INVESTIGATING USE OF ENVIRONMENTAL DNA (eDNA) IN MICHIGAN STREAMS TO DETECT FERAL SWINE (*Sus scrofa*)

Amberly N. Hauger

Master's Degree Thesis

Submitted to

University of Michigan - Flint

In partial fulfillment of the requirements

For the degree of

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Department of Biology

2019

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BY

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Invasive feral swine can damage ecosystems, disrupt plant and animal populations, and transmit diseases. Monitoring feral swine populations depends on expensive and/or labor intensive techniques such as aerial surveys, field surveys for sign, trail cameras, and landowner reports. Environmental DNA (eDNA) provides an alternative method for locating feral swine. From May 2017 to April 2018, domestic or feral swine DNA was artificially introduced into Bluff and Black Creeks in mid-Michigan. Water samples and data on various stream variables were collected at the location of DNA insertion and along a 400 m transect to investigate factors affecting swine DNA detection. Top models portraying presence of swine eDNA were selected based on Akaike's Information Criterion corrected for low sample sizes (AIC_c). Model-averaging was used to portray parameter estimates from competing models, which included season, collection method, distance downstream from DNA introduction, water temperature, water velocity, and stream type. Two variables significantly affected detection of swine DNA, season and distance from the insertion point. Detection of swine DNA and season had an inverse relationship; odds of detecting swine eDNA decreased 54% each time the seasons changed from winter to spring, spring to summer, and summer to fall. As distance increased downstream from the introduced source point, the odds of DNA detection increased by 111% every 100 m (sampled up to 400 m) beyond the insertion point. The research confirmed swine DNA can be detected in streams up to 400 m from the source point and was influenced by season. Future eDNA sampling for feral swine should occur in the winter or spring. The results provide an

important foundation for field application of utilizing eDNA as an alternative technique to monitor feral swine in northern regions of the US.

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Literature Review

In the early 1500s, domestic swine (*Sus scrofa)* were introduced into the US by Spanish explorer Hernando de Soto from Europe as a food source (Wood and Barrett 1979, USDA 2016). In 1890, 13 Russian boar from Germany were introduced into New Hampshire and confined to a 58 km enclosure as a personal wild game preserve (Mayer 2009). Russian boar have since been used as an alternative game species by ranchers and sportsman over the last 100 years, in both fenced and free ranging expanses (Fogarty 2007, Centner and Shuman 2015). Invasive feral swine, particularly at high densities, harm plant and animal populations, disrupt natural ecosystems, and transmit diseases to livestock, pets and humans (Hunter 2012). Feral swine damage agriculture, natural resources, and private property through direct consumption of crops and plant communities, and damage from rooting, foraging, wallowing and soil compaction. Areas affected by feral swine may become more susceptible to erosion and introduction of nonnative or invasive species (USDA 2015). At one time, the Michigan Department of Natural Resources (MDNR) estimated 1,000-3,000 feral swine in the state, with sightings reported in 72 of 83 counties (USDA 2017). Applied research, intensive lethal control, and legislation outlawing ownership of Russian boar in Michigan drastically reduced populations in core areas in the Lower Peninsula (D.R. Etter, MDNR, personal communication).

Four stages of species invasion exist; transport, establishment, spread, and impact (Lockwood et al. 2013). After an invasive species is transported from native range and introduced into a new location, the invasive species can either become established as a self-sustaining population or fail. Once the population is established, the species either remains in a localized area or spreads to new geographical locations. Feral swine exhibit

rapid spread, have a competitive advantage over native species, and can obtain self-sustaining populations easily; reflecting classic characteristics of an invasive species (Valéry et al. 2008, Snow et al. 2017). Thirty years ago, feral swine were established in only 17 states, but in the last three decades populations expanded throughout a large portion of the continental US and into Canada.

The preferred outcome for controlling invasive species once they are established is to remove individuals and limit spread. However, as the population is reduced, successful eradication relies on detection of individuals at low densities (Mehta et al. 2007, Jerde et al. 2011, Dejean et al. 2012). Historically, detecting invasive feral swine in Michigan relied on reports from hunters and the public, by contacting landowners, through aerial surveys, monitoring bait sites, intensive field searches for sign (e.g. tracks, feces, wallows, rooting) trapping, and using trail cameras (D.R. Etter, MDNR, personal communication). All of these methods are labor intensive and do not consistently produce reliable information about the status of feral swine in a particular area. Agencies responsible for controlling feral swine need alternative techniques to more efficiently detect and monitor landscapes for feral swine.

Environmental DNA (eDNA) provides an alternative surveillance method for species at low densities or in early stages of invasion (Dejean et al. 2012). eDNA is genetic material obtained directly from environmental samples (e.g. soil, sediment, water) without obvious signs of the biological source (Thomsen and Willerslev 2015). Potential sources of DNA include feces, hair, saliva, urine, feathers, and shed skin cells (Waits and Paetkau 2005). eDNA has been used successfully to detect Burmese pythons (*Python bivittatus)* in southern Florida waterways (Piaggio et al. 2014), and silver and bighead (Asian) carp (*Hypophthalmichthys nobilis* and *H. molitrix*) in the Great Lakes (Jerde et al.

2011). Advantages of using eDNA for surveillance include reduced field efforts, refinement of a specific search area, cost effectiveness, and non-invasiveness (i.e., does not require locating or handling live animals; Bohmann et al. 2014, Thomsen and Willerslev 2015). Environmental DNA provides wildlife managers the option to monitor invasive species populations beyond traditional methods with increased detection sensitivity, limiting both monitoring and analytical costs, and increasing specificity of target species identification (Darling and Mahon 2011).

Integration of molecular eDNA techniques with aquatic ecology allows for improved detection of rare or endangered species at low densities, as well as early invasions of non-native species into aquatic ecosystems (Thomsen et al. 2012, Goldberg et al. 2013, Jerde et al. 2013, Balasingham et al. 2016). Aquatic organisms, such as fish and amphibians, are at the forefront of eDNA development and detection techniques in controlled and field experiments (Jerde et al. 2011, Goldberg et al. 2013, Roussel et al. 2015). Balasingham et al. (2016) used eDNA to determine movement and retention of Atlantic salmon (*Salmo salar*) in a flowing water system two hours after source DNA was removed, providing an accurate spatial identification of target species location. Others examined relationships between eDNA concentration and biomass or abundance for both fish and amphibians. Takahara et al. (2012) and Pilliod et al. (2013) reported a positive correlation between biomass or abundance and eDNA concentrations in water samples for fishes and amphibians.

