

THESIS

MOLECULAR DETECTION OF FERAL PIGS USING ENVIRONMENTAL DNA

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## ABSTRACT

### MOLECULAR DETECTION OF FERAL PIGS USING ENVIRONMENTAL DNA

Feral pigs are considered one of the most widespread invasive vertebrate species worldwide (Chavarria et al. 2007). Since their introduction to North America, feral pigs have become one of the more pressing wildlife management problems in the United States (West et al. 2009). Feral pigs have omnivorous largely indiscriminant feeding habits, destructive rooting behavior, high fecundity, and contribute to pathogen spread, making them a destructive and dangerous invasive species. Due to the negative impacts pigs incur on ecosystems throughout the United States, measures for management and eradication are currently underway. Challenges to eradication efforts include immigration of feral pigs from surrounding areas, illegal transportation by humans, difficulty in detecting and removing the last few individuals, and the high fecundity of feral pigs. Feral pig populations must be reduced to zero for successful control; a few remaining individuals can reproduce leading to rapid repopulation (Barrett and Pine 1980, Choquenot et al. 1996, Bieber and Ruf 2005).

Application of environmental DNA (eDNA) detection allows for surveillance and management of invasive species that are difficult to monitor or detect by field observations alone (Ficetola et al. 2008, Jerde et al. 2011). Such techniques have been successfully applied to aquatic and semi-aquatic species (Ficetola et al. 2008, Thomsen et al. 2012a, Piaggio et al. 2014); applications to detect terrestrial mammals have only recently emerged as a possibility (Rodgers and Mock 2015). Environmental DNA techniques could provide an ideal approach for

detection and surveillance of feral pigs because pigs spend time drinking or wallowing daily and shed cells containing DNA into the water through these behaviors.

I conducted a series of experiments to optimize an assay for detection of eDNA of feral pigs in the field. My goals included optimizing a field protocol for collecting, storing, and transporting turbid water samples from the field, developing a method for efficient eDNA capture from the field sample, and, finally, developing an understanding of degradation of eDNA in turbid water systems and of sensitivity of the assay.

Methods of eDNA capture vary across studies. Understanding the differences in the efficiencies of various methods to concentrate, extract, and amplify eDNA is vital for best performance. Aquatic systems that are sampled for eDNA may vary in characteristics that can affect eDNA detection (i.e., turbidity, eDNA concentration, inhibitor presence). In Chapter 1, I present results of tests of combinations of concentration and extraction techniques and compare pre- and post- inhibitor removal methods on detectability of eDNA. I wanted to optimize a protocol without using filtration as a method of concentration because this method can be cumbersome in the field (Piaggio et al. 2014) and filters can clog with sediment from turbid water, as might be found in feral pig wallows. I tested three concentration methods (centrifugation, sodium acetate with ethanol precipitation, and resin beads), five methods of extraction (MagMAX-96 AI/ND Viral RNA extraction kit, the QIAamp DNA Micro kit, the CTAB protocol, the DNeasy *mericon* Food kit, and the PowerBiofilm® DNA isolation kit), and whether Zymo Inhibitor Removal Technology (IRT) columns effectively removed inhibitors without losing DNA. I also compared the sensitivity of conventional PCR and quantitative PCR to amplify low concentrations of DNA. Through this exercise, I determined that the best protocol to use for

capture of eDNA from turbid water samples includes the following steps: 1) concentrate the sample via centrifugation, 2) extract DNA using the DNeasy *mericon* Food kit, and 3) remove inhibitors post-extraction by using the Zymo IRT columns. My results show that quantitative PCR is more sensitive to low levels of eDNA than conventional PCR. I found drastic differences in efficiencies of various methods in each step of eDNA capture, emphasizing the importance of considering the appropriate protocol to use for the water system of interest.

With an optimized method for detection of eDNA in turbid water samples, the next step (Chapter 2) in developing a field technique was to test the sensitivity of the assay. One goal in Chapter 2 was to determine how long a pig must have contact with a water source and what behaviors are required (i.e., drinking or wallowing) for sufficient eDNA to be shed and detected. Through a series of pen studies, I found that my assay was sensitive enough to detect low levels of eDNA shed by a single pig in a wallow. Further, I found that I could detect low levels of eDNA shed through just nose and mouth contact with an automated waterer after just 15 minutes of contact.

My second goal in Chapter 2 was to develop an understanding of how long pig eDNA can persist in turbid water. By comparing the degradation of eDNA shed by a single pig compared to a group of 13 pigs, I demonstrated differences in persistence and developed a model to predict the probability of detection of eDNA depending on the amount of time of degradation and whether or not the wallow was used by a single pig or a group of pigs. These results can provide insight into how recently a feral pig was in an area monitoring changes in eDNA concentration and detection over time.

An obstacle that continues to hinder applications of eDNA monitoring in the field is the requirement of a cold chain of storage or transport for water samples containing eDNA. In Chapter 3, I tested the performance of a lysis buffer (i.e., Longmire's solution) for preservation of eDNA in water samples (15 mL) over the course of 56 days. Longmire's lysis buffer has been shown to effectively preserve eDNA collected on water filters (Renshaw et al. 2015), but this has not yet been attempted with "raw" water samples. I compared three ratios of Longmire's buffer to water sample (i.e., high, medium, and low concentrations of Longmire's) with a no treatment group (no method of preservation) and with a positive control group (cryogenic preservation). Water samples with the highest ratio of Longmire's lysis buffer added (5 mL lysis buffer: 15 mL sample water) were effectively preserved over the 56 days in the face of temperature changes and incident UV exposure, resulting in perfect detection of eDNA. Therefore, addition of Longmire's solution is a viable method of eDNA preservation for raw water samples and performs at the same level of detection as does typical cold chain storage.

Through this research, I have eliminated the need for time-consuming eDNA capture in the field (no filtering) and costly cold chain storage for collecting and transporting water (Longmire's solution preservation). These efficiencies can reduce the time and effort required to collect eDNA samples for feral pig detection in the field. I have tested the assay in a simulated field system to determine the detection limits and found that only minimal contact between the pig and water is required for a positive detection. This will be important for detecting new invaders, especially if wallowing behavior is reduced as a result of hunting pressure (Sodeikat and Pohlmeier 2003) or similar management practices. Lastly, through the degradation study, I found that eDNA persists in water for up to 56 days and that PCR-based

detection alone may not be the best approach to monitor wallows; instead, measuring the eDNA concentration in wallows over time allows us to determine whether a new invader arrives or if a pig was missed.

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## TABLE OF CONTENTS

ABSTRACT.....	ii
ACKNOWLEDGEMENT.....	vii
CHAPTER 1: CLEARING MUDDIED WATERS: OPTIMIZED CAPTURE, PURIFICATION, AND AMPLIFICATION OF ENVIRONMENTAL DNA FROM TURBID WATERS.....	1
SUMMARY.....	1
INTRODUCTION.....	2
FERAL PIGS.....	3
ENVIRONMENTAL DNA.....	4
METHODS.....	6
EDNA PURIFICATION (EXTRACTION).....	6
SAMPLING.....	7
PURIFICATION TECHNIQUES.....	8
EDNA CAPTURE OPTIMIZATION & INHIBITOR REMOVAL.....	10
SAMPLING.....	10
EDNA CAPTURE.....	11
INHIBITOR REMOVAL.....	13
CONVENTIONAL PCR OR QUANTITATIVE PCR.....	13
RESIN BEAD PILOT STUDY.....	14
STATISTICAL ANALYSIS.....	15
SEQUENCE VERIFICATION.....	16
RESULTS.....	17
EDNA PURIFICATION (EXTRACTION).....	17
EDNA CAPTURE OPTIMIZATION & INHIBITOR REMOVAL.....	17
CPCR OR QPCR?.....	18
RESIN BEAD PILOT STUDY.....	19
SEQUENCE VERIFICATION.....	19

DISCUSSION.....	19
CONCLUSION.....	22
CHAPTER 2: MOLECULAR DETECTION OF A TERRESTRIAL MAMMAL: AN OPTIMIZED APPROACH IN DETECTING LOW LEVELS OF FERAL PIG ENVIRONMENTAL DNA IN WATER SOURCES.....	31
SUMMARY.....	31
INTRODUCTION.....	31
INVASIVE SPECIES.....,,.....	32
MANAGEMENT.....,,.....	34
EDNA.....	35
METHODS.....	38
STUDY SITES.....	38
EVALUATING ASSAY SENSITIVITY.....	39
PERSISTENCE.....	40
EDNA CAPTURE.....	40
STATISTICAL ANALYSIS.....	41
ASSAY SENSITIVITY.....	41
PERSISTENCE.....	41
RESULTS.....	42
ASSAY SENSITIVITY.....	42
EDNA ACCUMULATION.....	42
PERSISTENCE.....	42
QUANTIFICATION OF EDNA DEGRADATION.....	42
DISCUSSION.....	43
CONCLUSION.....	45
CHAPTER 3: NO FILTERS, NO FRIDGES: A METHOD OF PRESERVATION OF ENVIRONMENTAL DNA.....	52
SUMMARY.....	52
INTRODUCTION.....	52
METHODS.....	55

RESULTS.....	57
DISCUSSION.....	58
CONCLUSION.....	59
REFERENCES.....	60
APPENDIX A.....	69

## Chapter 1: Clearing muddied waters: Optimized capture, purification, and amplification of environmental DNA from turbid waters

### Summary

Interest in detection of elusive species using environmental DNA (eDNA) is growing. However, understanding the differences in the efficiencies of various methods to concentrate, extract, and amplify eDNA is vital for best performance. Aquatic systems may vary in their characteristics such as turbidity, eDNA concentration, and inhibitor load that can affect eDNA capture efficiency. Application of eDNA detection of terrestrial invasive or endangered species may require sampling at intermittent water sources that are used for drinking and cooling; these water bodies may often be stagnant and turbid. I tested three concentration techniques, five methods of extraction, and whether or not inhibitor removal increased elution quality on turbid water samples containing feral pig eDNA. I determined that the best protocol for eDNA capture in a turbid water system was to concentrate the 15 mL water sample via centrifugation, extract DNA from the centrifuged pellet with the DNeasy *mericon* food kit, and remove inhibitors from the elution with Zymo Inhibitor Removal Technology columns. Further, I compared the sensitivity of conventional PCR (cPCR) to quantitative PCR (qPCR) by serial dilution of my extractions and found that qPCR was more sensitive in detecting low concentrations of eDNA. I show drastic differences in efficiencies of various methods in each step of eDNA capture, emphasizing the importance of considering the appropriate protocol to use for the water system of interest.

