow, shading, water temperature, and the natural history of the study organism. Turbid water will increase the amount of filters required and the time of filtration, and it will potentially inhibit the RT-PCR reaction. Water sample collection should be avoided during periods of high flow; the increase in volume would likely decrease the concentration of eDNA and could affect the distance of eDNA dispersal. The amount of stream shading and water temperature will have an effect on degradation of eDNA, and would likely degrade faster in streams with an open canopy, directly exposed to UV light and having high water temperatures. Natural history of the organism could also affect the eDNA availability, with perhaps higher eDNA concentrations during spawning events. These variables could affect the detectability of a species using eDNA analysis.

CONCLUSION

Environmental DNA analysis is an emerging technique of aquatic species detection. It is increasing in its application and growing in popularity. I assessed the ability of this technique to detect freshwater mussels in Kansas streams. The species of focus in the survey were the Cylindrical Papershell and Mapleleaf. I was successful in developing species-specific primers for both standard and Real-Time PCR assays for the Cylindrical Papershell. I was unsuccessful in developing species-specific primers for the Mapleleaf. Cylindrical Papershell DNA was detected in the environmental samples but due to contamination in the controls I was unable to determine the source of the DNA. The likely source of contamination was from the bottles and filter funnel that were autoclaved between samples. I attempted a new extraction technique of boiling the filters in TE buffer. The initial test with sample SH21 seemed to work, but no eDNA was detected in the samples collected in the spring of 2017 at sites where the Cylindrical Papershell was physically sampled in 2015.

Because of contamination in the 2015 water samples I am unable to determine if eDNA of the Cylindrical Papershell was detected. Because of the difference in seasons and the extraction technique of the 2017 samples, I was unable to determine if eDNA was absent or if the extraction technique was insufficient.

Nonetheless, advances were made in detecting Cylindrical Papershell eDNA. Through trial and error I discovered a method of extracting eDNA from filter samples with the Qiashredder/ Blood and Tissue Kit technique. The method was consistent and easily replicated. I learned that autoclaving is insufficient in degrading nucleotides, and

equipment must be washed in a 50% bleach solution between samples. The most useful contribution of this study was developing a species specific Cylindrical Papershell primers.

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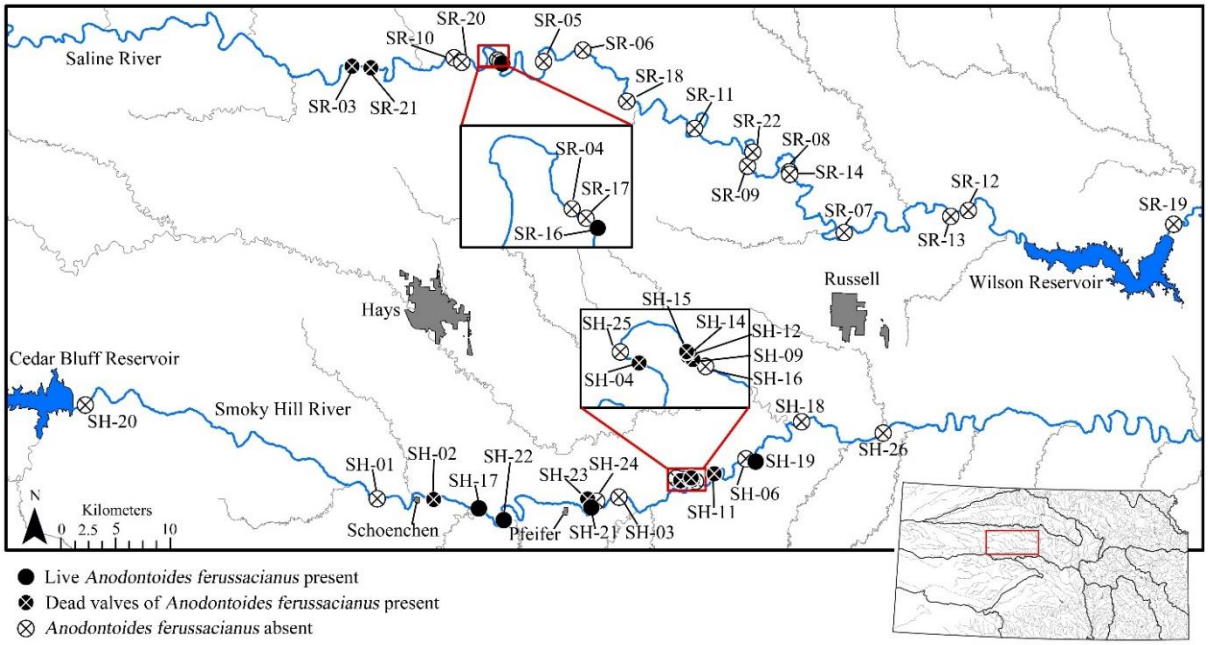


Figure 1. Survey area and sites sampled for freshwater mussels on the Saline and Smoky Hill rivers in Ellis, Russell, and Trego counties, Kansas during 2015 (Karlin et al. 2017).

Table 3. Qualitative survey data for live mussels collected in the Saline and Smoky Hill rivers in Ellis, Russell, and Trego counties, Kansas during 2015 (Karlin et al. 2017).