Few studies researched transportation and detection probability of mammalian eDNA in aquatic ecosystems (Roussel et al. 2015). One study in Europe used eDNA to survey six different threatened freshwater animal species (amphibians, fish, mammals, insects and crustaceans) including Eurasian otter (*Lutra lutra*), a semi-aquatic mammal,

throughout natural lakes, ponds, streams, and temporary pools (Thomsen et al. 2012). Results indicated a lower detection probability for mammals compared to five other taxonomic groups, potentially attributed to the semi-aquatic lifestyle and large territorial range of Eurasian otter (Thomsen et al. 2012). Foote et al. (2012) conducted research in both natural and controlled settings to detect harbor porpoise (*Phocoena phocoena*) eDNA in a marine ecosystem. Static acoustic monitoring devices recorded echolocation clicks to confirm porpoise presence that was then compared to eDNA data. Harbor porpoise eDNA was successfully detected in all samples collected from control locations, but results from samples collected from random field locations were less conclusive. Detection was less reliable in random field locations, and hence acoustic monitoring was deemed more successful for harbor porpoise detection (Foote et al. 2012). Terrestrial mammal eDNA was monitored from a drinking water source in a captive coyote (*Canis latrans*) research facility (Rodgers and Mock 2015). Coyote eDNA was successfully detected in drinking water sources, however when considering natural drinking water resources eDNA detection was affected by environmental factors and water source characteristics (Rodgers and Mock 2015). Additionally, rate of DNA degradation in aquatic systems is influenced by environmental factors and can vary from system to system (Barnes et al. 2014, Deiner and Altermatt 2014).

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Chapter 1

INVESTIGATING USE OF ENVIRONMENTAL DNA (eDNA) IN MICHIGAN STREAMS TO DETECT FERAL SWINE (*Sus scrofa)*

Introduction

Invasive feral swine (*Sus scrofa*) harm plant and animal populations, disrupt natural ecosystems, and transmit diseases to livestock, pets, and humans (Hunter 2012). Historically, detection of invasive feral swine in Michigan relied on reports from hunters and the public, by contacting landowners, and through aerial surveys, monitoring bait sites, intensive field searches for sign (e.g. tracks, feces, wallows, rooting), trapping, and trail camera monitoring. These methods are labor intensive and do not consistently produce reliable information about status of feral swine in a particular area.

Michigan researchers successfully captured feral swine, but it was difficult to locate and collar individuals. Corral trapping was the most successful technique, but involved intensive field scouting, movement of equipment to build corral traps, baiting, and monitoring swine activity with a trail cameras. Over three years of field research aimed at capturing feral swine in Michigan only 18 animals were captured and eight of those were collared. Density estimates of feral swine were lower than anticipated and resulted in few animals being captured (D.R. Etter, Michigan Department of Natural Resources, personal communication).

Expansion and establishment of invasive feral swine in Michigan would cause detrimental ecological and economic impacts in the state (Snow et al. 2017). Satellite telemetry data from feral swine research in Michigan documented large home-ranges, presence in a wide variety of cover types including agriculture and wetlands, and animals frequently crossing open water (D.R. Etter, Michigan Department of Natural Resources, personal communication). Using environmental DNA (eDNA) as a monitoring tool for

feral swine may allow wildlife managers to pinpoint remaining small groups of individuals across the landscape to focus eradication efforts. Agencies responsible for controlling feral swine need alternative techniques to efficiently detect and monitor landscapes for feral swine to prevent establishment and spread. Recent advancements in molecular techniques offer alternatives for documenting animal presence by utilizing a DNA signature in an environmental sample. However, the ability to detect and quantify animal DNA from freshwater is determined by the relationship between DNA excretion and degradation (Lindahl 1993), coupled with the ability to acquire that DNA through field sampling. Williams et al. (2016) developed laboratory techniques to detect swine eDNA from wild pig wallows in a controlled experiment conducted in a livestock facility. However, this technique has not been replicated in field conditions with variable water flow, chemistry, substrates, and environmental conditions.

This research utilized eDNA technology to parameterize detection of swine DNA in free-flowing aquatic systems. Water temperature, velocity, pH, and turbidity were evaluated relative to the detection of swine DNA (Table 1). The objectives of this study were to: (1) determine whether swine DNA could be detected in free-flowing streams; (2) parameterize detection probability of swine DNA in natural stream systems by season, distance, collection method, and selected stream variables; and (3) recommend sampling techniques for future feral swine eDNA surveillance in Michigan. Results from this research will assist in developing future feral swine monitoring protocols for wildlife managers and feral swine control agencies.

Methods and Materials

Study Sites

The study was conducted in two free-flowing streams (Bluff and Black Creeks) in Midland County, Michigan, from May 2017 to April 2018 on public and private lands (Figure 1). Lowland cover types included lowland shrubs, marshes, wetlands, beaver ponds, and swamp hardwoods interspersed with upland oak and aspen stands. The two watersheds have low topographic relief (199.4 m-195.7 m; MDNR 2016). Bluff Creek is characterized as uniform and free-flowing with little undergrowth, a full overstory canopy, and small floodplain with steep sloping hills that parallel the stream. Black Creek has a wide floodplain with small floodings, multiple water channels when the water table is high, and thick understory and brush. Permission for access to private lands was obtained from landowners and public land access was obtained from the Michigan Department of Natural Resources (MDNR; state land use permit #73-2017-013).

The study areas were selected due to similarity in water resources and forest cover features known to be used by feral swine in Michigan based on previous GPS research (D.R. Etter, MDNR, personal communication). Past research acquired 51,984 GPS points recorded over three years in the general area the study sites from eight collared feral swine. Marked feral swine were located within 400 m of creeks, streams, or rivers approximately 36% of the time. The data further suggested that collared feral swine crossed a creek, stream, or river, at least 888 times, or roughly every 1.2 days. Bluff and Black Creeks were selected as study sites because feral swine were documented to use similar habitats in Michigan and the two streams have contrasting characteristics and riparian areas.

Monitoring for Feral Swine

Absence of feral swine within the study sites was essential for study success because the objectives were to determine factors affecting detectability of feral swine eDNA from an introduced source at a known points in two streams. If feral swine were present in either watershed additional sources of eDNA could be introduced and results misrepresented. Previous research monitored the study area since 2013, with no evidence of feral swine reported to date (D.R. Etter, MDNR, personal communication). I also used other techniques to monitor for feral swine activity from November 2016 to April 2018. Surveys were conducted along each stream for feral swine sign (tracks, scat, rooting, wallowing; Mayer 2009) one week prior to DNA insertion and water sampling. Additionally, ten trail cameras were placed up and downstream from the point of DNA insertion (source point) at each study site (Bushnell® Trophy Cam HD Aggressor 14MP Trail Camera, Bushnell Outdoor Products, Overland Park, Kansas). Ten additional cameras were set in the Mud Creek watershed, located between Bluff and Black Creek watersheds, for additional surveillance. Cameras were spaced 400 m apart, over a total distance of 4000 m, and locations were based on wildlife river crossings, lowland areas, or beaver dams. Trail cameras were checked once every two weeks for photos of feral swine. A control water sample was collected at time=0 (T=0; source point of swine DNA introduction) from each stream before swine DNA was artificially introduced to also confirm absence of swine DNA.

Field Sampling Methods and Materials

Sampling locations for eDNA within each watershed were determined by the midpoint of the trail cameras. This process also provided equal distribution of cameras up and downstream of the source point. The midpoint was at the 200 m mark of the eDNA sampling transect (Figure 2). If a road or bridge occurred at the midpoint, the

location was moved upstream so the midpoint ended greater than or equal to 100 m away from the man-made structure.