## Introduction

The need for effective ways to assess biodiversity and to detect and monitor invasive or endangered species has fueled the advancement of methods for identifying DNA shed from an organism into the environment (Bohmann et al. , Ficetola et al. 2008, Folloni et al. 2012, Goldberg et al. 2015). Successful implementation of environmental DNA (eDNA)-based detection requires successful capture of eDNA, even when it may not be common in the environment (Ficetola et al. 2008, Goldberg et al. 2011, Jerde et al. 2011, Thomsen et al. 2012a, Rees et al. 2014). Water samples can contain varying amounts of target DNA due to several factors including the size of the shedding organism, the volume and intensity of secretion or shedding, and the rate of degradation of eDNA in the water (Dejean et al. 2011, Takahara et al. 2012, Thomsen et al. 2012b, Goldberg et al. 2013, Pilliod et al. 2014). Further, abiotic features of aquatic systems including turbidity, temperature, pH, size, and flow rates can affect persistence of the target eDNA (Barnes et al. 2014). Beyond the difficulties posed by capture of eDNA, laboratory purification and successful amplification of the target region(s) can be difficult. Each step requires optimization for sensitivity and specificity, along with sustained efforts to monitor for and minimize contamination because eDNA samples are inherently of low quality and low quantity.

The abiotic and biotic factors that may affect one's ability to detect a target organism using eDNA influence the choice of method for capturing and isolating DNA from environmental samples. Multiple methods are available for each step in the capture (concentration), purification (extraction), and amplification processes. Most eDNA studies have been performed

in clear water marine (Foote et al. 2012, Thomsen et al. 2012a, Kelly et al. 2014) or freshwater (Martellini et al. 2005, Ficetola et al. 2008, Dejean et al. 2011, Goldberg et al. 2011, Jerde et al. 2011, Piaggio et al. 2014) systems. The detection of terrestrial invasive and/or endangered species will rely on sampling at intermittent water sources that are used for drinking and cooling which may often be small, stagnant, and turbid. Turbid water poses a set of challenges to successful eDNA detection. DNA released into the environment can be absorbed in soils and sediments by the formation of cationic bridges between the phosphate groups of the nucleic acids and clay surfaces, protecting the DNA from degradation (Nielsen et al. 2006, Nielsen et al. 2007, Pietramellara et al. 2008). This phenomenon was observed by Turner et al. (2015) in their finding that fish eDNA was more concentrated in sediments of experimental ponds and natural rivers compared to surface water. Stagnant water systems may also contain inhibitors, humic substances that may be co-extracted with DNA that interfere with PCR amplification, making detection more difficult (Wetzel 1992, Matheson et al. 2010, McKee et al. 2015). Stagnant, turbid water sources may have eDNA bound up in colloidal particulate matter and may contain inhibitors that would affect the ability to detect the eDNA. My goal in this study was to determine the best practices for capture, purification, and amplification of eDNA shed from an invasive, terrestrial mammal, feral pigs, into turbid water.

### ***Feral Pigs***

Feral pigs are considered one of the most widespread invasive vertebrate species worldwide (Chavarria et al. 2007, West et al. 2009). Due to the negative impacts of feral pigs on agricultural and natural ecosystems and their continued expansion, management efforts are being focused on reducing feral pig populations (United States Department of Agriculture). A

major challenge to eradication involves detecting the last few individuals during an eradication effort, new invaders on the front, or small populations transported by humans (Hone 1983, Saunders and Bryant 1988, Choquenot et al. 1996, West et al. 2009). An accurate, highly sensitive method of detection of feral pigs is needed to help managers discover individuals when numbers are low and before the population increases to an unmanageable point.

Feral pigs spend time each day drinking or wallowing in water (Taylor 1993, Jay et al. 2007) during which they shed cells containing DNA into the water source. In many areas across this species' invasive range in the United States, such water sources are small, stagnant, and turbid bodies of water. An eDNA detection technique could provide an ideal approach for detection and monitoring of this invasive species by sampling these water bodies. However, the development of a feral pig eDNA assay is challenging due to the inherent characteristics of wallows: muddy and stagnant. An optimized approach for concentration, purification, inhibitor removal, and amplification of eDNA from this system is critical for invasive species management and monitoring success.

### ***Environmental DNA (eDNA)***

Multiple approaches have been applied to the capture of eDNA from aquatic systems (Figure 1-1). Concentration of DNA using precipitation with sodium acetate and ethanol (Valiere and Taberlet 2000, Ficetola et al. 2008), centrifugation (Caldwell et al. 2007, Foote et al. 2012), and filtration (Goldberg et al. 2011, Jerde et al. 2011, Pilliod et al. 2013a) have all been shown to successfully capture eDNA from bodies of water. Though precipitation and centrifugation constrain the volume of the water samples, filtration methods often get clogged, hindering the capture of eDNA, particularly in turbid waters (Piaggio et al. 2014). Resin beads have been used

to successfully capture virus particles in wastewater by offering an anionic exchange area and attracting particles with a negative surface charge (Perez-Mendez et al. 2014). Because DNA is also charged, resin beads may efficiently capture small fragments of DNA from large quantities of water which could be ideal for eDNA collection in the field. The comparison of each of these methods for eDNA capture in turbid waters has not been performed and was one objective of this study.

Extraction methods for purification also vary among eDNA studies. These methods exploit different techniques for purifying DNA including chloroform-based protocols (Debroas et al. 2009, Turner et al. 2014, Renshaw et al. 2015), physical disruption (Jerde et al. 2011, Amberg et al. 2013), and column-based techniques (Ficetola et al. 2008, Goldberg et al. 2013, Piaggio et al. 2014). Different combinations of concentration and extraction methods also produce varied results (Deiner et al. 2015, Goldberg et al. 2015). The choice of an optimal concentration and extraction combination is likely dependent on the biology of the system from which the eDNA is collected. Turbid water may require a different method for purifying eDNA compared to freshwater or marine systems; I tested multiple extraction techniques in combination with various capture methods to identify the optimal protocol for my system.

An inhibitor removal step post-extraction can remove humic substances that may be co-extracted with DNA and interfere with downstream PCR reactions (Matheson et al. 2010, McKee et al. 2015); such a step may also help optimize eDNA detection from turbid waters. The trade-off of performing an inhibitor removal step post-extraction is that there is a risk of losing some DNA in a sample that already contains a low quantity of eDNA. This might make the difference in reporting a false negative or not (Darling and Mahon 2011). I suspected that an



inhibitor removal step would improve my efficiency in eDNA amplification due to the expected turbidity of feral pig water samples. Diluting the sample can also dilute inhibitors but inhibitor removal columns (e.g., Zymo Research) have been shown to be superior to dilution in other eDNA studies (Farrington et al. 2015, McKee et al. 2015). To improve efficiency in capturing and detecting eDNA from turbid water in this study, I tested a kit-based inhibitor removal system (Zymo Research, Irvine, California, USA).

Lastly, amplification success of purified eDNA samples may vary depending on the PCR approach and analysis. It has been suggested that quantitative PCR (qPCR) is more sensitive than analysis of conventional PCR (cPCR) on a genetic analyzer or gel (Wilcox et al. 2013, Nathan et al. 2014) for detecting eDNA. In the current study, I performed a robust comparison of the sensitivity of my feral pig eDNA assay across both platforms (qPCR and fragment analysis with cPCR) in order to optimize the sensitivity of my assay for detecting low concentrations of eDNA in turbid water.

## **Methods**

Laboratory work was performed at the USDA-APHIS National Wildlife Research Center in Fort Collins, Colorado, USA. Extractions were performed in a lab where only low quantity/low quality DNA was processed. All PCR and post-PCR procedures were completed in separate rooms. To minimize contamination of the samples, equipment, benchtops, pipettors, and fume hoods were cleaned with a 10% bleach solution before and after any procedure.

### ***eDNA Purification (extraction)***

One of my goals was to test various purification techniques in combination with three different capture methods. To reduce the total number of combinations to be tested, I first

used a concentration technique (addition of sodium acetate with ethanol precipitation) that was shown to be useful for eDNA capture in other studies (Valiere and Taberlet 2000, Ficetola et al. 2008, Piaggio et al. 2014) along with 5 purification methods (the MagMAX-96 AI/ND Viral RNA extraction kit, the QIAamp DNA Micro kit, the CTAB protocol, the DNeasy mericon Food kit, and the PowerBiofilm® DNA isolation kit); I then selected the top two best-performing techniques. I evaluated these two techniques (detailed below) in combination with two additional capture methods (three total: sodium acetate and ethanol precipitation, centrifugation, and resin beads) to identify the overall best combination for the capture and purification of eDNA from turbid waters.

### ***Sampling***

Water was collected from a 95 L tub that served as a water source for a single feral swine sow in captivity at the USDA-APHIS/Colorado State University Wildlife Research Facility. Water was collected on March 3, 2015 and again on March 25, 2015 by submerging a single sterilized 2L Nalgene® bottle and filling it. Water was murky and turbid with mud, fecal matter, and some food pellets.

The collected water was mixed using a magnetic stir bar and plate and then subsampled into thirty 50 mL centrifuge tubes. Subsamples were numbered in order of collection and then randomly assigned to each of the five extraction techniques using a random number generator. These subsamples were stored overnight at -80°C. All eDNA capture and purification steps were performed within a 12-hour period the day after the initial collection. Three replicates of each extraction method were performed for each subsample. To monitor for contamination, I

included one negative control, containing only extraction reagents, with each set of extractions for all methods.