Site		*Anodontoides ferussacianus*	*Lasmigona complanata*	*Leptodea fragilis*	*Potamilus ohiensis*	*Pyganodon grandis*	*Quadrula quadrula*	*Toxolasma parvus*	*Utterbackia imbecillis*	Total	CPUE	Species Richness	Simpson Diversity Index
SR-03		-	-	-	-	-	-	-	-	-	-	-	-
SR-04		-	-	-	-	-	1	19	-	20	9.76	2	0.01
SR-05		-	-	-	-	-	-	-	-	-	-	-	-
SR-06		-	-	-	-	-	-	-	-	-	-	-	-
SR-07		-	-	-	-	-	-	-	-	-	-	-	-
SR-08		-	-	-	-	-	1	-	-	1	0.33	1	0.00
SR-09		-	-	-	-	-	-	-	-	-	-	-	-
SR-10		-	-	-	-	-	-	-	-	-	-	-	-
SR-11		-	-	-	-	-	-	-	-	-	-	-	-
SR-12		-	-	-	-	-	-	-	-	-	-	-	-
SR-13		-	-	-	-	-	-	-	-	-	-	-	-
SR-14		-	-	-	-	-	-	-	-	-	-	-	-
SR-16		4	-	-	-	-	2	-	-	6	1.24	2	0.56
SR-17		-	-	-	-	-	-	-	-	-	-	-	-
SR-18		-	-	-	-	-	-	-	-	-	-	-	-
SR-19		-	3	-	-	-	2	-	-	5	1.43	2	0.60
SR-20		-	-	-	-	-	-	-	-	-	-	-	-
SR-21		-	-	-	-	-	-	-	-	-	-	-	-
SR-22		-	-	-	-	-	2	-	-	2	1.00	1	0.00
SH-01		-	-	-	-	-	-	-	-	-	-	-	-
SH-02		-	-	-	-	-	-	-	-	-	-	-	-
SH-03		-	-	2	1	-	34	-	-	37	18.50	3	0.15
SH-04		-	-	-	1	-	12	-	-	13	6.50	2	0.15
SH-06		-	-	1	-	-	30	-	-	31	15.50	2	0.06
SH-09		-	-	-	-	-	9	-	-	9	4.50	1	0.00
SH-11		-	-	1	1	-	82	-	-	84	10.50	3	0.05
SH-12		-	-	-	-	-	9	-	-	9	3.60	1	0.00
SH-14		-	-	-	-	-	11	-	-	11	5.50	1	0.00
SH-15		-	-	-	-	-	8	-	-	8	4.00	1	0.00
SH-16		-	-	-	-	-	53	-	-	53	17.67	1	0.00
SH-17		2	-	-	1	3	3	-	-	9	2.25	4	0.81
SH-18		-	-	3	2	-	-	-	-	5	0.87	2	0.60
SH-19		1	-	17	16	-	61	-	-	95	13.57	4	0.53
SH-20		-	-	-	-	-	-	-	-	-	-	-	-
SH-21		4	-	5	6	1	19	-	1	36	9.00	6	0.68
SH-22		3	-	-	-	-	5	-	13	21	5.25	3	0.57
SH-23		-	-	-	2	-	9	-	-	11	2.75	2	0.33
SH-24		-	-	1	1	-	1	-	-	3	0.60	3	1.00
SH-25		-	-	5	1	-	233	1	3	243	34.71	5	0.08
SH-26		-	-	-	15	-	4	-		19	5.99	2	0.35
Saline River	Total	4	3	-	-	-	8	19	-	34	0.75	4	0.63
	CPUE	0.09	0.07	-	-	-	0.18	0.42	-	-	-	-	-
Smoky Hill River	Total	10	-	35	47	4	583	1	17	697	9.37	7	0.29
	CPUE	0.13	-	0.47	0.63	0.05	7.83	0.01	0.23	-	-	-	-

Table 4. Thermal cycle program (MCRE 52-L) for standard PCR analysis.

Step	Temperature	Time
1.	95°C	3:00
2.	95°C	1:00
3.	55°C	1:00
4.	72°C	2:00
5.	GOTO 2	34X
6.	72°C	10:00
7.	4°C	0:00

Table 5. Master mix solution used during standard PCR reactions for eDNA survey.

Component	µl per well
5X Buffer	4
dNTP	1
Primers	2
Taq	0.2
MgCl2	2
H2O	9.8
eDNA	1

Table 6. NanoDrop results for muscle tissue samples from freshwater mussels collected in the Saline and Smoky Hill rivers in Kansas.

Sample ID	Conc.	A260	A280	260/280
Mapleleaf 1	121.6	2.431	1.194	2.04
Mapleleaf 2	120.8	2.416	1.197	2.02
Fragile Papershell 1	57.5	1.149	0.577	1.99
Fragile Papershell 2	21.1	0.422	0.209	2.02
Pink Papershell	216.5	4.331	2.153	2.01
Cylindrical Papershell	104.5	2.090	1.024	2.04
Lilliput	303.3	6.066	2.864	2.12
Paper Pondshell 1	90.7	1.814	0.916	1.98
Paper Pondshell 2	125.9	2.519	1.273	1.98
White Heelsplitter 1	120.3	2.407	1.305	1.84
White Heelsplitter 2	140.7	2.814	1.517	1.86
Giant Floater 1	116.0	2.319	1.181	1.96
Giant Floater 2	159.5	3.191	1.623	1.97

Table 7. Master mix solution used during real-time PCR reactions for eDNA survey.

Component	µl per well
SYBR Green	5
Primers	0.5
H2O	2.5

Figure 2. Program (eDNA _Sybr_50Cycle) used during Real-Time PCR Assays.

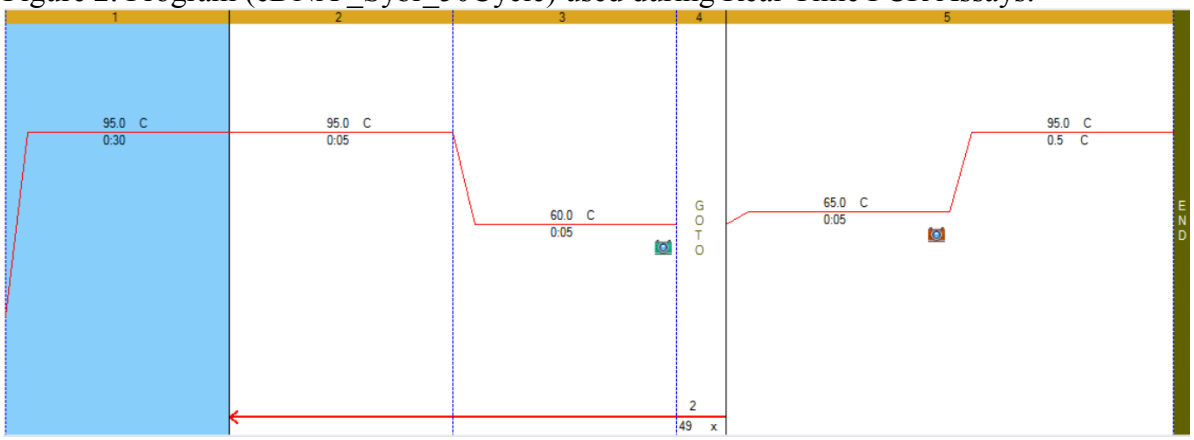
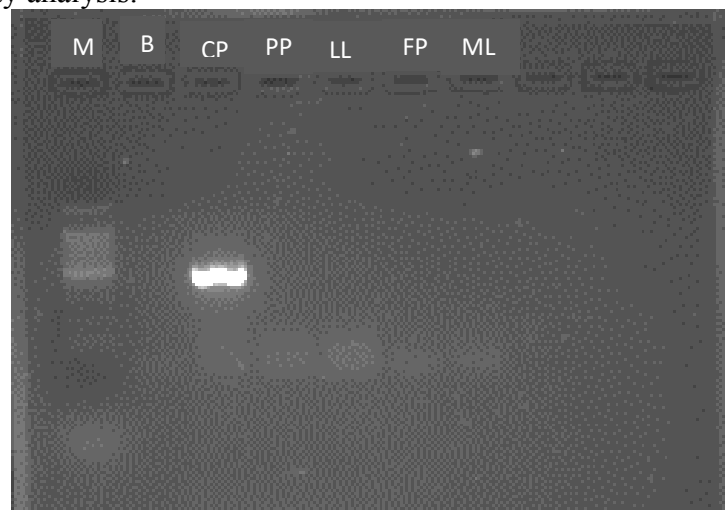
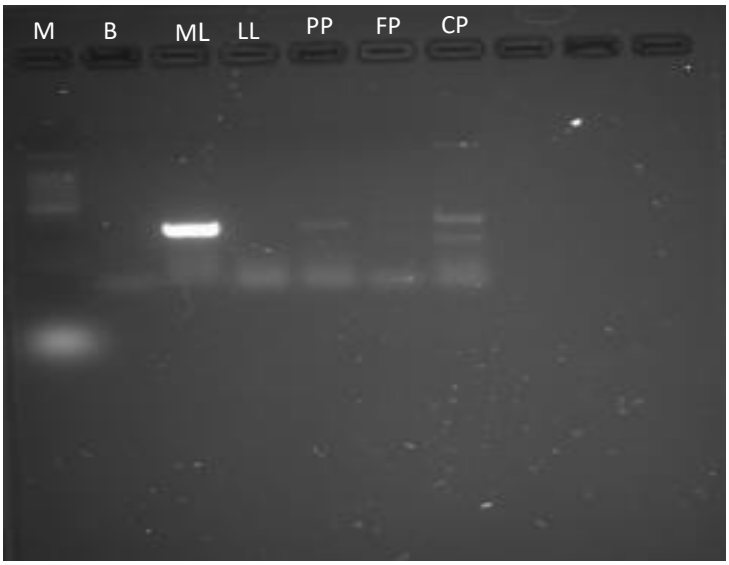