T-posts were placed on either side of the stream to mark the location for source point swine DNA artificial insertion. A rope pulley system was assembled between the T-posts. Additionally, one T-post was placed every 100 m for a total of 400 m to mark the start location of each 100 m segment. Flagging was used along the stream bank to mark 10 m increments within each segment and recorded with a GPS (Garmin GPS 12 XL, Garmin Ltd., Olathe, Kansas; Figure 2).

Currently, feral and domestic swine DNA cannot be differentiated on the molecular level in eDNA samples (A.J. Piaggio, National Wildlife Research Center, personal communication). Therefore, introduced swine (feral or domestic) DNA specimens (legs and/or hides) were acquired from United States Department of Agriculture – Wildlife Services (USDA) in Michigan or from a swine processing facility. Each specimen was thawed for 24 hours. The swine specimens were attached with zip ties to the rope pulley system between the T-posts at the source point. The chosen swine specimens (leg/hide) were used to simulate swine crossing a stream while introducing DNA into the watershed. Swine specimens were introduced into the stream by pulling them through the water four times to simulate all four legs of a feral swine walking across the stream. The swine specimens were used only once and disposed of by MDNR.

Water samples were collected during each DNA exposure trial to examine seasonal variation in stream transportation of eDNA. Seasons were designated as winter (December 21-March 20), spring (March 21-June 20), summer (June 21-September 20), and fall (September 21-December 20). Samples were collected every 2-3 weeks with at least 18 days between each DNA insertion based on reported DNA preservation and

degradation (Dejean et al. 2011, Thomsen et al. 2012, Piaggio et al. 2014). Water samples were collected from the middle of the stream water column (Goldberg et al. 2013) using a telescopic dipper (1.5-3.5 m Conbar Telescopic Dipper, Forestry Suppliers Inc., Jackson, Mississippi). The ladle was dipped in an upstream motion while collecting the minimum amount of water needed (~50 ml) and the remaining stream water was placed in an 18.9 L bucket. To avoid cross contamination of samples between subsequent samples and each 100 m segment, the telescopic dipper was sterilized with a 20% bleach solution then rinsed multiple times with distilled water into the 18.9 L bucket. The dipper was dried with paper toweling. The telescopic dipper and handle were also sterilized prior to sampling to limit contamination between study sites. The displaced water amount was recorded and discarded away from the stream after sample collection was completed.

Nalgene bottles were the only collection method used at the start of this research project based upon previous published research as of May 2017 (Piaggio et al. 2014, Williams et al. 2017). After the Central Michigan University (CMU) laboratory processed the first round of water samples they made a recommendation to use Sterivex filters as a collection method. The change was made to increase detection probability, limit contamination, and easier to implement in the field. Collection processes were updated in September 2017 and fully implemented by October 2017 using the sampling procedure described below.

Prior to swine DNA being manually introduced into streams, 10 (50 ml or 500 ml total) samples were collected in Nalgene bottles (Thermo Scientific™ Nalgene™ 60 mL Wide-Mouth HDPE Packaging Bottles with Closure, Fisher Scientific, Pittsburgh, Pennsylvania); and designated T=0, to serve as the control sample at the swine DNA

introduced source point (0 m; Figure 2). Sample locations were recorded using a GPS to ensure sampling occurred at the same location throughout the entire study. To determine eDNA detection at the source point immediately after DNA insertion, 10 (50 ml) water samples were collected, and designated T=1. Two hours later another 10 samples were collected at the source point and designated T=2. Two Sterivex filters (Sterivex-GP capsule filter pore size 0.22 μm, Millipore Sigma, Darmstadt, Germany) were also used to run 250 ml of water through each filter (500 ml total) for T=0, T=1, and T=2. Sampling continued downstream and one 50 ml sample was collected every 10 m for a total of 400 m using Sterivex filters (Figure 2). Each 100 m segment used two Sterivex filters, filtering five 50 ml samples for a total of 250 ml of water per Sterivex filter. Additional supplies required to collect water with Sterivex filters included 50 ml and 3 ml syringes, Longmire's lysis buffer solution from CMU, and luer lock ring plugs and tip caps.

Water samples collected in Nalgene bottles from source points T=0, T=1 and T=2 were individually labeled, stored in baggies, placed in a field cooler with ice, and immediately frozen at the Sanford, Michigan field station until delivered to CMU. Sterivex filter water samples had the remaining water pushed through the filter (Spens et al. 2017), were filled with Longmire's lysis buffer (Renshaw et al. 2015) to preserve DNA captured in the filter, capped, labeled individually, and then wrapped with parafilm. Filters were stored at room temperature at CMU until laboratory analysis and later stored long term in an ultracold freezer (-80°C). If T=0 sample results indicated contamination (i.e., presence of swine DNA), they were verified by rerunning remaining water samples corresponding to the specific sampling event.

Stream Measurements

Water temperature, stream velocity, pH, and turbidity were measured during each DNA trial (Table 1). Water temperature, pH, and turbidity was collected from shore using the telescopic pole and dipper during collection of water samples to detect DNA. The stream was entered to measure velocity after collection of water samples. The temperature and pH meter (Oakton pH 5+ and 6+ Meters, pH/Temperature/mV, Oakton Instruments, Vernon Hills, Illinois) was calibrated prior to sampling each day per instructions, The temperature and pH data were taken at the source point and at every 100 m segment downstream (Figure 2). Stream velocity was measured using a flow probe (Global FP111 Flow Probe, Geo Scientific Ltd., Vancouver, British Columbia Canada), using the average velocity function. Average velocity was determined by moving the flow probe smoothly and evenly back and forth from top to bottom of the flow for 20-40 seconds. Because we had to wait to measure stream characteristics until after sample collection, it is possible there may have been changes in flow from the beginning of a sampling event to the end. Sampling the 400 m transect took an average of 4 hours and 45 minutes. Turbidity was monitored using a turbidity meter (Oakton Turbidity Meter, Oakton Instruments, Vernon Hills, Illinois) by collecting a small water sample (>10 ml) in a small glass vial and placing it into the meter. The turbidity meter was calibrated before each sampling event and was measured in nephelometric turbidity units (NTU). Turbidity was collected at the source point and every 100 m downstream from the middle of the stream (Figure 2).

Laboratory Sample Processing

DNA extraction and digital droplet polymerase chain reaction (ddPCR) (BioRad QX200 Droplet Digital PCR system, BioRad Laboratories Inc., Hercules, California) was processed at CMU under supervision of Dr. Andrew R. Mahon. Swine DNA extraction,

amplification, and detection from water samples collected in Nalgene bottles followed protocol similar to Williams et al. (2017). A centrifuge was used to concentrate DNA within a sample and this DNA was pooled by segment (T=0, T=1, T=2, 0-100 m, 101-200 m, 201-300 m, and 301-400 m; Caldwell et al. 2007, Williams et al. 2016). The DNA supernatant was decanted and the remaining DNA pellet was extracted using DNeasy Mericon Food Kit (Qiagen, Hilden, Germany) with the 200 mg manufacture's protocol. Swine DNA extraction, amplification, and detection from Sterivex filters followed the protocol developed by Spens et al. (2017). DNA was extracted using an optimized protocol from the DNeasy blood and tissue kit (Qiagen, Hilden, Germany; Spens et al. 2017).