### ***Purification Techniques***

Each 50 mL subsample was aliquoted into three replicates of 15 mL each. Subsamples were concentrated by sodium acetate precipitation (Valiere and Taberlet 2000) with a centrifugation modification (Piaggio et al. 2014). Sodium acetate and ethanol were added to each of the subsamples and stored overnight in a -20°C freezer and then centrifuged (Turner et al. 2014). The resulting pellet was dried for 5 minutes by inverting the 50 mL tubes (Piaggio et al. 2014).

Extraction methods that were compared included the MagMAX-96 AI/ND Viral RNA extraction kit (Applied Biosystems, Foster City, California), the QIAamp DNA Micro kit (Qiagen, Hilden, Germany), the CTAB (cetyltrimethyl ammonium bromide) protocol (Coyne et al. 2001, Turner et al. 2014), the DNeasy *mericon* Food kit (Qiagen), and the PowerBiofilm<sup>®</sup> DNA isolation kit (MoBio, Carlsbad California). The MagMAX-96 AI/ND Viral RNA extraction kit is a magnetic bead/total nucleic acid extraction method. For use with this kit, the pellet was resuspended in 200 µL of water and extraction was performed via an automated robot (King Fisher 96 Extraction Robot). For the QIAamp DNA Micro kit, the pellet was resuspended in 300 µL of Qiagen buffer ATL and extracted according to protocol for automated purification of DNA on a QIAcube (Qiagen) programmed to follow the Forensic Case Work Samples protocol (Piaggio et al. 2014). The CTAB protocol for extraction of aqueous eDNA precipitated/pelleted from water was followed with some modifications (Turner et al. 2014). Recipes for the CTAB extraction buffer (100mM Tris-HCl, 1.4M NaCl, 1% polyvinylpyrrolidone, 2% cetyl trimethyl ammonium

bromide, 20 mM EDTA), Sevag (24:1 chloroform:isoamyl alcohol), and LoTE buffer (10mM Tris, 0.1mM EDTA) were used from Turner et al. 2014. The pellet/CTAB/Sevag mixture was centrifuged at 3250g instead of 3220g due to centrifuge limitations of speed settings. The DNA pellet was allowed to air dry overnight to ensure that all ethanol from the previous step evaporated because ethanol can interfere with downstream processes such as PCR. The tubes were left open overnight with a Kimwipe™ (Kimtech, Pleasant Prairie, Wisconsin) covering the tops of the tube. The pellet was resuspended in Lote buffer according to Turner's protocol (Turner et al. 2014). The standard protocol for 200 mg of sample was used for the DNeasy *mericon* Food kit. For the PowerBiofilm® DNA isolation kit the pellet was resuspended after concentration in 350 µL of BF 1 solution and moved into extraction; I used a vortex adapter for the bead beating step.

All extracted DNA from each of the five techniques was stored in a -80°C freezer until PCR analysis. To verify that DNA was captured and purified from each sample for each extraction method, DNA concentrations were measured using a Qubit® 2.0 fluorometer (Life Technologies, Carlsbad, California). For this portion of the study, I used conventional PCR and fragment analysis on an ABI 3130 (Life Technologies) to test for successful feral swine DNA capture and purification.

Primers were designed using AlleleID (ver. 7.0; Premier Biosoft) for a target fragment of the mitochondrial D-loop region of *Sus scrofa* for 101 basepairs (bp) in length (*SusScrofa*DF 5' CAAGCATTCCATTCGTATG; *SusScrofa*DR 5' CGCATATTTGTATGTTTGTG). After the primers were designed, I performed a BLAST search to assess specificity using the National Center for Biotechnology Information website (Benson et al. 2013). Primers were also tested in the lab for

cross-reactivity with DNA extracted from sheep, cow, deer, and dog tissue using the DNeasy Blood & Tissue kit (Qiagen). *SusScrofa*DF was labeled with fluorescent dye 6-FAM for visualization of fragments on an ABI3130xl genetic analyzer (Life Technologies, Carlsbad, California).

Each PCR was a 25  $\mu$ L reaction containing 2.5  $\mu$ L 10x Amplitaq buffer (Life Technologies), 0.5 mM  $MgCl_2$ , 0.4 mM dNTP, 5% DMSO, 0.25  $\mu$ L of each primer (10 $\mu$ M), 0.5 U Amplitaq (Life Technologies), 18  $\mu$ L distilled water, and 1  $\mu$ L of DNA extract. The thermocycling program optimized for amplification involved 15 min at 95°C; 60 cycles of 30s at 94°C, 45s at 54°C, 1 min at 72°C; and the final extension time was 7 min at 72°C. Each PCR run included a negative control and extraction negative control to monitor for contamination. Each extracted water sample amplification was replicated three times to account for heterogeneity associated with low quality/low quantity DNA assays (Taberlet et al. 1996, Ficetola et al. 2008). Fragment analysis was accomplished on an ABI3130xl genetic analyzer with a Liz500 size standard. A bin was created in Gene Mapper 4.0 (Life Technologies) to identify the target peak at 96 bp. This shift from the 101 bp target amplicon was likely due to the fluorescent dye label on the forward primer causing a mobility shift of the amplified fragment on the analyzer (Sutton et al. 2011, Technologies 2016). A positive detection from a water sample was confirmed if at least one out of the three PCR replicates was positive.

### ***eDNA Capture Optimization and Inhibitor Removal***

#### ***Sampling***

Water was collected from the same 94.6 L tub used by a single feral swine sow in captivity as used in my previous step. Water was collected on May 6, 2015 by submerging a

single sterilized 2L Nalgene bottle in the tub and filling it. Water quality was similar to previous sampling with suspended particulates causing turbidity.

The water was homogenized and subsampled as described above. A total of sixty 15 mL subsamples were collected from the water sample. Subsamples were numbered in order of collection and randomly assigned to 6 different treatments (10 each) using a random number generator.

### *eDNA Capture*

To determine the most efficient capture and purification combination, the best two extraction techniques (CTAB and DNeasy *mericon* Food kit; Figure 1-2) were paired with three different concentration techniques. The 6 treatments were as follows: 1) centrifugation concentration in combination with the CTAB purification, 2) sodium acetate/ethanol concentration and CTAB purification, 3) resin bead concentration and CTAB purification, 4) centrifugation concentration and DNeasy *mericon* food kit purification, 5) sodium acetate/ethanol concentration and DNeasy *mericon* food kit purification, and 6) resin bead concentration and DNeasy *mericon* food kit purification (Figure 1-2). I included one negative control, which contained extraction reagents only, with each set of extractions for all treatments to monitor for contamination.

For concentration by centrifugation, Caldwell et al's protocol (2007) was followed with a modification of resuspending the pellet in the appropriate lysis buffer after decanting the supernatant.

Concentration with resin beads involved adding 0.25 g of Amberlite IRA-900 anion exchange resin (Sigma-Aldrich, St. Louis, Missouri) into each of the 15 mL samples and shaking

for 2 hours at room temperature (23° C) (Perez-Mendez et al. 2014) on a C24KC Refrigerated Benchtop Incubator Shaker (Eppendorf/New Brunswick Scientific, Hamburg, Germany) at a speed of 225 RPM, rapid enough to allow the beads to gently swirl in the sample water. The water was then decanted and the resin beads were resuspended in the lysis buffer of the appropriate protocol for each treatment. For the CTAB extraction, the resin beads were treated as if they were a pellet and the beads in lysis buffer were incubated according to protocol (Perez-Mendez et al. 2014). For the DNeasy *mericon* food kit, the resin beads were treated as if they were the starting food material. After resuspending the resin beads in the food lysis buffer, everything was transferred to a 2 mL tube and the rest of the manufacturer's protocol was followed (Qiagen).

The sodium acetate and ethanol precipitation followed Valiere and Taberlet's protocol: 1.5 mL of 3M sodium acetate and 33 mL absolute ethanol (Valiere and Taberlet 2000) with a modification of eliminating overnight incubation of the mixture (Piaggio et al. 2014), an adjustment of centrifuge speed due to centrifuge limitations, and drying the pellet for 5 minutes after decanting the supernatant (Piaggio et al. 2014, Turner et al. 2014).

Any additional modifications used for the CTAB protocol and the DNeasy *mericon* Food kit are included in the previous section describing extraction methods. All DNA extractions for each treatment were carried out on the same day. Purified DNA from each sample was analyzed using the previously described PCR amplification and fragment analysis. These 60 purified DNA samples were also used in another test to assess if inhibitor removal would increase sensitivity.

### *Inhibitor Removal*

Samples from the 6 capture/purification treatments were also run through an IRT column (Zymo Technology) to determine if inhibitors were affecting PCR performance. Conventional PCR and fragment analysis as described previously were used for amplification and visualization. Findings were compared to the results generated without the IRT column inhibitor treatment.

### ***Conventional or Quantitative PCR?***

In order to compare sensitivity of conventional PCR (cPCR) to real time quantitative PCR (qPCR), serial dilutions were made from the Zymo-treated extractions that had been successfully amplified using cPCR with fragment analysis, and then amplified using both cPCR and qPCR protocols (Figure 1-3). Eighty percent of elutions from the optimized extraction process were diluted and run against the standard curve. The cPCR program and recipe described above were used with the modification of using 5 µL of DNA as PCR template to keep the amount of DNA consistent for both PCR assay types. The primers described earlier were imported into AlleleID (ver. 7.0; Premier Biosoft) to create a compatible Taqman probe (5'-/56-FAM/AAACCAAAACGCCAAGTACTTAATTAC/3BHQ\_1/-3') with a FAM label and Black Hole Quencher Dye (IDT) for qPCR. Each qPCR reaction was a 30 µL reaction containing 15 µL Taqman environmental mastermix (Life Technology), 1 µL of each primer (10 µM), 1 µL of the probe (2.5 µM), 1 µL BSA, 6 µL distilled water, and 5 µL of DNA extract. The real time thermocycling program optimized for amplification involved 10 min at 95°C; 50 cycles of 95°C for 15 sec and 1 min at 52°C. I used an internal positive control (IPC), which was a synthetic gene of my chosen amplicon (gBlocks® Gene Fragments, IDT). I developed a standard curve



from 1:10 serial dilutions of the IPC ( $1 \times 10^7$  copies/ $\mu\text{L}$  to 100 copies/ $\mu\text{L}$ ) to evaluate my qPCR. The IPC was diluted down to 1 copy/ $\mu\text{L}$  and incorporated into the standard curve to use to calculate the relative DNA concentration of my eDNA extractions.