Figure 3. Results for gel electrophoresis of PCR product for Cylindrical Papershell primer specificity analysis.



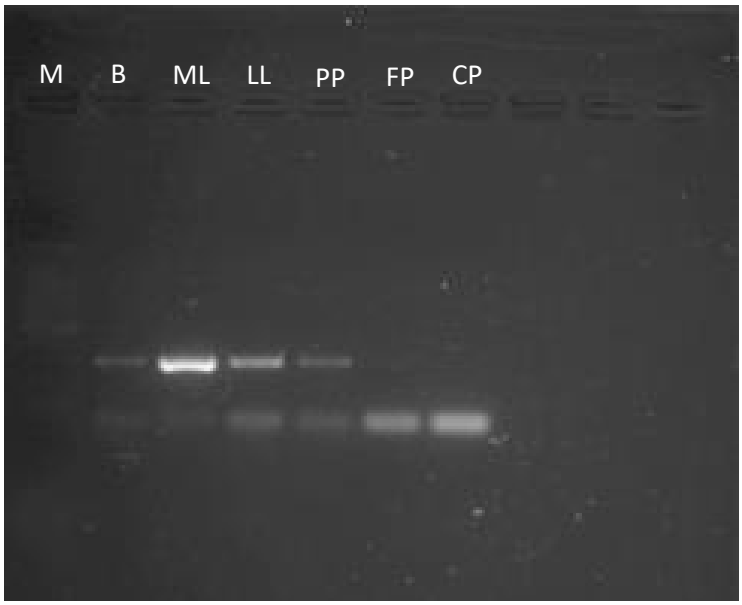
M= Marker, B= Blank, CP= Cylindrical Papershell, PP= Pink Papershell, LL= Lilliput, FP= Fragile Papershell, ML= Mapleleaf.

Figure 4. Results for gel electrophoresis for PCR product for Mapleleaf primer specificity analysis.



M= Marker, B= Blank, ML= Mapleleaf, LL= Lilliput, PP= Pink Papershell, FP= Fragile Papershell, CP= Cylindrical Papershell.

Figure 5. Results for gel electrophoresis for PCR product for Mapleleaf primer specificity-2.



M= Marker, B= Blank, ML= Mapleleaf, LL= Lilliput, PP= Pink Papershell, FP= Fragile Papershell, CP= Cylindrical Papershell.

Figure 6. RT-PCR assay of Cylindrical Papershell DNA dilutions.The straight line is 10ng/µL diluted to 0.1pg/µL represented by the diamonds. Displaying results for 1 µL of primer per well.

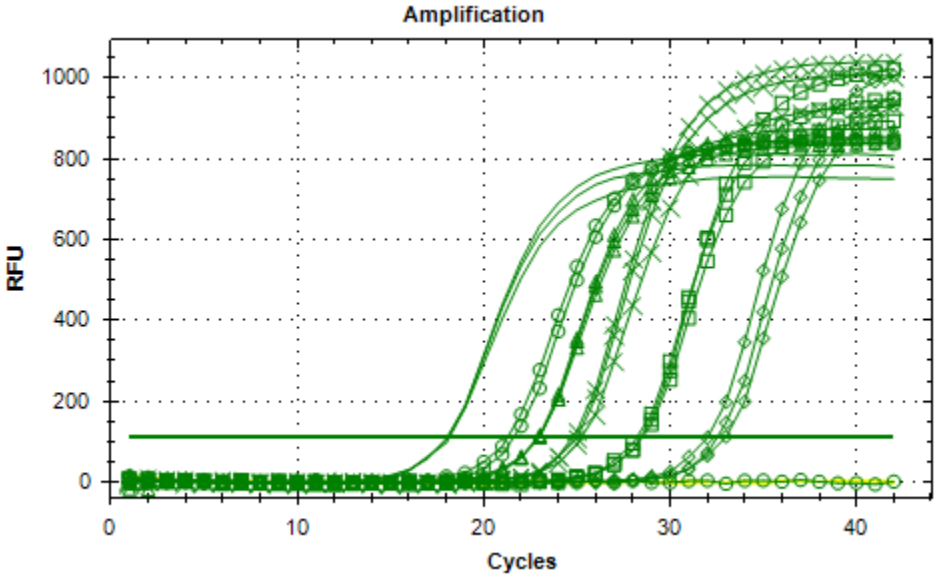


Figure 7. Standard curve for Cylindrical Papershell primer assay.

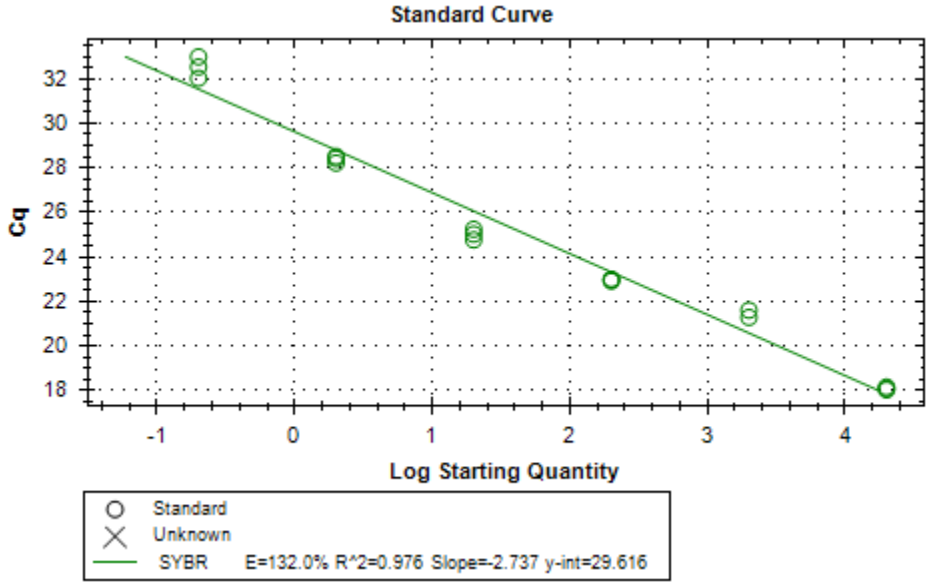


Figure 8. RT- PCR assay for Cylindrical Papershell primer specificity. Cylindrical Papershell DNA is represented in green. The straight line is 10 ng/µL and the circles are 10 pg/µL.