DNA extraction from Sterivex filters was a two day process in which DNA was extracted from the Longmire's lysis buffer and the enclosed filter. During the second day of the extraction protocol, the DNA found in both the Longmire's lysis buffer and enclosed filter was combined and frozen until processed by ddPCR. *Sus scrofa* primers and the probe for ddPCR was developed by the National Wildlife Research Center (NWRC), Fort Collins, Colorado (Williams et al. 2017). Both Nalgene bottle and Sterivex filter samples underwent DNA extraction individually, but DNA from individual samples within a segment (T=0, T=1, T=2, 0-100 m, 101-200 m, 201-300 m, and 301-400 m) was pooled per collection method. A subsample of pooled DNA from each segment then underwent amplification via ddPCR. Not all DNA extracted from each individual sample was used in pooling of segments; the remaining DNA was saved and frozen at -80°C. Amplification of single species-specific primers and probes using ddPCR was used to detect the concentration of swine DNA in the water samples. DNA presence was measured by the amount of target DNA copies per microliter (copies/µl).

Every PCR plate included a positive swine control, which was collected from a Michigan Russian boar euthanized during control efforts in another research project.

Contaminated T=0 bottle samples were initially rerun with remaining water from a specific sampling event, but when samples were rerun, they would have been processed through at least 2 freeze-thaw cycles. The T=0 contaminated bottles samples were removed from analysis because of two or more freeze-thaw cycles which could alter DNA results (Takahara et al. 2015). Contaminated T=0 filter samples were re-run to verify if the control sample was positive or negative for swine DNA during a specific sampling event. If the re-run sample came back positive, it was deemed contaminated, and the entire sampling event was removed from the analysis. If the re-run filter sample was negative, it was deemed negative (Mahon et al. 2013).

Data Analyses

For this study a positive ddPCR result contained swine DNA copies >0. Percentage of positive swine DNA detections was plotted against distance intervals (T=0 and every 100 m segment thereafter) to graphically represent effect of distance on DNA detection.

Pearson's correlation coefficients were calculated between, distance, water temperature, velocity, pH, turbidity, and discharge to identify redundant variables. Correlation coefficients >0.5 were deemed as correlated and removed redundant variables from modeling. Season was treated as ordinal variables; 1=winter, 2=spring, 3=summer, and 4=fall, and collection method represented Nalgene Bottles and Sterivex filters. Distance downstream from the introduced swine DNA source point (0 m; T=0) included T=0 (or 0 m), 100 m, 200 m, 300 m, 400 m. All analyses were completed in R Studio (R Studio Version 1.1.419, Boston, Massachusetts).

Generalized linear mixed-effects models were used to relate swine DNA detection (binary response; yes or no) by season, collection method, distance, water temperature, velocity, pH, and turbidity. Stream was included as a random effect because Bluff and Black Creeks represented variable habitat types and stream characteristics. A priori candidate models were created from uncorrelated independent variables and used Akaike's Information Criterion corrected for small sample size (AICc) to rank models. Models within two ΔAICc values were defined as competing and AICc weight for each model was reported (w_i=probability a model is the top model supported by the data given a set of alternative models; Burnham and Anderson, 2002). Parameters were significant if 95% confidence intervals (CIs) did not overlap 0. Model-averaging of competing models was conducted using MuMIn in R (Bartoń 2015). Exponentiated coefficient estimates and upper and lower 95% CIs for the top-ranked model were evaluated to aid in explaining how response variables affected detection of swine DNA.

Results

Swine specimens were introduced for 17 stream trials between May 2017 and April 2018; 919 total samples were collected from Bluff and Black Creeks (Table 2). One trial from late summer of 2017 was removed from regression modeling for Black Creek because the stream lacked flowing water. Therefore, sampling in September 2017 for this trial only included data from stream locations T=0, T=1, and T=2. Nine additional sampling events were excluded from analyses due to contamination of the control (T=0) samples (Nalgene bottles N=8, Sterivex filter N=1).

Velocity and discharge were highly correlated (R=0.84) and discharge was removed from the analyses because it was a derived value. Six models competed (Table 3) and model-averaging included season, collection method, distance, water temperature,

velocity, and stream. Season significantly affected probability of detecting swine DNA (Table 4). Seasons with cooler or cold weather increased swine DNA detection (Figure 3). For each change in season from winter to spring to summer to fall, odds of detecting swine eDNA decreased 54% (Table 4).

Swine DNA was successfully detected 29% of the time at 100 m and 53% at 400 m (9/17) downstream from the introduced source point in Bluff and Black Creeks (Figure 4). Distance was a significant variable in the model (Table 4). For every one 100 m increase in distance downstream (sampling occurred up to 400 m), DNA detection increased 111% (Table 4, Figure 5). Swine DNA at T=1 proved DNA was being shed during stream introduction, however DNA was not detected at T=1 for 7/17 sampling events. Of the seven sampling events, five had positive swine DNA detected downstream of T=1.

Swine DNA was detected at 400 m ranging from 221 to 346 minutes after swine DNA introduction. Of the 9/17 events when DNA was detected at 400 m, three sampling events occurred when swine DNA was not detected at T=1.

Discussion

Swine DNA was successfully detected in both Bluff and Black Creeks at all distances up to 400 m. Swine eDNA was successfully detected in a controlled wallow experiment by Williams et al. (2016) in Colorado, but detection of swine eDNA in moving water systems has not been published. Season and distance are important variables that affect detection of swine DNA in free-flowing streams.

The study results specify a higher detection of swine DNA when sampled in the winter and spring (Figure 3). Stewart et al. (2017) reported increased eDNA in localized areas when breeding occurred in the spring, but eDNA was more evenly distributed

during the summer for Yangtze finless porpoises *(Neophocaena asiaeorientalis*). In amphibian and fish species, detection of DNA is higher during breeding and birthing season due to more individuals in an area (Laramine et al. 2015, Spear et al. 2015, de Souza et al. 2016). Higher detections of swine DNA detections may also occur when water temperatures are colder. Strickler et al. (2015) determined water temperature at ~5°C in an open freshwater environment extended DNA detection for bullfrogs (*Lithobates catesbeianus*) when compared to controlled experiments at 25°C and 35°C.

The odds of detecting swine DNA increased as sampling occurred further downstream from the introduced DNA source. Swine DNA was detected 9/17 times at the 400 m segment in Bluff and Black Creeks (Figure 4). Diener and Altermatt (2014) found eDNA up to 9.1 km downstream for two species of planktonic crustacean (*Daphnia longispina* and *Unio tumidus*) and Jane et al. (2015) reported caged brook trout (*Salvelinus fontinalis*) eDNA was detected 239.5 m after introduction.