Each cPCR and qPCR included a “no template” negative control of PCR reagents and the extraction negative controls to monitor for contamination. Each extracted water sample was replicated in qPCR and cPCR three times. The PCR approach that successfully amplified DNA to the lowest dilution was determined to be the most sensitive in detecting low concentrations of target eDNA.

### ***Resin Bead Pilot Study***

I performed a small experiment to test if resin beads could be an effective method of eDNA capture from larger volumes of turbid water. I suspected that they may perform better with larger volumes of water than my initial assessment because they have been successfully used in the past to capture virus particles from 50 mL and 1L water samples (Perez-Mendez et al. 2014). To test this hypothesis, three 18.9 L buckets were filled with 10 L of water collected from a 50L tub that served as a waterer for two captive boars. A fourth bucket was filled with 10L of tap water to serve as a control. I added 0.5 g of Amberlite resin beads and a magnetic stir bar to each bucket and spun the water on a stir plate for 2 hours at a rate quick enough to keep the beads afloat and dispersed through the water sample. At the end of two hours, the beads were allowed to settle and the majority of the water was decanted. A small volume of water was left in with the beads and a pipette with a 25 mL pipette tip was used to transfer the beads into a 50 mL centrifuge tube (i.e., “falcon” tube). The remaining water collected in the transfer of the beads was removed and the DNA on the beads was immediately extracted using the

CTAB extraction protocol for extraction of aqueous eDNA precipitated/pelleted from water (Turner et al. 2014) with the modifications described above. The CTAB extraction protocol was chosen because it was the only extraction method that yielded any positive detections with resin bead concentration with the 15 mL sample volume. Each sample was amplified in triplicate with qPCR after inhibitor removal.

### ***Statistical Analysis***

Detection of species using eDNA is likely imperfect (Schmidt et al. 2013); occupancy modeling approaches account for imperfect detection in estimating whether or not sites are “occupied” or used by a species (MacKenzie et al. 2006). I used an occupancy approach to estimate the probability of detection ( $p$ ) for each method in each optimization step: extraction, concentration, and inhibitor removal. Encounter histories were constructed from the set of three PCR replicates where cPCR results were coded as ‘1’ for a positive detection if at least one peak was observed on the genetic analyzer at the appropriate size (accounting for dye shift) and ‘0’ otherwise. Similarly, qPCR reactions were considered positive if the threshold cycle of amplification (Ct value) was below 40 (coded as ‘1’) and negative (‘0’) otherwise. In all analyses, occupancy was set as a fixed parameter with a value of 1.0 because all water samples were exposed to pigs and “occupied” by pig eDNA. Data were analyzed using the occupancy approach with detection  $<1$  as implemented in Program MARK (White and Burnham 1999). Candidate models in each model set were ranked by their  $\Delta AICc$  values (MacKenzie et al. 2006) and estimates of the probability of detection reported here come from the top-ranked model from each set.

I developed an *a priori* candidate set of occupancy models for the extraction method analysis; this set included models incorporating the effect of extraction method, sampling period, and the additive combination of the two. Sampling period was included because differences in water quality or amount of eDNA shed into the water on each sampling date could affect the probability of detection. The *a priori* set of candidate models to evaluate the optimal concentration of eDNA and inhibitor removal included models with the method of extraction, concentration method, inhibitor removal, and the additive combination of all three factors on the probability of detection of eDNA.

For all optimization steps, the single method with the highest probability of detection (p) was carried on to the next step of optimization. However, I carried over the top two purification techniques (DNeasy mericon Food Kit and CTAB) into the concentration/inhibitor removal optimization step (Figure 1-2). For the analysis comparing the sensitivity of cPCR with qPCR, the *a priori* set of candidate models included models with an effect of the dilution of the eDNA extraction, the amplification method, and the additive combination of the two. Finally, the results of the resin bead pilot study are portrayed as the proportion of qPCR positive detections for each of the three 10 L water samples.

### ***Sequence Verification***

Twenty percent of PCR products from the optimized eDNA capture method that produced a positive detection were randomly chosen to be sequenced for verification that the target sequence was amplified. Sequencing of PCR products was completed using ABI BigDye chemistry (Life Technologies). Cycle sequencing purification was accomplished through the PrepEase (USB) protocol. Sequences were imported into Sequencher (ver.5.1; Gene Codes

Corp.) and a BLAST search was performed using the National Center for Biotechnology Information (Benson et al. 2013).

## **Results**

### ***eDNA Purification (extraction)***

The performance of extraction methods varied between the two collection periods (March 3, 2015 and March 25, 2015). In the first collection period, all methods except the MagMAX-96 AI/ND Viral RNA extraction kit (Life Technologies) produced at least one positive detection. In the second period, only two methods (CTAB and DNeasy *mericon* food kit) produced any positive detections.

The best supported model of detection probability for eDNA purification method included the effects of collection period and extraction method, carrying almost all of the Akaike weight ( $\omega_i = 0.99$ ) (Table 1-1). The probability of detection ( $p$ ) of eDNA was highest for the CTAB extraction protocol (Period 1: 0.222, Period 2: 0.074) and DNeasy *mericon* Food Kit (Period 1: 0.222, Period 2: 0.019) so these methods were carried into the next step of optimization (Table 1-2). These methods produced positive detections in both water collection periods where the other methods only performed with some success in one collection period. The probability of detection for both the CTAB extraction method and DNeasy *mericon* Food Kit was lower in the second collection period than the first (Table 1-2).

### ***eDNA Capture Optimization and Inhibitor Removal***

The best supported model, with an Akaike weight of 1.0, evaluating concentration techniques and the effect of inhibitor removal was the model with the additive combination of concentration, extraction, and inhibitor removal (Table 1-3). Detection varied across

concentration and extraction methods and was overall higher when inhibitor removal techniques were used (Table 1-4). The highest probability of detection was 0.70; the combination of techniques leading to this level of detection was centrifugation, DNeasy *mericon* Food Kit, and Zymo IRT column (Table 1-4).

### ***cPCR or qPCR?***

Detection probabilities, as a measure of assay “efficiency”, were compared between cPCR with fragment analysis and qPCR to assess the sensitivity of each to low concentrations of eDNA. Both cPCR and qPCR performed equally well on the environmental samples when the template DNA was 5  $\mu\text{L}$ . However, qPCR was more sensitive than cPCR when the DNA from environmental samples was diluted. Conventional PCR lost sensitivity (i.e., fewer positive detections) as early as the  $10^{-1}$  dilution. Quantitative PCR retained perfect detection until the  $10^{-3}$  dilution (Figure 1-3). The best supported model carried all of the Akaike weight (1.0); the combination of factors included in this model were the level of dilution and PCR method.

I observed that detection sensitivity began to decline once samples fell below 1 copy/ $\mu\text{L}$ . The average DNA concentration of my undiluted eDNA elutions was estimated as 1335.125 copies/ $\mu\text{L}$ . To relate this to copy number per sample collected, I calculated how many copies/L of sample water I collected using the quantification of eDNA shedding rates (Klymus et al. 2015, Lance 2016). The estimated eDNA that could be collected in a 1L sample from the artificial wallow system, assuming perfect efficiency throughout the assay, was  $1.367 \times 10^7$  copies/L.

### ***Resin Bead Pilot Study***

I successfully detected eDNA from resin beads when I collected larger amounts of water (10L). The proportion of qPCR positives varied across the three 10L water samples ranging from a probability of detection of 0.00 to 0.67 (Table 1-5).

### ***Sequencing verification***

The randomly selected PCR products were all confirmed as a portion of the D-loop region of *Sus scrofa* thus confirming that these were not false positives.

### **Discussion**

Through this demonstration it is clear that techniques in concentration, extraction, and amplification of eDNA have varying efficiencies in detecting eDNA from turbid water samples. Through comparing various methods at each stage of eDNA sample processing, I have determined that the optimal protocol for eDNA detection in turbid water samples is to concentrate a 15 mL water sample by centrifugation, extract the eDNA using the DNeasy mericon food kit, follow up with Zymo IRT columns to remove any inhibitors, and amplify the DNA with my qPCR assay.

In the first step of optimization, the two sample collection periods varied in detection probability; this is likely due to differences in water quality at the two times of collection. The water in the first period of collection may have had fewer inhibitors, perhaps more DNA was shed by the pig, or environmental conditions varied enough between the two collection periods to cause a difference in eDNA detectability.

Both the CTAB and DNeasy *mericon* food kit are chloroform based extraction methods that showed the most promise purifying eDNA samples; chloroform based techniques for DNA

extraction have withstood the test of time and continue to be the preferred method in some studies (Garber and Yoder 1983, Debroas et al. 2009, Oh et al. 2011, Turner et al. 2014, Renshaw et al. 2015). Both protocols use cetyltrimethylammonium bromide (CTAB) which can either form complexes with nucleic acids in low salt conditions or with cellular inhibitors in high salt conditions (Qiagen). I chose to carry these two methods on to the next step of optimization due to their consistent performance across both water collection periods.

The eDNA capture and inhibitor removal steps were analyzed simultaneously. Prior to inhibitor removal with Zymo IRT columns, it appeared that the addition of sodium acetate with ethanol precipitation paired with the DNeasy *mericon* food kit extraction provided the optimal method for eDNA capture from turbid samples. However, after inhibitor removal, the combination of centrifugation concentration and DNeasy *mericon* food kit extraction had a slightly higher probability of detection (Table 1-4). One drawback to centrifugation using 50 mL falcon tubes is the need for a centrifuge that can accommodate them. I selected a fixed angle rotor that could hold 12 falcon tubes (50 mL) and spin at the required 9000 x g (Beckman); most large rotors are swing style and cannot spin above 3220 x g. Because the sodium acetate with ethanol precipitation concentration technique performed nearly as well as centrifugation, this may be an acceptable alternative when a centrifuge is not available.