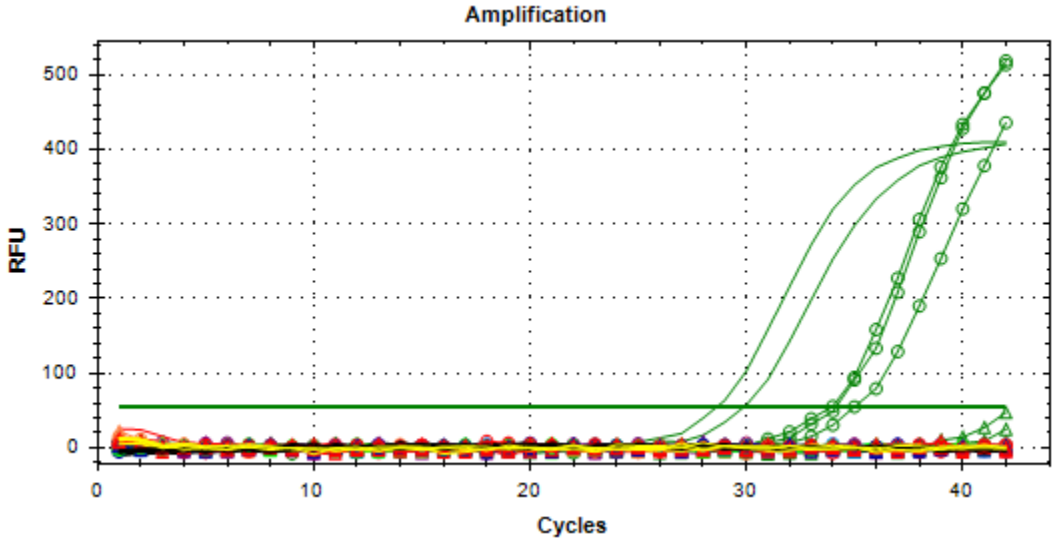


Figure 9. RT-PCR assay for Mapleleaf DNA dilutions. The straight line is 10ng/µL diluted to 0.1pg/µL represented by the diamonds. Displaying results for 0.5 µL of primer per well.

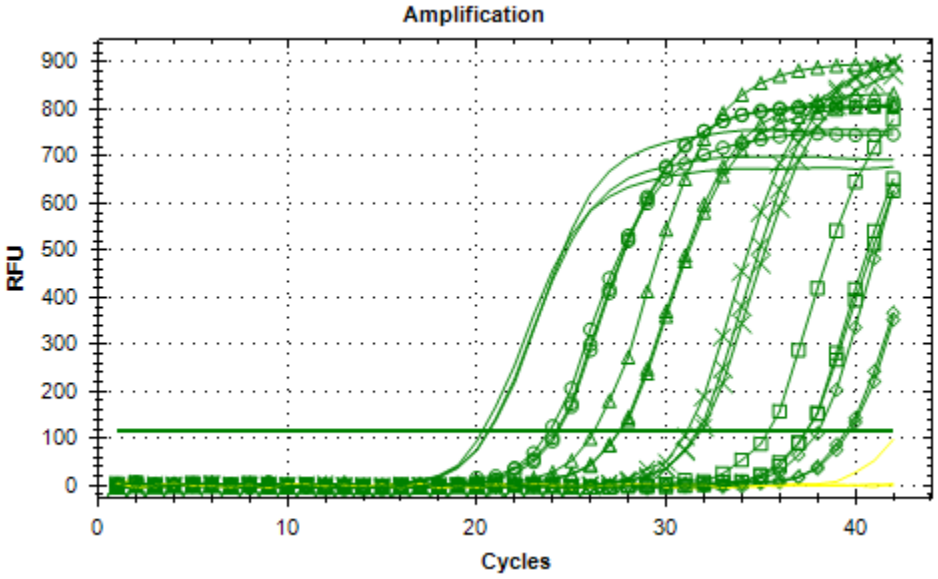


Figure 10. Standard Curve of RT-PCR Analysis for the Mapleleaf primer.

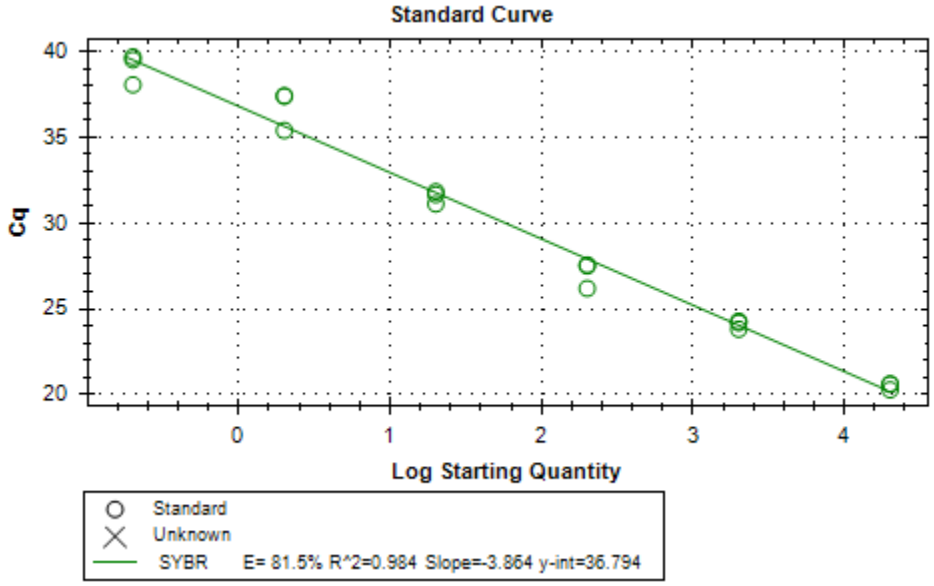


Figure 11. RT-PCR assay for Mapleleaf primer specificity. Mapleleaf DNA represented in pink, Paper Pondshell DNA represented in purple, Fragile Papershell represented in green,White Heelsplitter represented in maroon, and Giant Floater represented in blue.