Limitations

Utilizing eDNA for a terrestrial mammal and simulating swine crossing a water resource while shedding DNA, creates an entirely new set of obstacles for wildlife and molecular biologists. Contamination of control samples (T=0) potentially occurred from undetected feral swine or hobby farms in the watersheds, swine DNA staying in the stream longer than anticipated, equipment contamination, and/or proximity DNA (Goldberg et al. 2016). Nshimyimana et al. (2018) reported porcine markers in 90% of the 63 sampled Michigan watersheds over three seasons due to agricultural manure applications. However, the sampled streams did not overlap with this eDNA study. The measurement (T=1) immediately after the introduction of swine DNA was intended to determine if DNA was present or absent in the stream; but seven times swine DNA at

T=1 was not detected. The movement of swine DNA in the streams may have been swifter than expected during several sampling events.

Conclusions

Feral swine research in Michigan reported one GPS collared adult male moved 143 km (0.77 km/hr) in one week during November, 2015 (D.R. Etter, Michigan Department of Natural Resources, personal communication). This encompasses a large area to search and locate an individual or group of feral swine. Collecting water samples across the landscape to determine presence of feral swine in an area may be more efficient and cost-effective to determine feral swine locations and focus eradication efforts. Swine DNA can be detected in streams up to 400 m from the source point and winter or spring are the recommended seasons to collect water samples to detect swine DNA; but ice, snowmelt, and/or seasonal flooding can make sample collection difficult. However, the impact of understanding swine DNA movement after source introduction provides an important foundation for future research and eDNA application for feral swine surveillance in the northern regions of the US.

Table 1. Range and average of Bluff and Black Creek stream variables plus a literature summary for selected stream variables used in model selection.

Variable	Bluff Creek Range; Average	Black Creek Range; Average	Literature
Season Winter December 21-March 20 Spring March 21-June 20 Summer June 21-September 20 Fall September 21-December 20	-	-	Few studies researched how season directly effects DNA detection. However, in amphibian and fish species, target species DNA is higher during breeding seasons due to an increase in the number of individuals in an area (Laramine et al. 2015, Spear et al. 2015, de Souza et al. 2016).
Temperature (°C)	0.20-22.90; 9.65	0.00-26.20; 11.45	Strickler et al. (2015) determined water temperature at ~5°C in an open freshwater environment extended DNA detection for bullfrogs (*Lithobates catesbeianus*) when compared to controlled experiments at 25°C and 35°C.
Velocity (m/s)	0.00-1.00; 0.24	0.00-0.33; 0.10	Velocity and discharge were measured because moving water can influence DNA detection (Pilliod et al. 2013, Deiner and Altermatt 2014, Jane et al. 2015).
pH	7.06-8.42; 8.04	5.84-7.70; 6.66	Water pH was sampled because Barnes et al. (2014), Strickler et al. (2015), and Lance et al. (2017) determined pH had an effect on DNA and detection; however, it was not clear which pH levels are ideal for DNA detection.
Turbidity (NTU)	1.04-17.12; 3.93	0.90-15.06, 4.06	Turbidity was measured because Williams et al. (2016) determined turbid waters influence DNA detection.

Table 2. Number (N) of water samples collected from May 2017 to April 2018 in support of environmental DNA (eDNA) analyses for detecting swine in Bluff and Black Creeks, Michigan, USA.

	Sampling Events[1]	Total N Collected	Total N Run by ddPCR	Positive N	Negative N
Bluff Creek	10	527	80	18	63
Black Creek	7	392	74	30	44
Total	17	919	154	48	107

[1]Number of days samples were collected in the field.

Table 3. Candidate models potentially describing detection of swine DNA in Bluff and Black Creeks, Michigan, USA. Data includes AICc values, AICc differences (ΔAICc), number of parameters (K), and AICc weights for each model. Models are presented in ascending order based on ΔAICc.

Rank	Model	K	AICc	ΔAICc	AICcWt[1]	CumWt[2]
1	Season + Method + Distance + Temperature + Velocity + (Stream)[3]	7	84.16	0.00	0.18	0.18
2	Season + Method + Distance + (Stream)	5	84.73	0.58	0.14	0.32
3	Season + Method + Distance + Temperature + (Stream)	6	84.84	0.68	0.13	0.45
4	Season + Method + Distance + Temperature + Velocity + pH + (Stream)	8	85.03	0.88	0.12	0.57
5	Season + Method + Distance + Temperature + Velocity + pH + Turbidity + (Stream)	9	85.26	1.10	0.11	0.68
6	Season + Method + Distance + Velocity + (Stream)	6	85.39	1.24	0.10	0.78
7	Season + Method + Distance + Velocity + pH + (Stream)	7	86.34	2.18	0.06	0.84
8	Season + Distance + (Stream)	4	86.57	2.41	0.06	0.89
9	Season + Method + Distance + pH + (Stream)	6	86.57	2.42	0.05	0.95
10	Season + Method + Distance + Temperature + Velocity + pH + Turbidity + Distance*Velocity[4] + (Stream)	10	87.85	3.69	0.03	0.98
11	Season + Distance + Velocity + (Stream)	5	88.27	4.11	0.02	1.00

[1] AICc Weight
[2] Cumulative Weight
[3] (Stream) = Random Effect
[4] Distance*Velocity = Interaction

Table 4. AICc model-averaged regression coefficient estimates and upper and lower 95% confidence limits for models fit to detect swine DNA. The exponentiated estimates and upper and lower 95% confidence limits are also shown to facilitate understanding expected change in the response variables while other variables are held constant.

	Coefficient Estimate	95% CI	Exponentiated Coefficient Estimate	Exponentiated 95% CI
Season[1]	-0.77	-1.43 - -0.08	0.46	0.24-0.93
MethodFilter	0.97	-1.65-3.33	2.63	0.19-28.05
Distance[1]	0.75	0.03-1.19	2.11	1.03-3.30
Velocity	-1.70	-6.58-1.06	0.18	0.00-2.89
Temperature	-0.66	-0.24-0.05	0.52	0.79-1.05