I recommend using a post-extraction inhibitor removal step. My results showed a strong effect of the Zymo IRT columns in cleaning up elutions as shown by a higher probability of eDNA detection when this step was included (Table 1-4). This is most obvious for the centrifugation concentration and DNeasy *mericon* food kit combination: the probability of detection increased from 0.40 without inhibitor removal to 0.70 with inhibitor removal (Table 1-4). In some cases,

eDNA was only detected after inhibitor removal (e.g., centrifugation concentration and CTAB extraction).

I also recommend using qPCR as the amplification method for eDNA detection. This method was most effective in detecting low quality or low quantity eDNA samples. While both techniques produced a probability of detection of 1.0 after the PCR recipe contained 5  $\mu$ L of template eDNA, the difference in sensitivity became apparent with sample dilution. In my eDNA extractions, DNA quantity ranged from 517 copies/ $\mu$ L to 2011 copies/ $\mu$ L suggesting stochasticity or eDNA clumping (Furlan et al. 2015) in my environmental samples, despite the effort to homogenize. This finding suggests that further optimization in sampling scheme should occur before applying this test in the field for management questions.

I did not successfully capture eDNA from 15 mL water samples using the resin bead concentration method in a pilot experiment. Nevertheless, once I increased the volume of sample water to 10L, resin beads showed promise in capturing eDNA. During the transfer of resin beads for extraction, some silt was collected and may have introduced inhibitors to the extracts (resulting in  $p < 1.0$ ), which may explain variable detection probabilities. A suggestion to improve this concentration technique is to use a filter or screen to separate the resin beads from debris at the transfer step.

Recently, occupancy modeling has been applied to eDNA surveillance to estimate the probabilities of detection and site occupancy for amphibians (Pilliod et al. 2013b), reptiles (Hunter et al. 2015), and emerging pathogens (Schmidt et al. 2013). I chose an occupancy approach to account for imperfect detection using an eDNA method. Factors that could influence the ability to detect pig eDNA using my assay may include the level of turbidity



(inhibitors), amount of eDNA shed into the system, or environmental factors. In order for this assay to be useful, its limits of detection should be explored, as I have done here. By comparing the probability of detection for each step in optimization, a robust method of eDNA capture in my turbid water system was established. Omitting equipment costs and PCR reagents, this method would cost ~\$5.04 per sample (\$3.00 per sample for DNeasy *mericon* food kit, \$2.04 per sample for Zymo IRT). The sample collection, concentration, purification, inhibitor removal, and amplification steps can be completed all within a single day, which allows for a fast turn-around time when presented with a pressing wildlife conservation or management question.

## **Conclusion**

This research provides an optimized method for eDNA capture from samples collected from a turbid water system. I showed dramatic differences in the probability of detection with each option for each step of eDNA capture and amplification. Comparing efficiencies of concentration, extraction, inhibitor removal-post extraction, and amplification techniques can reduce biases in capturing eDNA (Deiner et al. 2015, Goldberg et al. 2015). Understanding the biology of the system from which eDNA is collected can influence the choices made for each step of the process.

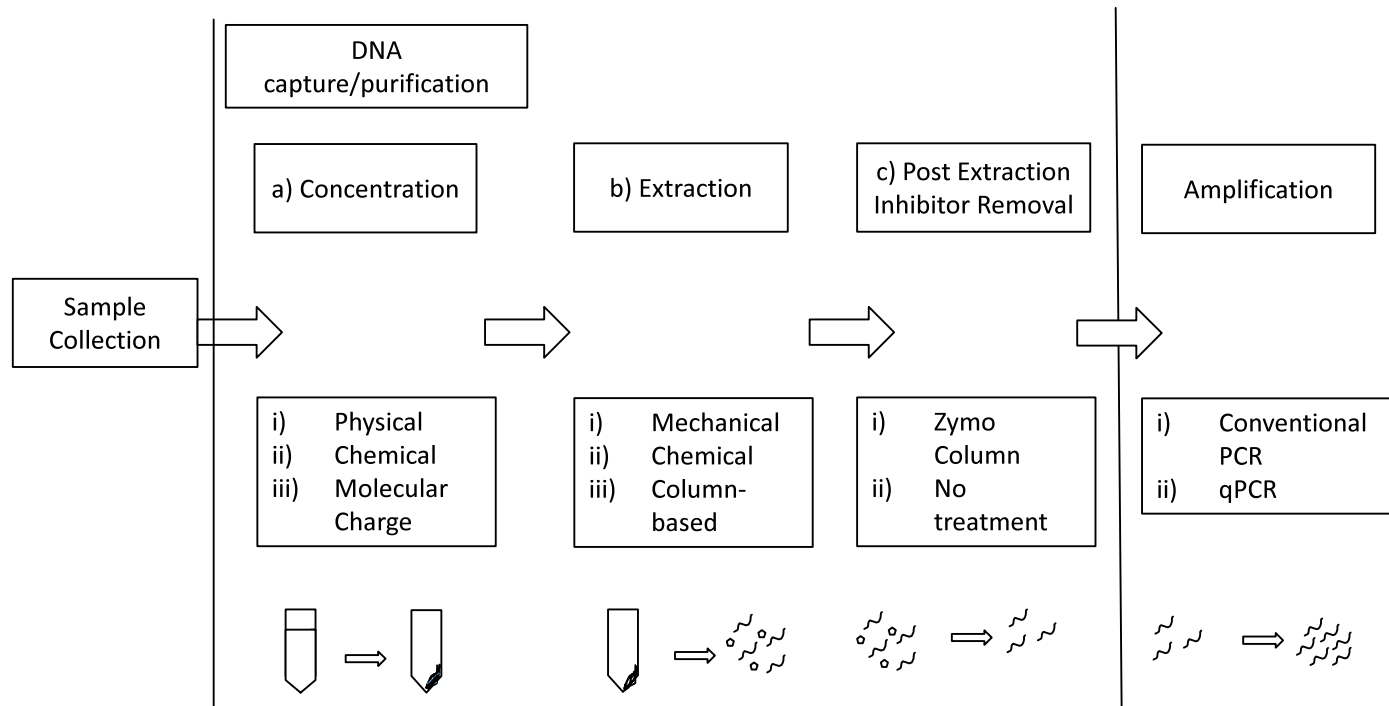


Figure 1-1: The process of eDNA capture involves concentration, extraction, inhibitor removal, and PCR amplification of a water sample. Each step of processing eDNA has multiple options that are optimal for the system that the water is collected from. The concentration step (a) captures the eDNA into a form that extraction methods can work with (transformation of water to pellet in a centrifuge tube). The extraction method (b) then purifies the DNA captured in the pellet with varying efficiencies (curved lines represent DNA, pentagons are inhibitors). An inhibitor removal post-extraction step (c) may effectively remove inhibitors while also potentially causing a loss of DNA. Finally, either conventional PCR or quantitative PCR can be chosen for the amplification technique, which may differ in sensitivity to low quantities of DNA.