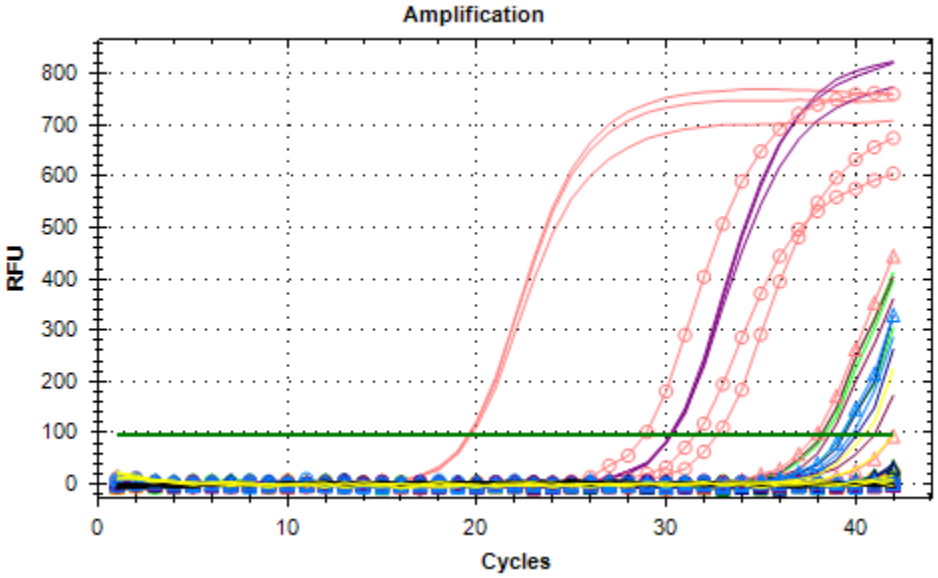


Figure 12. Results for gel electrophoresis of SR16-2 PCR product.

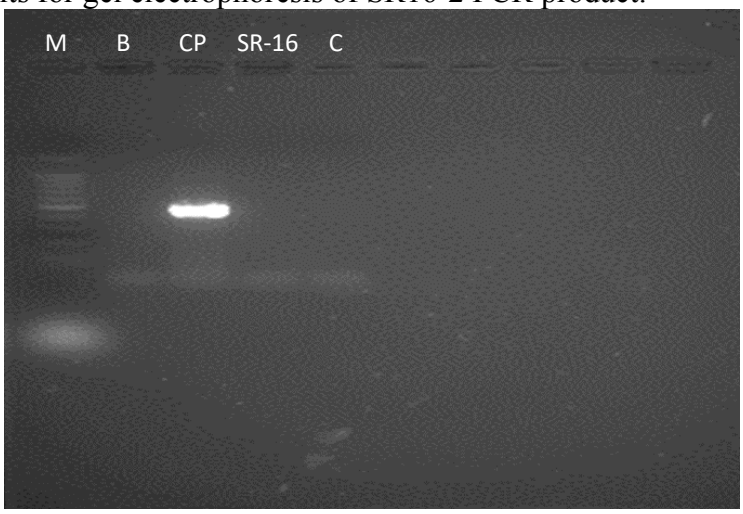


Figure 13. Results for gel electrophoresis of SH25-1 PCR product.

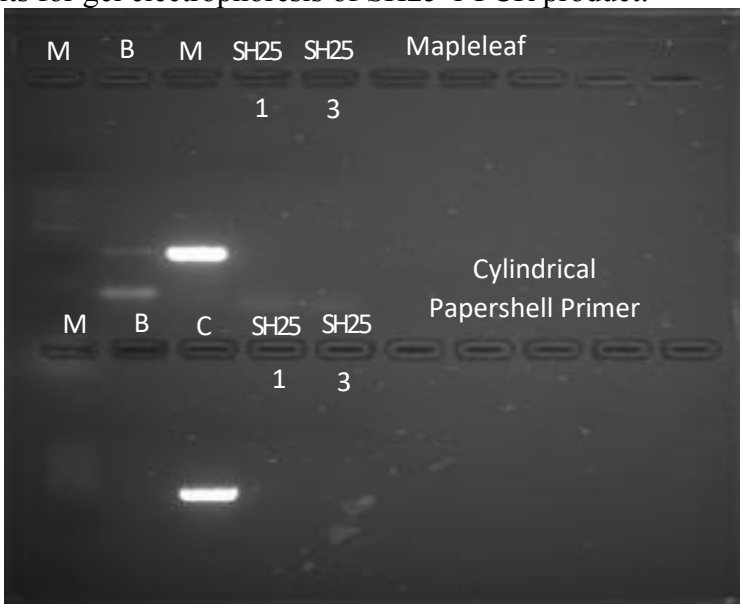


Figure 14. Results for gel electrophoresis of SH25-1 degradation PCR Product.

Figure 15. RT-PCR assay of eDNA using the Cylindrical Papershell primer. Known Cylindrical Papershell DNA represented in green, SR16-C in maroon, SH19-C in blue, and SH 17-C in orange.

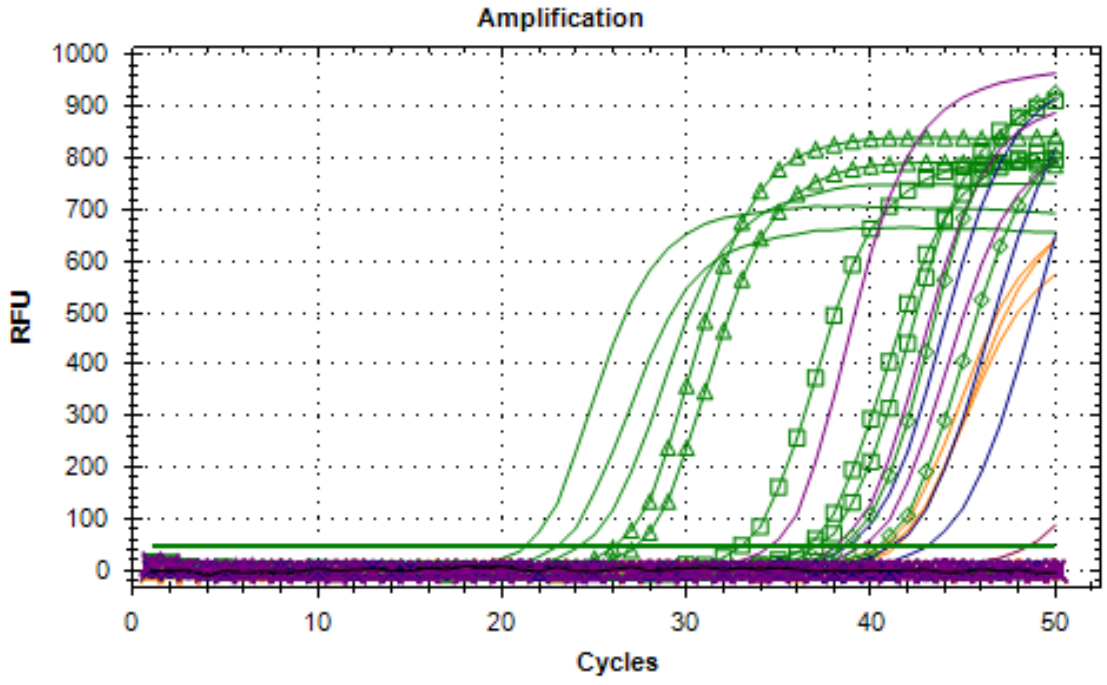


Figure 16. RT-PCR assay of eDNA samples spiked with Cylindrical Papershell DNA and diluted, unspiked eDNA samples using the Cylindrical Papershell Primer. Unspiked SH22 was amplified, as well as spiked SH17 and SH22.