[1] = Net negative or positive effect on the detectability of swine DNA

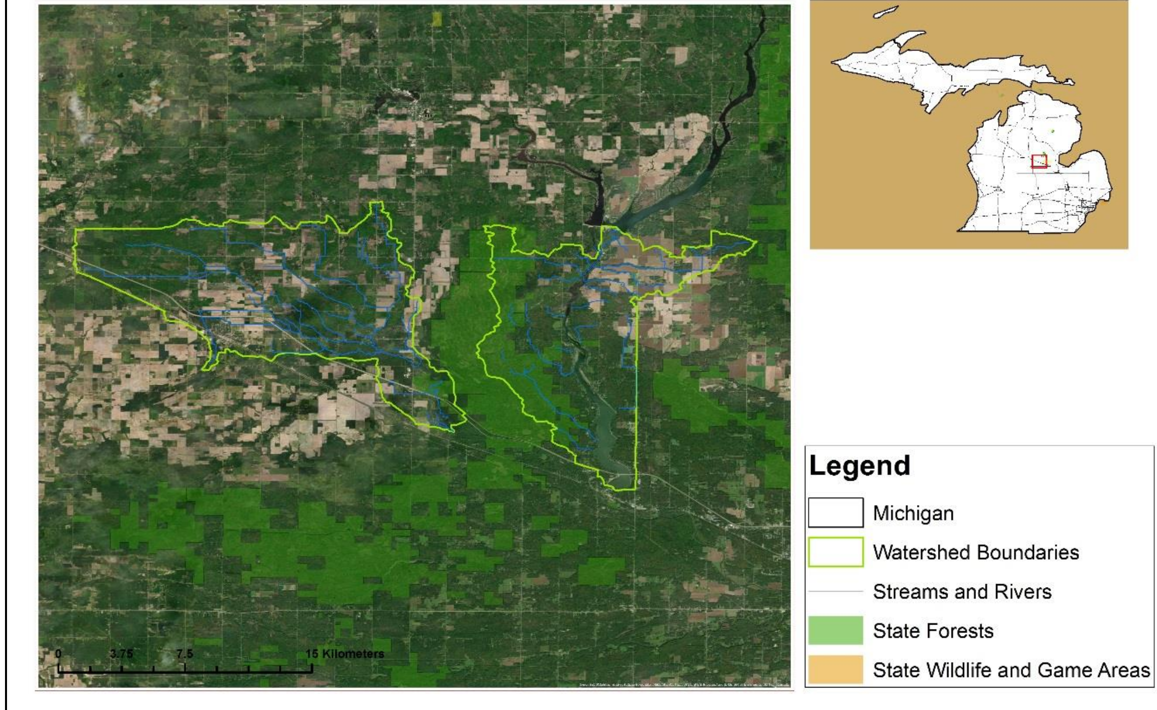

Figure 1. Locations of Bluff and Black Creeks, Michigan, USA.

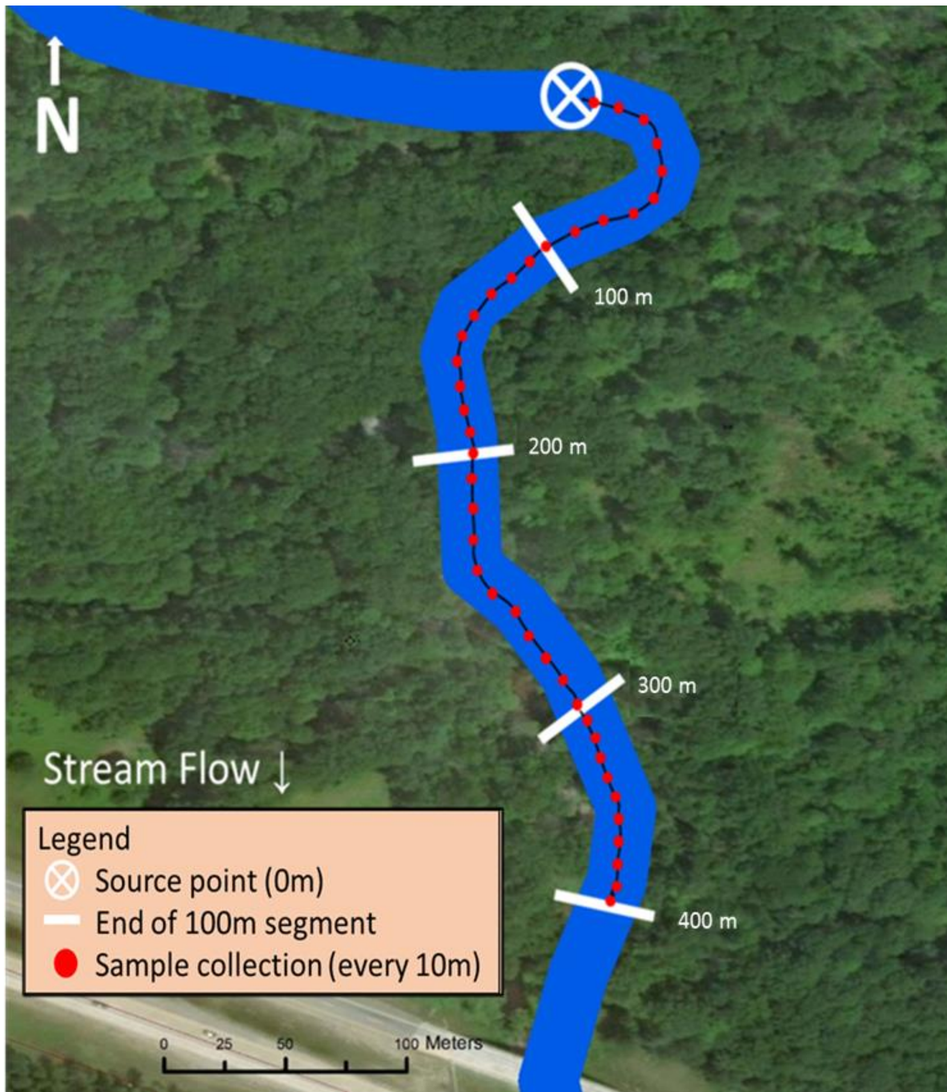

Figure 2. Sampling procedure schematic used in Bluff and Black Creeks, Michigan, USA.