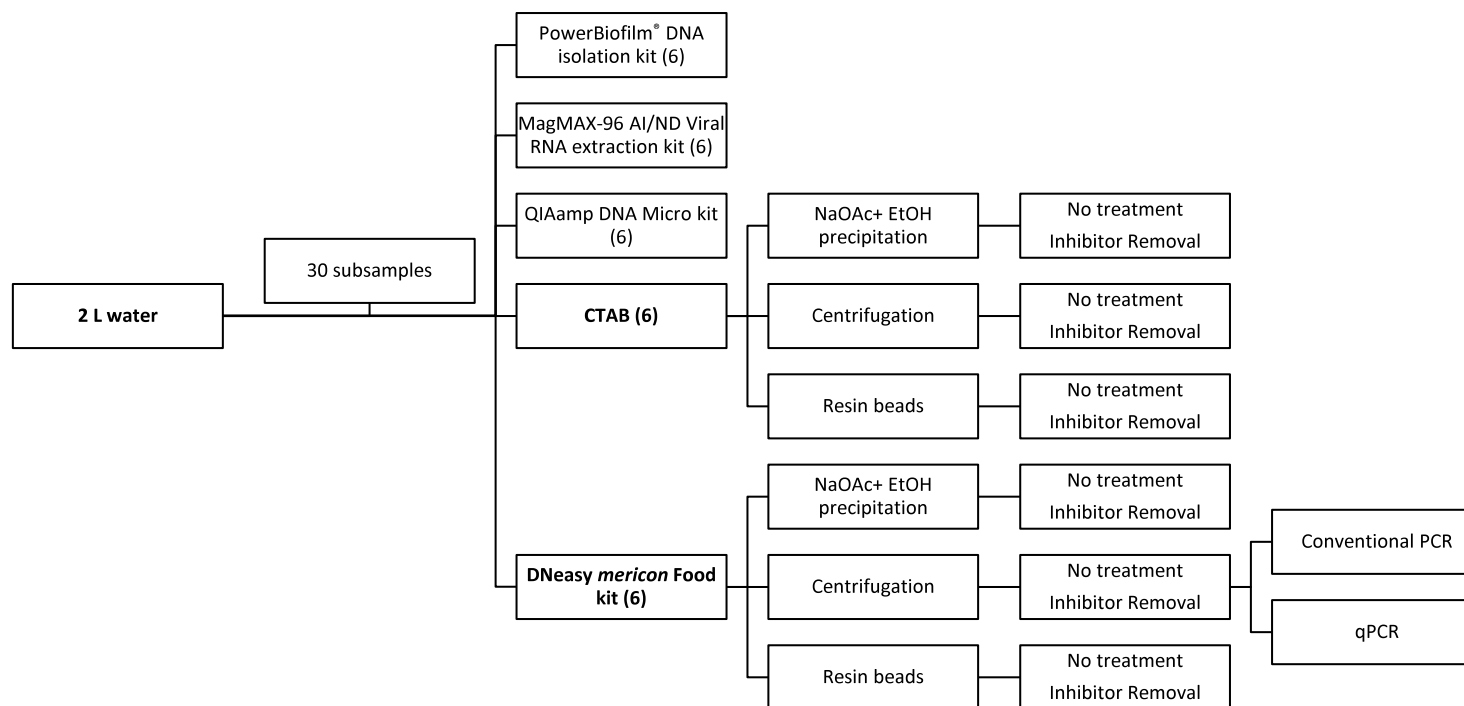


Figure 1-2: Schematic for the study design of my eDNA capture optimization experiment. For each of two collection dates, 2 L of water was collected from a 94.6 L tub serving as a waterer for a single feral swine sow (A). The 2 L of water was subsampled (B) and randomly assigned to an extraction kit (C). Each subsample was aliquoted into three replicates of 15 mL sample water, extracted, and run in triplicate on conventional PCR. Next, the DNeasy *mericon* Food kit and CTAB protocol were compared with various concentration techniques (D). A second sample collection of 1 L of water was collected from a 94.6 L tub serving as a waterer for a single feral swine sow. The 1 L sample was subsampled and randomly assigned to a concentration and extraction method combination (E). After analyzing concentration and extraction success with conventional PCR for each sample, the samples were cleaned with a Zymo IRT column and reanalyzed (F). The samples that were passed through the IRT columns from the centrifugation and food kit extraction combination were used to determine sensitivity of each PCR instrument (conventional PCR and quantitative PCR).

Table 1-1: Model ranking of occupancy models evaluating the probability of detecting eDNA from turbid water using various DNA extraction techniques (“extraction”) and the effect of water collection date (“period”: March 3, 2015 and March 25, 2015). The number of parameters (K) in each model, the small sample sized-corrected AIC values (AICc), the AICc differences (delta AICc), the Akaike weight for each model, and deviance are reported for each candidate model.

<b>Model</b>	<b>K</b>	<b>AICc</b>	<b>Delta AICc</b>	<b>Akaike weight</b>	<b>Deviance</b>
p(extraction+period)	10	254.5759	0.0000	0.99998	48.9670
p(period)	2	276.8575	22.2816	0.00001	88.4825
p(extraction)	5	283.1330	28.5571	0.00000	88.4810

Table 1-2: The probability of detection of eDNA detection in turbid water samples estimated based on the best model (Table 1-1) of collection period and extraction method. The CTAB protocol and DNeasy mericon Food kit were the only extraction methods that performed in both water collection periods.

Collection Period and Extraction Method	Probability of Detection (p)
Period 1 CTAB	0.222
Period 1 QIAamp DNA Micro kit	0.222
Period 1 DNeasy mericon Food kit	0.222
Period 1 MagMAX-96 AI/ND Viral RNA	0.000
Period 1 PowerBiofilm®	0.056
Period 2 CTAB	0.074
Period 2 QIAamp DNA Micro kit	0.000
Period 2 DNeasy mericon Food kit	0.019
Period 2 MagMAX-96 AI/ND Viral RNA	0.000
Period 2 PowerBiofilm®	0.000

Table 1-3: Model ranking of occupancy models evaluating the probability of detecting eDNA from turbid water using DNA concentration (“concentration”), extraction technique (“extraction”), and inhibitor removal (“IRT”). The number of parameters (K) in each model, the small sample sized-corrected AIC values (AICc), the AICc differences (delta AICc), the Akaike weight for each model, and deviance are reported for each candidate model.

<b>Model</b>	<b>K</b>	<b>AICc</b>	<b>Delta AIC</b>	<b>Akaike weight</b>	<b>Deviance</b>
p(concentration+extraction+IRT)	12	215.7695	0.0000	1	58.4847
p(concentration)	3	310.6033	94.8338	0	174.0275
p(extraction)	2	320.6637	104.8942	0	186.1922
p(IRT)	2	327.2747	111.5052	0	192.8032

Table 1-4: The probability of detection estimated based on the best model:  $p(\text{concentration} + \text{extraction} + \text{IRT})$ . Bolded is the highest probability of detection though concentration by centrifugation, extraction by DNeasy *mericon* Food kit, and IRT treatment. (NT: No treatment; IRT: Inhibitor Removal Technology)

Concentration + Extraction + Inhibitor Removal Models	Probability of Detection (p)
Cent + CTAB + NT	0.000
Cent + Food + NT	0.100
Resin + CTAB + NT	0.000
Resin + Food + NT	0.000
NaOAc/EtOH+CTAB + NT	0.000
NaOAc/EtOH + Food + NT	0.400
Cent + CTAB + IRT	0.367
<b>Cent + Food + IRT</b>	<b>0.700</b>
Resin + CTAB + IRT	0.067
Resin + Food + IRT	0.00
NaOAc/EtOH+CTAB + IRT	0.00
NaOAc/EtOH + Food + IRT	0.667

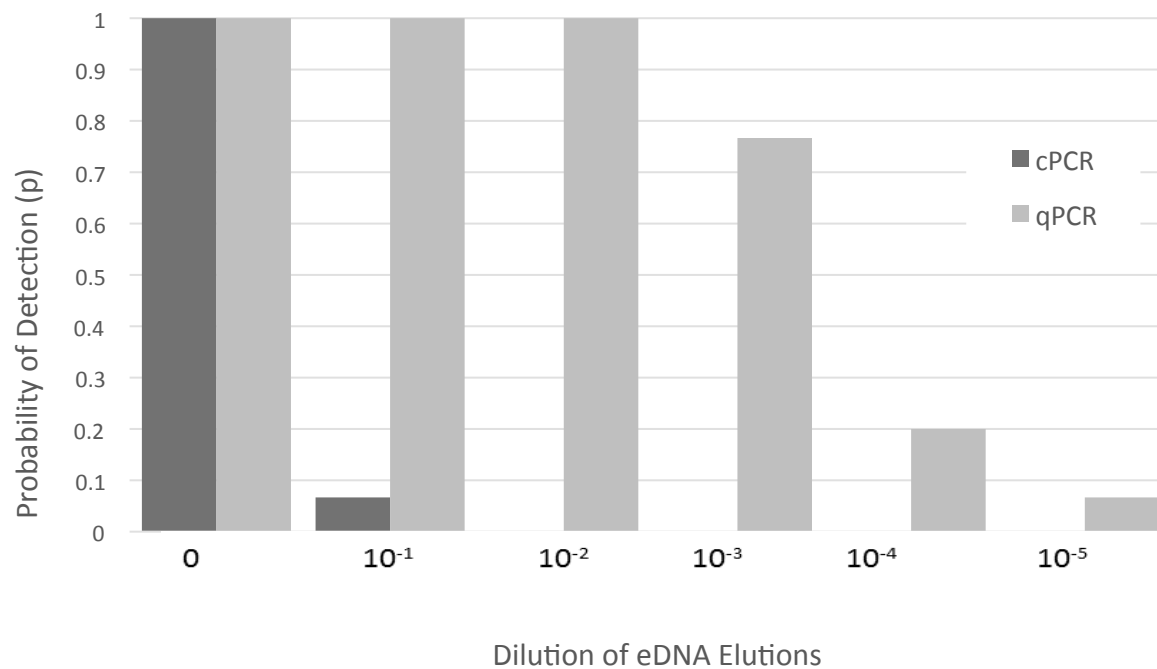


Figure 1-3: Sensitivity, or the ability to detect low levels of eDNA, of cPCR and qPCR amplification methods across dilutions of eDNA sample. Fragment analysis on cPCR lost sensitivity after 10<sup>-1</sup> dilution. Real time qPCR provided positive detection consistently until the 10<sup>-3</sup> dilution and then gradually lost sensitivity.



Table 1-5: Detection probabilities for eDNA samples concentrated using resin beads in three 10L samples of turbid water collected from a pig waterer. Samples were extracted using the CTAB method followed by cleaning the elutions with inhibitor removal technology and amplification using qPCR.

10L Bucket ID	Proportion of qPCR positive detections
1	0.667
2	0.333
3	0

## Chapter 2: Molecular detection of a terrestrial mammal: An optimized approach in detecting low levels of feral pig environmental DNA in water sources

### **Summary**

The application of environmental DNA (eDNA) detection techniques to invasive species can provide a method of surveillance that outperforms traditional observation methods. Feral pigs are a destructive invasive species with a rapidly expanding distribution across the United States. Use of an eDNA detection technique would benefit management efforts by providing a sensitive method for detection of even small numbers of individuals. I show that my method of molecular detection is sensitive enough to detect very low quantities of eDNA shed by a terrestrial mammal that has limited interaction with water. My method can be used to detect feral pigs in low numbers and with minimal contact and can be an important tool for the management of this invasive species. This study also provides a level of understanding of how long eDNA may persist in turbid water samples.

### **Introduction**

Few habitats remain that have not been affected by invasive species introduced in some way by humans due to globalization (Nuwer 2014). Whether this introduction is via the pet trade, agriculture, or transportation networks (e.g., ballast water in ships, cargo containers), the threat of invasive species spreading to naïve areas, with attendant negative impacts, is growing. In the U.S. alone, there are approximately 50,000 invasive species and their economic impacts are estimated to be upwards of \$120 billion per year (Pimentel et al. 2005). Effective detection

and management strategies are necessary to limit successful invasions and preserve native wildlife and ecosystems.

### ***Invasive Species***

Biotic invaders are species that migrate, or are transported, to a new range in which subsequent generations reproduce, spread, and persist in the environment, often causing a negative impact on the newly colonized environment and biota (Mack et al. 2000). Not all biotic immigrants are successful in invading a naïve area as movement from a native range and establishment in a new area is not always successful (Mack 1995, Jeschke and Strayer 2005). Life history traits such as high reproductive rate and rapid growth are core characteristics of successful, resilient invaders (Duncan et al. 1999, Sakai et al. 2001, Blackburn et al. 2009) but dependence on humans for rapid dispersal is also important (Jeschke and Strayer 2006).