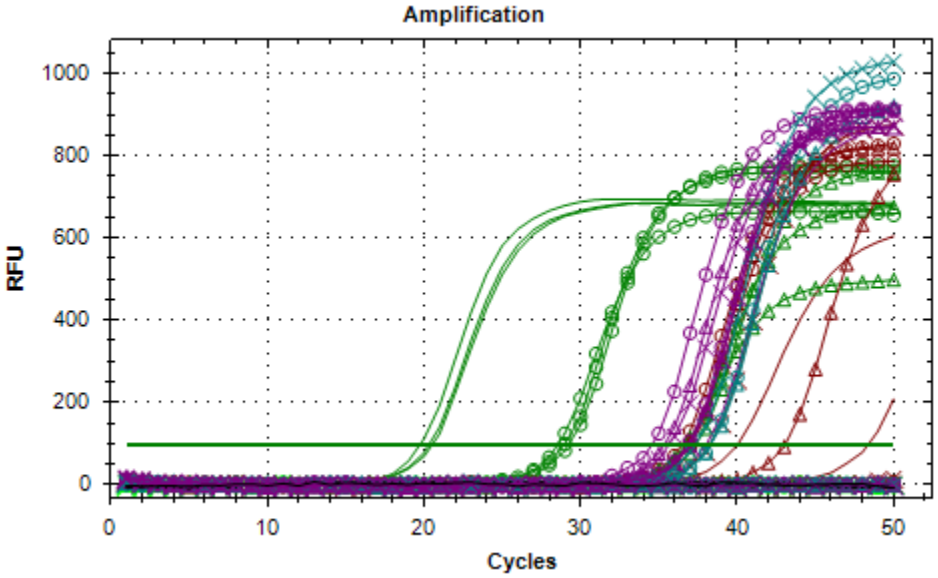


Figure17. RT-PCR assay of eDNA pooled dilution samples using the Cylindrical Papershell Primer.

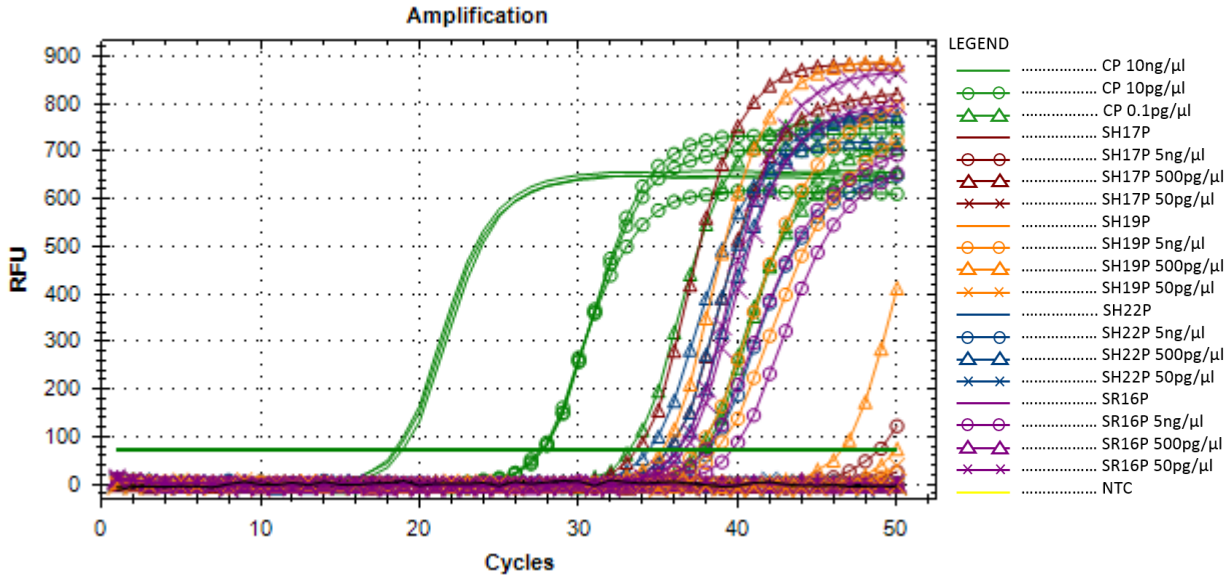


Figure18. RT-PCR assay of eDNA SH21 B-I samples using the Cylindrical Papershell primer. DNA is represented in green, SH21 eDNA in purple.

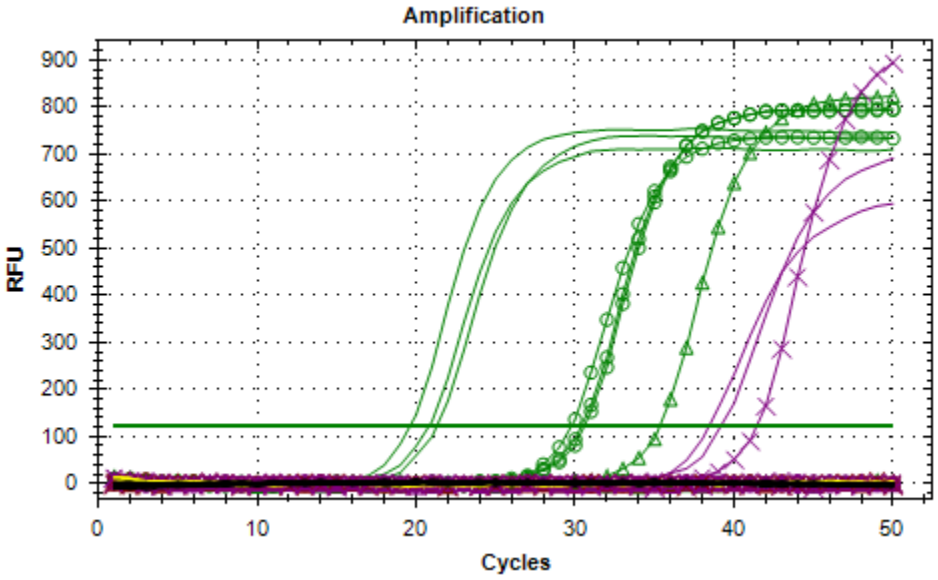


Figure 19. RT-PCR assay of 2017 eDNA samples using the Cylindrical Papershell Primer. Cylindrical Papershell DNA is represented in green and no eDNA was amplified.