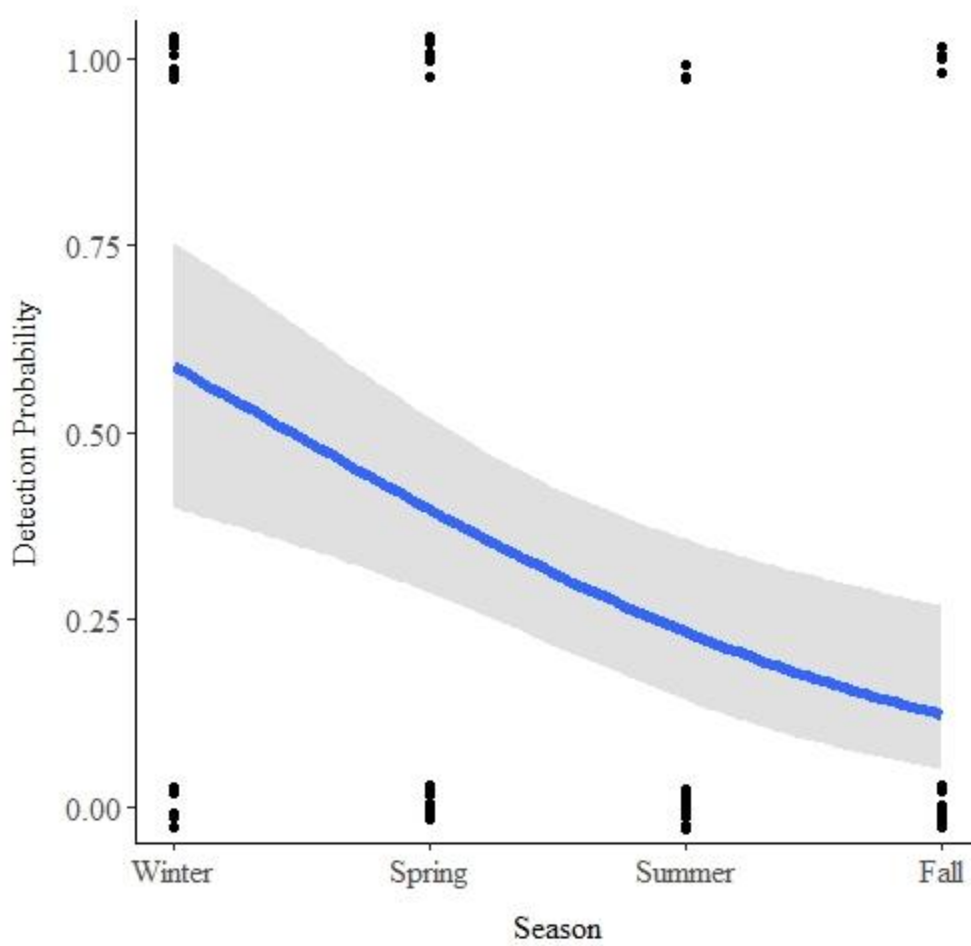

Figure 3. Detection probability of swine DNA by season for Bluff and Black Creeks using a general linear model with a 95% confidence interval.

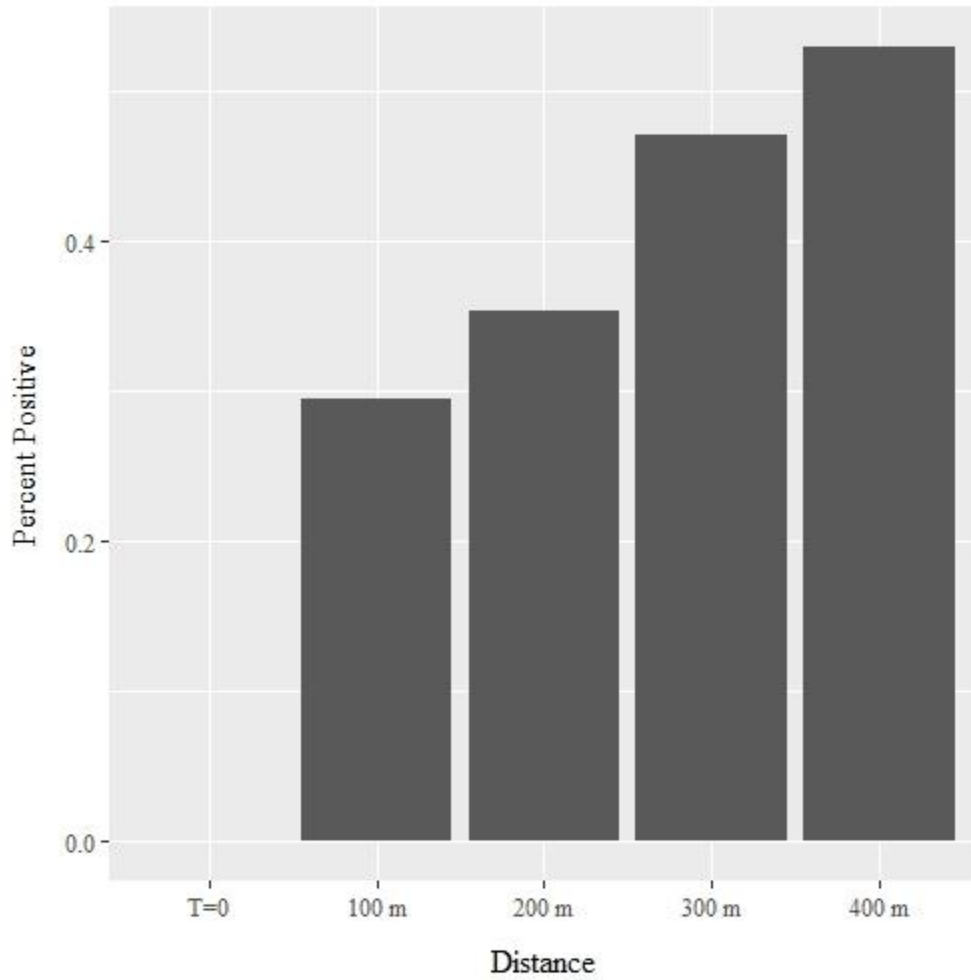

Figure 4. Percentage of positive digital droplet polymerase chain reaction (ddPCR) results detecting swine DNA for each stream segment analyzed from Bluff and Black Creeks, Michigan, USA.

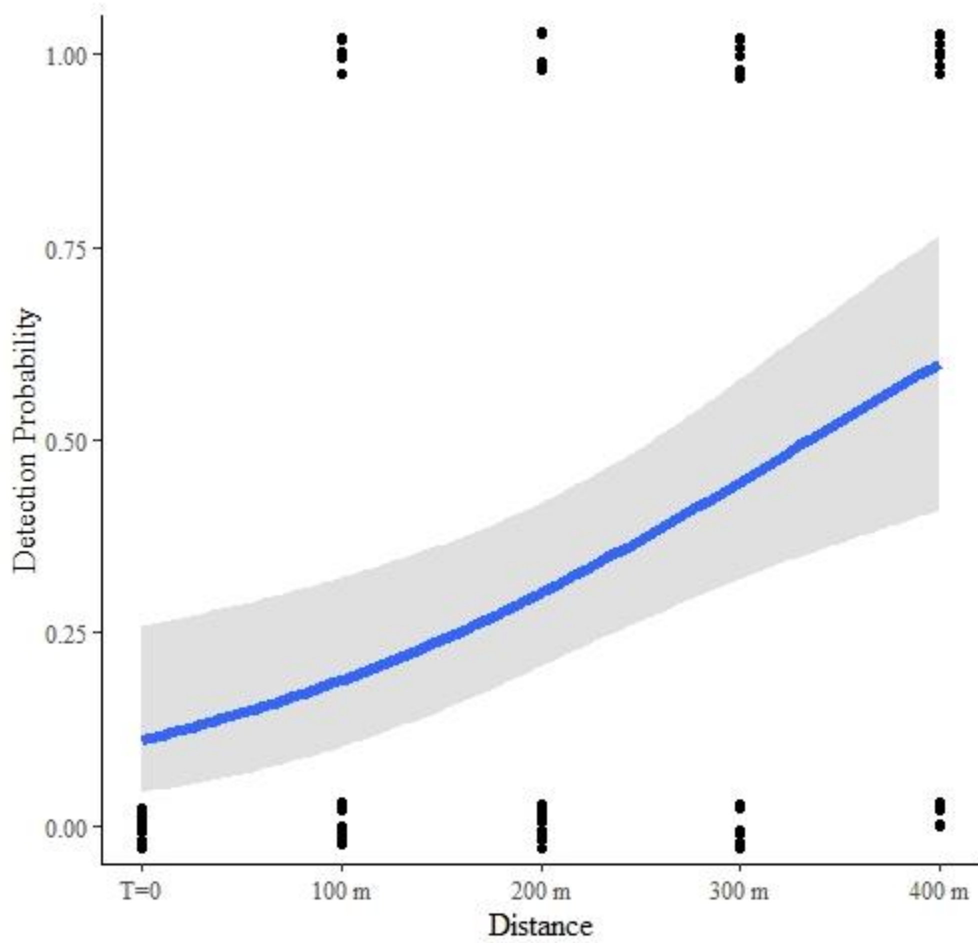

Figure 5. Detection probability of swine DNA at sampling locations T= 0 and each stream segment for Bluff and Black Creeks using a general linear model with a 95% confidence interval.