Feral pigs have many qualities of a successful invader: they are extremely adaptive, reproduce rapidly, and anthropogenic dispersal of this species is well-documented (West et al. 2009). Genetic data from various locations across Eurasia revealed multiple centers of independent domestication of feral pigs (Larson et al. 2005). Feral pigs have inhabited the mainland of the U.S. since the early 1500's after being introduced to Florida as domesticated European pigs (Towne and Wentworth 1950, Mayer and Brisbin 1991). Pigs were popular livestock for American settlers due to their adaptability and ability to survive in the wild (West et al. 2009) and were an important source of food and lard (Towne and Wentworth 1950). Mitochondrial DNA sequences of wild pigs from the U.S. suggest a strong association between introduced pigs and European domestic breeds, thus reflecting the known history of human

colonization and settlement of the U.S. (McCann et al. 2014). After the introduction of domesticated pigs, free-range livestock management practices and escapes or release from enclosures led to the establishment of wild, or feral, pig populations across the country (Taylor 1993). Strong evidence exists that the range of feral pigs continues to expand into new areas (West et al. 2009); feral pigs are currently found in 35 states across the country (S.C.W.D. 2014).

Aside from human-assisted movements, characteristics of feral pigs that have made them a successful invasive species include high reproductive rates (Taylor et al. 1998, Waithman et al. 1999) and that they are opportunistic generalists (Fogarty 2007, West et al. 2009). Feral pigs can inhabit a multitude of habitat types, including harsh, seemingly uninhabitable regions such as deserts and northern latitudes with long winters (Adkins and Harveson 2007, West et al. 2009, Wyckoff et al. 2012, S.C.W.D. 2014). Due to their adaptability, suitable habitats occur throughout most of the country. Landscape features that can impact feral swine movement or habitat preference include the availability of shelter, food, and water (Graves 1984, Saunders and Bryant 1988, Sodeikat and Pohlmeier 2003). Hunting interests prompted illegal trade and translocation of pigs throughout the U.S., further contributing to rapid range expansion (Taylor 1993, McCann et al. 2014).

Feral pigs can be considered ecosystem engineers due to the changes they catalyze on a landscape (Jones et al. 1997). Pigs alter the composition and structure of vegetation communities by reducing plant survival through rooting behaviors, wallowing, and trampling (Taylor 1993, Hone 2002). Further, they can disperse the seeds of invasive weeds via excretion after consumption (Lynes and Campbell 2000). Diet analysis shows that pigs will eat almost any organic substance (Schley and Roper 2003). The influence of feral pigs through consumption of

native flora and fauna is of concern for wildlife managers in general but especially at protected areas such as wildlife refuges, national forests, and parks (Singer et al. 1981, Campbell and Long 2009, Hess et al. 2010). Feral pigs are generalists, which may allow them to have a competitive advantage over other species with more specialized diets (Springer 1977, Wood and Barrett 1979, Sweeney et al. 2003). Other impacts to ecosystems that are caused by feral pigs include pathogen shedding into water sources (Hampton et al. 2006, Jay et al. 2007), pathogen spillover (Wu et al. 2012), and viral reassortment (Kida et al. 1994, Hall et al. 2008). Due to their behavioral plasticity, omnivorous feeding habits, rooting behavior, high fecundity, and potential to contribute to pathogen spread, feral pigs have become a destructive and dangerous invasive species.

### ***Management***

There are currently efforts across the U.S. to reduce feral pig populations. Despite these control efforts in many states, feral pig populations continue to grow. Challenges to eradication efforts include immigration of feral pigs from surrounding areas, movement by humans, difficulty in detecting and removing the last few individuals, and the high fecundity of feral pigs. Feral pig populations must be reduced to zero for successful control because a few remaining individuals can reproduce leading to rapid repopulation (Barrett and Pine 1980, Choquenot et al. 1996). Application of environmental DNA (eDNA) detection techniques allows for surveillance and management of invasive species that are difficult to monitor or detect by field observation (Ficetola et al. 2008, Jerde et al. 2011, Piaggio et al. 2014, Tréguier et al. 2014). Detection of invasive species using eDNA is likely to be more efficient than observational monitoring after an intensive eradication program or in the initial stages of an invasion because

the probability of visually detecting a few remaining individuals is likely very low (Jerde et al. 2011, Pilliod et al. 2013a).

### ***eDNA***

Environmental DNA is DNA that is released from an organism into the environment and can be detected in cellular or extracellular forms (Darling and Mahon 2011, Jerde et al. 2011). Sources of eDNA include feces, urine, mucus, saliva, gametes, and shed skin or hair (Ficetola et al. 2008, Taberlet et al. 2012). Environmental samples vary in the amount of DNA present due to many factors: the relative volume of sample to target DNA, size of the organism, as well as the volume or intensity of secretion or shedding (Ficetola et al. 2008, Klymus et al. 2015). Depending on conditions, DNA may persist for various lengths of time in the environment (Ficetola et al. 2008, Dejean et al. 2011, Barnes et al. 2014). Conditions that are likely to affect degradation of eDNA include exposure to UVB radiation, pH, heat, and endo- and exonucleases in the aquatic environment (Ficetola et al. 2008, Pilliod et al. 2014). Another influence on DNA in the environment is microorganisms that digest and break down DNA (Dejean et al. 2011). Other challenges associated with eDNA detection include the presence of inhibitors and the sensitivity and specificity of lab assays. Inhibitors are humic substances that may be co-extracted with eDNA and inhibit the performance of conventional PCR or quantitative PCR (Tsai and Olson 1992, Matheson et al. 2010, Albers et al. 2013) such that the eDNA detection assay does not perform as expected (McKee et al. 2015).

The design and implementation of eDNA detection methods for invasive species monitoring must be rigorously controlled through good laboratory practices and the

development of assays with high sensitivity and specificity to prevent errors in detection.

Despite the fact that DNA begins to degrade as soon as it is shed, and is typically found in low concentrations in the environment, eDNA detection has been an effective tool for identification of recently introduced aquatic or semi-aquatic invasive species (e.g., (Ficetola et al. 2008, Darling and Mahon 2011, Piaggio et al. 2014)). Application of eDNA has largely been restricted to aquatic species, limiting conservation and management efforts with this method. The concept of using eDNA in water sources to detect terrestrial wildlife has been tested (Rodgers and Mock 2015), but not yet optimized in terms of detection and degradation thresholds.

Environmental DNA techniques could provide an ideal approach for detection and monitoring of feral pigs. Feral pigs daily spend time drinking or wallowing in water bodies (Taylor 1993, Jay et al. 2007) for thermoregulation and protection from insects and parasites (Graves 1984, Heinken et al. 2006, Campbell and Long 2009). Through drinking behaviors, saliva containing cells with DNA are shed into the water (Rodgers and Mock 2015) while wallowing behaviors can lead to shedding of epithelial cells; urine and feces can also be a source of eDNA shed into the environment (Valiere and Taberlet 2000, Beja-Pereira et al. 2009). However, molecular detection techniques using eDNA are susceptible to sources of error. False positives can occur if target DNA is present in the environment and the organism is not. This may be caused by the inability to distinguish DNA from living or dead organisms (Mountfort and Hayden 2006) or whether DNA was introduced via another source or persisted in the environment after the individual relocated (Darling and Mahon 2011). Specificity of the primers is also important for reducing false positives (Darling and Mahon 2011) which are errors that can occur when primers cross react with non-target sequences if the similarity between target

and potential non-targets present in the system is high (Raut et al. 2007, Pelt-Verkuil et al. 2008). False negatives occur when target DNA is present in the sample but is not detected. Applying eDNA detection methods to monitoring of invasive feral pigs should be sensitive enough to detect low quality or low quantity DNA, thus minimizing false negatives. This error can be avoided by designing an assay with substantial sensitivity, by including replicates so that detection rates can be estimated directly, and by optimizing eDNA capture methods (Darling and Mahon 2011).

Here, I test the sensitivity of an eDNA assay I developed for the detection of feral pigs (Chapter 1). One goal of this study is to determine how long a pig must have contact with a large, temporary water source or what behaviors are required (i.e., drinking/contact with snout vs wallowing/whole body contact) to shed sufficient DNA in water for reliable detection. The second goal is to develop an understanding of how long pig eDNA can persist in water, providing insight into how recently a pig visited the water source. An understanding of pig DNA persistence in water could also be useful in surveillance of areas of new invasion by providing a time frame of when feral pigs were likely last in the area. Using my persistence data, I hope to develop a model to determine the appropriate amount of time to wait in order for traces of DNA from past pig populations to degrade beyond detectability. Through a series of careful experiments, we are one step closer to implementing eDNA monitoring in the field for detecting invasion or monitoring success in an eradication effort.



## **Methods**

Laboratory work was performed at the USDA-APHIS National Wildlife Research Center in Fort Collins, Colorado, USA. Extractions were performed in a lab where only low quantity/quality DNA was processed. All PCR and post-PCR procedures were completed in separate rooms. Equipment, benchtops, pipettors, and fume hoods were cleaned with 10% bleach before and after any procedure.

### ***Study sites***

Feral pigs were held in captivity at the USDA-APHIS/Colorado State University Wildlife Research Facility and were used for this study with all necessary IACUC reviews and approvals. I had two enclosures, one with 13 pigs (hereafter, “Group”) and another enclosure with a single pig (“Boar”).

To develop an understanding of the behavior of eDNA shed by feral pigs into the environment, I built artificial wallows to mimic conditions I might find in the wild. Artificial wallows were constructed by placing an 1135 L tub flush to the ground in each of the pig enclosures (Group: June 26, 2014; Boar: July 9, 2014). Tubs had never been exposed to pigs prior to use. Cinder blocks were added to make the wallows shallow and accessible for pigs to enter and leave with minimal effort. I then filled the water tubs with the cinder blocks to a final volume of approximately 800 L each (Group: 757L, Boar: 852L).