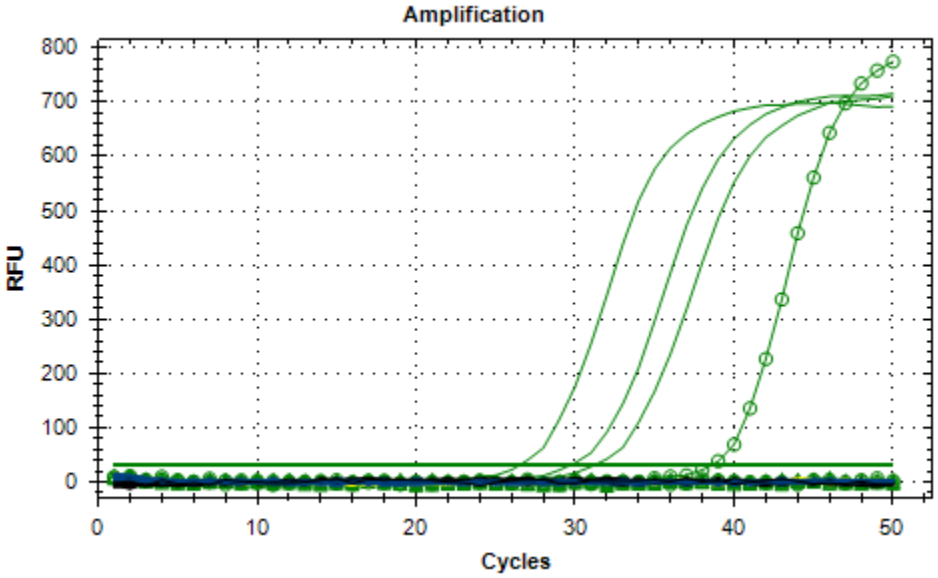


Figure 20. Hydrograph for the Smoky Hill River near Schoenchen, Kansas during 2015 water collection.

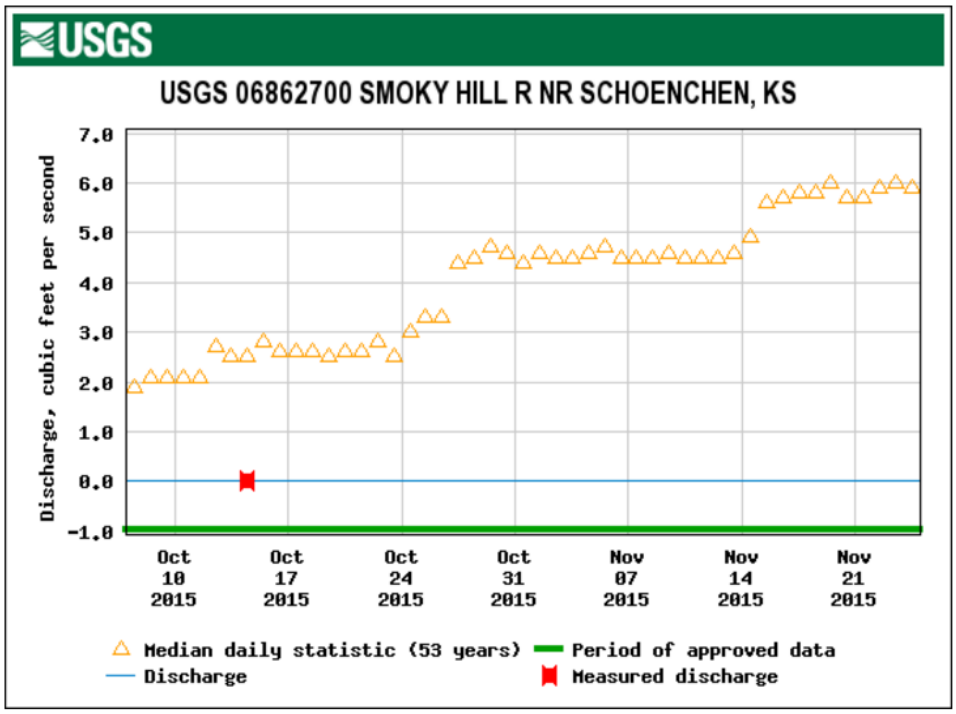


Figure 21. Hydrograph for the Smoky Hill River near Russell, Kansas during 2015 water collection

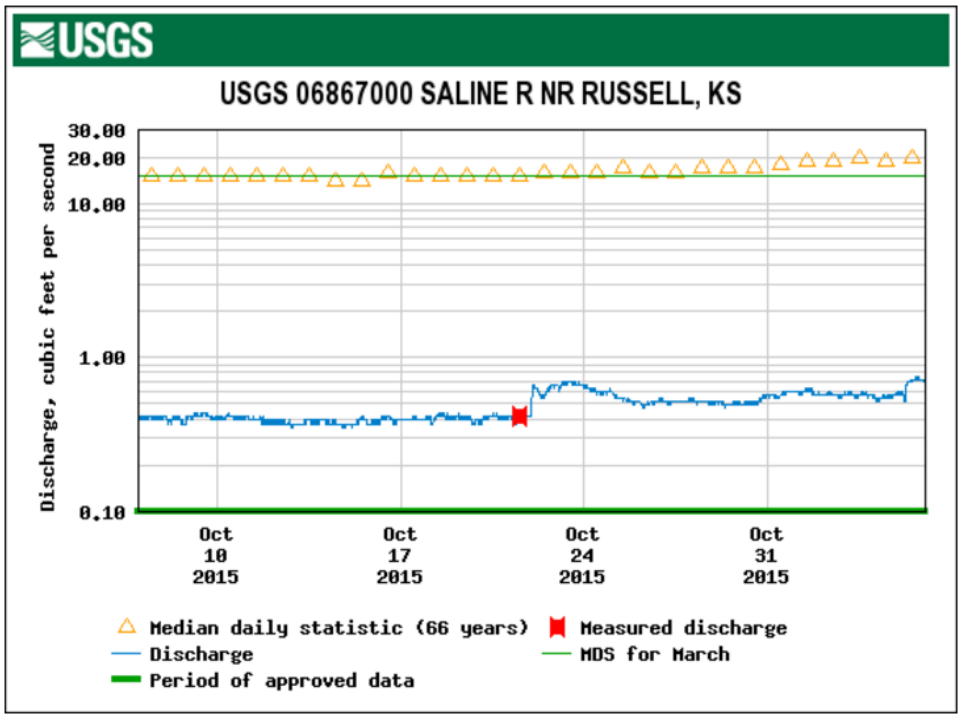
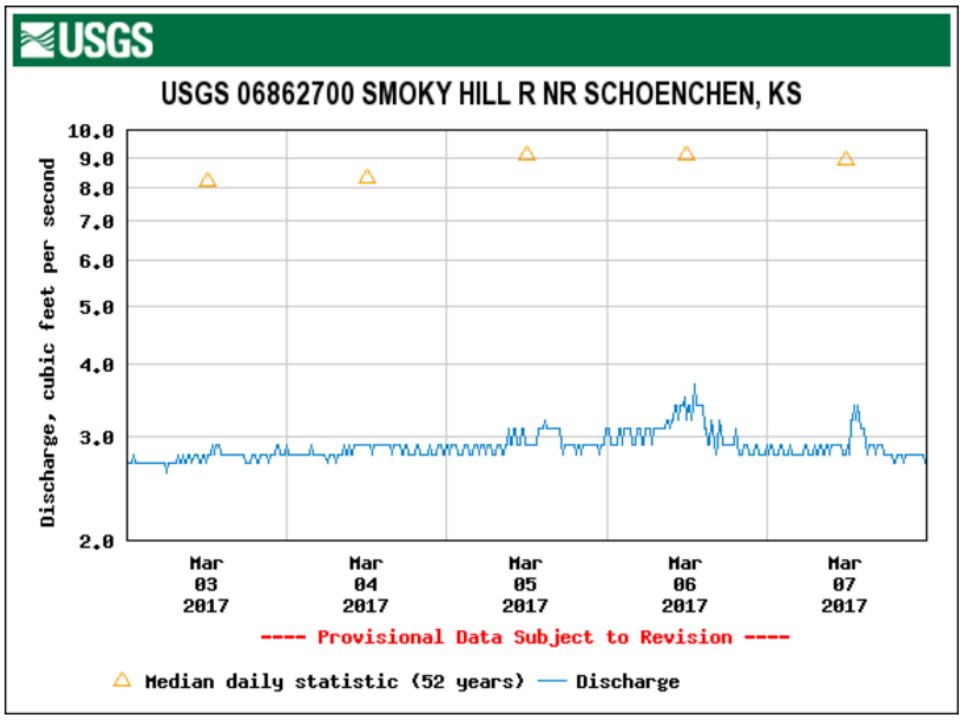