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Appendix I. Comparison of Nalgene bottles and Sterivex filters for eDNA sampling of Bluff and Black Creeks, Michigan, USA.

We used two different collection methods, Nalgene bottles and Sterivex filters, during this research project (Appendix Table 1.). Paired T-test results indicated filters detected swine DNA better than bottles with a mean ($\bar{x}$) difference of -0.028 (t= -2.52, p=0.02, CIs=-0.50 to -0.05). Detection probability with 95% CI was graphed using least square means of Nalgene bottles and Sterivex filters (Appendix Figure 1). The field use of Nalgene bottles required a large cooler carry a large number of samples (10 bottles per sampling segment = 70 bottles per sampling event) while traversing along the streams and through dense forest habitats. The samples also needed to be frozen within 24 hours or less after collection. This timeline could be difficult when working in remote field locations for many hours especially in extreme heat. Our sampling days averaged 7.5 hours, with the longest day at 10.5 hours. One study determined that DNA was no longer detectable in streams 11.5 hours after the target species Atlantic salmon (*Salmo salar*) was removed (Balasingham et al. 2016). The bottles also take up a large amount of storage space in the freezers and laboratory. Contamination can occur more easily with Nalgene bottles by DNA proximity because the bottles are open to the environment. Sterivex filters were easier to use, required less filters per sampling session (2 filters per sampling segment = 14 filters total per sampling session), are a closed system to prevent contamination, DNA is preserved easily with Longmire's buffer solution, and can be kept at room temperature for up to 150 days (Wegleitner et al. 2015). Stream turbidity did affect the number of filters that may need to be used in a specific sampling event, but that is easily adjusted. Initially it may appear that bottles are cheaper to purchase than filters, but the final costs are similar due to less filters to process in the lab and the ease of using filters in the field are highly recommended (Appendix Tables 2 and 3.).

Appendix Table 1. Total number (N) of water samples collected in Nalgene bottles and Sterivex filters from Bluff and Black Creeks.

	Sampling Events[1]	Total N Collected	Nalgene Bottles	Sterivex Filters
Bluff Creek	13	813	520	293
Black Creek	14	831	583	248
Total	27	1644	1103	541

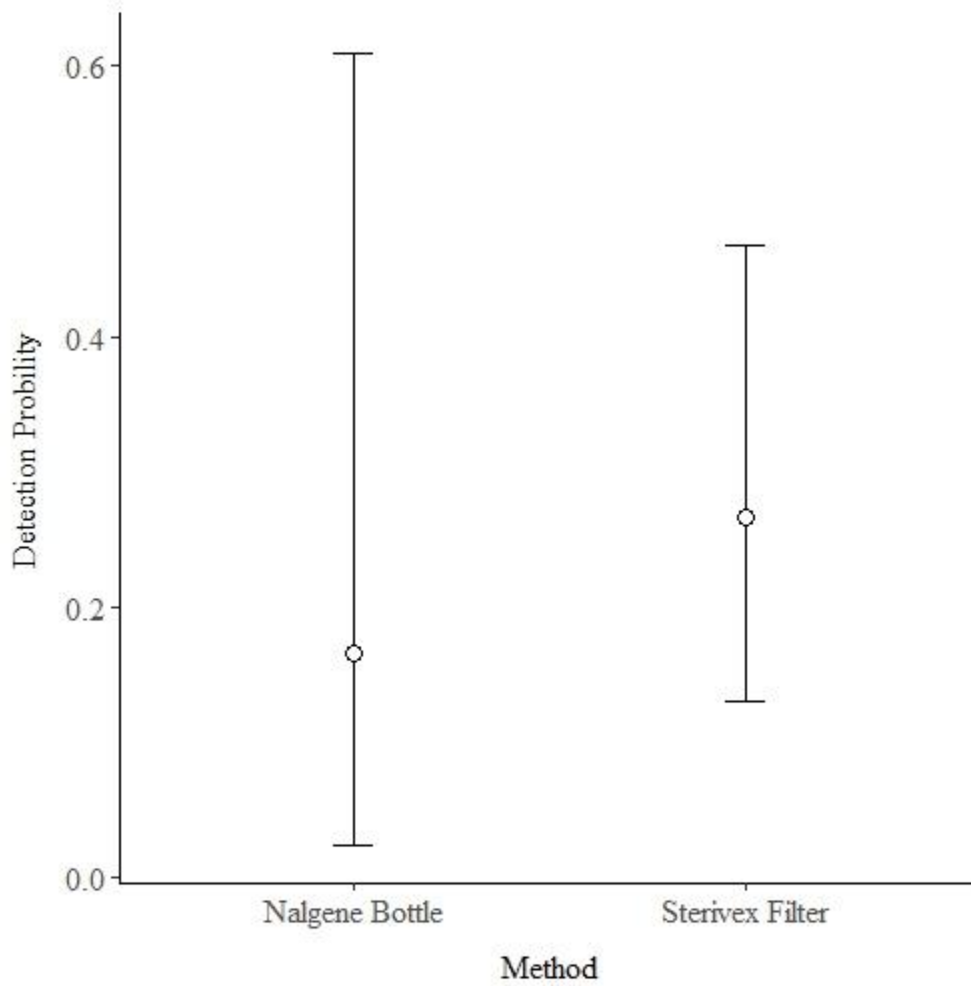
[1] Total number of days samples were collected in the field.

Appendix Table 2. Cost of Nalgene bottles and Sterivex filters at individual and bulk prices, university or non-university purchase.

		University	Non-University
Individual Prices	Nalgene Bottle	$0.55	$0.79
	Sterivex Filter	$9.99	$12.16
Bulk Prices	Nalgene Bottle	$0.55	$0.79
	Sterivex Filter	$9.74	$11.84

Appendix Table 3. Estimated cost of Nalgene bottles and Sterivex filters used for the University of Michigan – Flint swine eDNA research project.

	Equipment Cost per Sample	Cost to Run ddPCR	N Samples	Final Cost
Nalgene Bottle	$0.55	$7.50	1103	$8,873.95
Sterivex Filter	$12.88	$5.50	541	$9,941.45



Appendix Figure 1. Detection probability of water collection method (Nalgene bottles or Sterivex filters) using the least square means method with a 95% confidence interval for Black and Bluff Creeks, Michigan, USA.

References

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