Figure 22. Hydrograph for the Smoky Hill River near Schoenchen, Kansas during 2017 water collection.



Appendix 1. Extracted eDNA samples, extraction technique used, and NanoDrop results. Water samples were collected from the Saline and Smoky Hill Rivers, Kansas.

Sample ID	Date of Collection	Date of Extraction	DNA Extraction Technique	DNA Conc. (ng/µl)	260/280
SR16-2	10/28/2015	4/11/2016	MP Fast DNA Spin	-7.8	1.69
SR16-2-1	10/28/2015	4/18/2016	Q. Dneasy B&T	255.9	1.54
SR16-2-2	10/28/2015	4/18/2016	Q. Dneasy B&T	176.3	1.53
SH25-1-1	11/03/2015	5/2/2016	Q. Dneasy B&T/ QS	188.9	1.85
SH25-1-3	11/03/2015	5/02/2016	Q. Dneasy B&T/ QS	47.6	1.74
SH21-C	11/02/2015	5/12/2016	Q. Dneasy B&T/ QS	-12.5	1.97
SH21-1	11/02/2015	5/12/2016	Q. Dneasy B&T/ QS	410.9	1.67
SH21-2	11/02/2015	5/12/2016	Q. Dneasy B&T/ QS	885.2	1.72
SH21-3	11/02/2015	5/12/2016	Q. Dneasy B&T/ QS	682.5	1.42
SH25-C	11/03/2015	5/16/2016	Q. Dneasy B&T/ QS	-10.3	2.44
SH25-2	11/03/2015	5/16/2016	Q. Dneasy B&T/ QS	67.0	1.74
SH25-3	11/03/2015	5/16/2016	Q. Dneasy B&T/ QS	159.7	1.70
SH18-C	10/27/2015	7/21/2016	Q. Dneasy B&T/ QS	-12.0	2.00
SH18-1	10/27/2015	7/21/2016	Q. Dneasy B&T/ QS	308.7	1.94
SH18-2	10/27/2015	7/21/2016	Q. Dneasy B&T/ QS	364.5	1.88
SH18-3	10/27/2015	7/21/2016	Q. Dneasy B&T/ QS	277.4	1.94
SH6-C	10/13/2015	7/22/2016	Q. Dneasy B&T/ QS	-10.3	2.24
SH6-1	10/13/2015	7/22/2016	Q. Dneasy B&T/ QS	72.4	1.47
SH6-2	10/13/2015	7/22/2016	Q. Dneasy B&T/ QS	228.3	1.72
SH6-3	10/13/2015	7/22/2016	Q. Dneasy B&T/ QS	169.3	1.79
SH3-C	10/21/2015	7/12/2016	Q. Dneasy B&T/ QS	-9.9	2.08
SH3-1	10/21/2015	7/12/2016	Q. Dneasy B&T/ QS	481.8	1.75
SH3-2	10/21/2015	7/12/2016	Q. Dneasy B&T/ QS	387.4	1.75
SH3-3	10/21/2015	7/12/2016	Q. Dneasy B&T/ QS	331.7	1.85
SH17-C	10/07/2015	8/31/2016	Q. Dneasy B&T/ QS	-19.8	2.21
SH17-1	10/07/2015	8/31/2016	Q. Dneasy B&T/ QS	606.2	1.80
SH17-2	10/07/2015	8/31/2016	Q. Dneasy B&T/ QS	207.9	1.55
SH17-3	10/07/2015	8/31/2016	Q. Dneasy B&T/ QS	508.3	1.73
SH19-C	10/13/2015	9/09/2016	Q. Dneasy B&T/ QS	-17.6	2.52
SH19-1	10/13/2015	9/09/2016	Q. Dneasy B&T/ QS	171.8	1.56
SH19-2	10/13/2015	9/09/2016	Q. Dneasy B&T/ QS	225.4	1.55
SH19-3	10/13/2015	9/09/2016	Q. Dneasy B&T/ QS	122.3	1.62
SH22-C	11/24/2015	9/13/2016	Q. Dneasy B&T/ QS	-14.6	2.01
SH22-1	11/24/2015	9/13/2016	Q. Dneasy B&T/ QS	167.0	1.68
SH22-2	11/24/2015	9/13/2016	Q. Dneasy B&T/ QS	215.6	1.73
SH22-3	11/24/2015	9/13/2016	Q. Dneasy B&T/ QS	85.2	1.66
SR16-C	10/28/2015	10/04/2016	Q. Dneasy B&T/ QS	-24.1	1.77
SR16-1	10/28/2015	10/04/2016	Q. Dneasy B&T/ QS	114.5	1.67
SR16-2	10/28/2015	10/04/2016	Q. Dneasy B&T/ QS	147.5	1.67
SR16-3	10/28/2015	10/04/2016	Q. Dneasy B&T/ QS	178.0	1.66
SH11-C	10/26/2015	12/13/2016	Q. Dneasy B&T/ QS	-0.1	0.30
SH11-1	10/26/2015	12/13/2016	Q. Dneasy B&T/ QS	69.8	0.49
SH11-2	10/26/2015	12/13/2016	Q. Dneasy B&T/ QS	84.5	1.80
SH11-3	10/26/2015	12/13/2016	Q. Dneasy B&T/ QS	31.2	1.47
SH21-1	11/02/2015	2/27/2017	Boiled in TE Buffer	38.6	1.53
SH21-2	11/02/2015	2/27/2017	Boiled in TE Buffer	39.5	1.54
SH1-2017-pool	3/3/2017	3/03/2017	Boiled in TE Buffer	55.8	1.54
SH22-2017-pool	3/4/2017	3/04/2017	Boiled in TE Buffer	51.2	1.47
SH21-2017-pool	3/6/2017	3/06/2017	Boiled in TE Buffer	69.9	1.48
SH19-2017-pool	3/7/2017	3/07/2017	Boiled in TE Buffer	61.3	1.54