### ***Evaluating Assay Sensitivity***

I used the Boar wallow to determine how much eDNA was needed to accumulate in the wallow for a positive detection. Three 60 mL samples were collected in Nalgene bottles from the tub immediately after filling it, serving as time zero samples. From then on, the pig was free to interact with the water in the wallow. Every 15 minutes for two hours, three 60 mL water samples were collected from the tub. Pig behavior and interactions with the water in the wallow were recorded (Appendix A). After the two hour sampling event in which the water samples collected were stored in a cooler, all samples were stored in a -80°C freezer until processing.

I also tested the sensitivity of the assay to detect pig DNA by allowing a single pig to have only minimal contact with the water source. This was accomplished by limiting the pig to snout/mouth contact only with galvanized steel waterers (Little Giant, Miller Manufacturing, Glencoe, MN). The sample collection part of this study was carried out at the USDA NWRC Mississippi Field Station in Starkville, Mississippi.

An automated waterer was used consisting of a large tank outfitted with a bowl at its base; the bowl was filled with 1.48 L of water and was refilled by pressure from the animal on a metal paddle situated on the back of the bowl. This waterer had never been used and was placed in a pen with a single pig. Three 60mL samples were collected in Nalgene bottles at time zero and every 15 minutes for 2 hours. The pig was allowed to drink from the waterer at will and, after samples were collected, the waterers automatically refilled to capacity after each sampling event. Pig behavior with the water was documented (Supplementary Data). Samples

were shipped from the field station to the lab (NWRC, Fort Collins, CO) on dry ice and then immediately stored in a -80° freezer until processing.

### ***Persistence***

Artificial wallows were left in both the Group and Boar pens for one week after installation in which the pigs actively used the water source. The wallows were turbid from dirt and mud introduced by the pigs. After one week of use, eighty-seven 60 mL samples were collected in Nalgene bottles from each of the wallows. A table was set up in an enclosed building with open sides allowing for temperature fluctuation and some exposure to UV; the 87 samples were placed on the table to allow for environmental degradation of the eDNA over time. Three of the 87 samples were taken at the start of the time series to serve as time zero controls, then 3 samples were collected from the table every 12 hours over the next two weeks to measure eDNA degradation over time. All samples were carried in a cooler from the outdoor enclosure into the lab (~5 minute walk) and stored in a -80°C freezer until processing.

### ***eDNA capture***

All samples were processed using my optimized eDNA capture and qPCR protocols previously developed for feral swine eDNA detection (Chapter 1). I included a negative control in each set of extractions to monitor for contamination.

Each PCR set included a “no template” negative control including only PCR reagents to monitor for contamination. Each extracted water sample was run in triplicate via qPCR. A water sample was considered “positive” only if all three qPCR replicates were positive and a replicate was considered positive if the Ct value was below 40 cycles.

## ***Statistical Analysis***

### *Assay Sensitivity*

I did not apply a statistical test for detection proportions for the boar and waterer detection studies, I simply recorded the number of successful samples (i.e. all qPCR replicates positive) per time point. I analyzed the accumulation of eDNA over 2 hours in naïve water by using a robust regression, allowing for inclusion of outliers that I felt were of biological importance. I included all measured DNA concentrations that resulted in a positive time point sample (all 3 qPCR replicates were positive).

### *Persistence*

For the persistence experiment, I used logistic regression to determine if hours of degradation affected detection of eDNA. This was performed on the proportion of qPCRs positive (total qPCRs = 9 per time point) over each of the 12-hour collection time points. I then combined the degradation data from both the Boar and Group of pigs and tested a series of logistic models. The *a priori* candidate set of models included the effects of hour of degradation, whether or not the eDNA was shed from a group of pigs, and the interaction of group eDNA shed and hour on detection of degrading eDNA; models were ranked by AIC. Using the best model, I estimated the amount of time necessary for previously shed eDNA to degrade, assuming no new introduction of pig occurs. I also modeled the decline in the concentration of eDNA in water over time to see how degradation time affects eDNA. I used a robust regression to account for the outliers that I thought were of biological significance. Statistical analyses were conducted in R x64 3.1.2.

## Results

### ***Assay Sensitivity***

I amplified the 101 bp fragment of the *Sus Scrofa* D-loop region from all water samples collected from the Boar wallow and waterer study (3/3 positive qPCRs for each of 3 samples collected) for each time point from 15 minutes to 2 hours (Table 2-1).

### ***eDNA Accumulation***

Time had a statistically significant effect on the concentration of DNA for the Boar trial ( $p=0.0012$ ) (Figure 2-1). Minutes of eDNA accumulation had a positive effect on the concentration of DNA measured ( $\beta=1.57$ ,  $SE=0.39$ ).

### ***Persistence***

There was a statistically significant effect of time on the detectability of degrading DNA for the Boar trial ( $p=0.00962$ ) but not for the Group trial ( $p=0.2307$ ) (Figure 2-2). The best supported model included hours of degradation, size of population (single pig or group), and an interaction of size (single pig or group) and hours of degradation (Table 2-2). Below is the equation I derived to estimate the number of hours to wait in order to reach the desired probability of detection (Equation 2-1, Table 2-3) using the estimates of  $\beta$  for each parameter.

$$\text{Equation 2-1: } \text{logit}(p) = 3.35837 - 0.03277 (\text{Hour}) - 0.16191 (\text{Group}) + 0.02554 (\text{Group} * \text{Hour})$$

### ***Quantification of eDNA Degradation***

Degradation time had a statistically significant effect on the concentration of eDNA measured in each replicate at each sample for the Group samples ( $p < 0.0005$ ) but not for the

Boar ( $p=0.01311$ ) (Figure 2-2). Hours of degradation had a negative effect on the measured DNA concentration in the water samples for the Group ( $\beta=-0.12$ ,  $SE = 0.02$ ) and for the Boar ( $\beta=-0.01$ ,  $SE = 0.00$ ).

## Discussion

I have demonstrated that eDNA shed by feral pigs can be detected in water after only 15 minutes of exposure by a single pig even through minimal contact (i.e., drinking). Therefore, eDNA detection offers a promising tool to monitor habitats for new invaders either transported from afar or on the invasion front. Hunting pressure may change pig behavior and cause them to be on the move and only interacting with water sources to drink rather than wallow (Sodeikat and Pohlmeyer 2003, Gaston 2008). The sensitivity of the assay to low amounts of eDNA could allow it to be a powerful tool for surveillance for the invasion front.

The tests for persistence of eDNA in the wallow samples showed that the number of individuals shedding into the water affected how long degrading eDNA could be detected (Figure 2-2). I did not run the persistence study long enough for the group eDNA to completely degrade and to be no longer detectable. However, combining data from the boar and group studies allows for an estimate of when to expect eDNA to degrade beyond detection from a group of pigs (group size of 13) (Figure 2-3). For example, the probability of detecting eDNA shed from a group of pigs would be less than 0.001 about 58 days (1397 hours) after the group of pigs had been removed. Understanding the relationship between the size of the population of pigs (single, few, many) to how long eDNA may persist in the environment is useful for management purposes. My results show if a lone pig was removed from an environment where

it was thought to be solitary, and 13 days (314 hours) later pig eDNA was detected (detection probability  $<0.001$ , Equation 1) the eDNA is likely from a new invader and not remnant eDNA from the removed pig. If a group (approximately 13 pigs) was eradicated from an area, I recommend, based on these results, that managers wait about 2 months (58 days; Table 2-2) before resampling the wallow for missed individuals or new invaders.

More emphasis should be placed on changes in eDNA concentration over time due to degradation rather than detection alone. Though I observed positive PCRs in the later time points, the DNA concentrations of the samples decreased throughout the duration of the trial. If this method were applied in a management setting, samples collected regularly after an eradication attempt could provide useful information on whether or not new invasions by pigs were occurring. This method would require continuous sampling but may not require intensive field work if samples were collected every few weeks or every month for monitoring an area in question.

In both the detection and persistence studies, DNA concentrations at each time point showed a great deal of variation. Detection of environmental DNA is inherently stochastic, in that it is not distributed homogeneously in the environment and the probability of detection varies. Recently, Furlan et al. (2015) found that eDNA detection was dependent upon the concentration, dispersion, and survey method. They found that DNA was not dispersed evenly in water samples, rather they were spatially clumped throughout the site causing some samples to contain few or no DNA copies where DNA was in fact shed into the system. I observed similar between-sample variation, although I did not assess whether it was due to clumping in the wallow or occurred during the extraction process. The outliers in the eDNA accumulation graph

(Figure 2-1) could be attributed to this phenomenon. To tackle this issue, many studies have started to apply occupancy approaches to estimate the probability of detection of eDNA and factors contributing to uncertain detection (Pilliod et al. 2013b, Hunter et al. 2015, Schmelzle and Kinziger 2016). Occupancy approaches take into account detection uncertainty, whether it is due to eDNA clumping or heterogeneity of capture efficiency, allowing for estimates of occupancy in the face of varying detection methods or other sources of heterogeneity (MacKenzie et al. 2006, Schmidt et al. 2013, Furlan et al. 2015).

## **Conclusion**

I have demonstrated that an eDNA assay can be sensitive enough to detect low quantities of eDNA shed by a terrestrial mammal that has limited interaction with water. This study also provides estimates of how long pig eDNA can persist in turbid water environments depending on if it is shed from a single pig or group of pigs. This method will be useful in a management application for detecting new invaders and determining efficiency of eradication efforts. Finally, we recommend using an occupancy approach to estimate something while acknowledging imperfect detection due to the performance of the method due to variability among wallows.



Table 2-1: Success in detecting feral pig eDNA from two water sources with different methods of eDNA shedding (wallow: full body interaction possible, waterer: nose/mouth contact only). Success in samples collected at each time point (total =3) producing 3/3 positive qPCRs are shown.

eDNA Accumulation Time (Minutes)	Positive Sample (3/3 positive qPCRs) <b>Wallow</b>	Positive Sample (3/3 positive qPCRs) <b>Waterer</b>
0	0	0
15	3/3	3/3
30	3/3	3/3
45	3/3	3/3
60	3/3	3/3
75	3/3	3/3
90	3/3	3/3
105	3/3	3/3
120	3/3	3/3

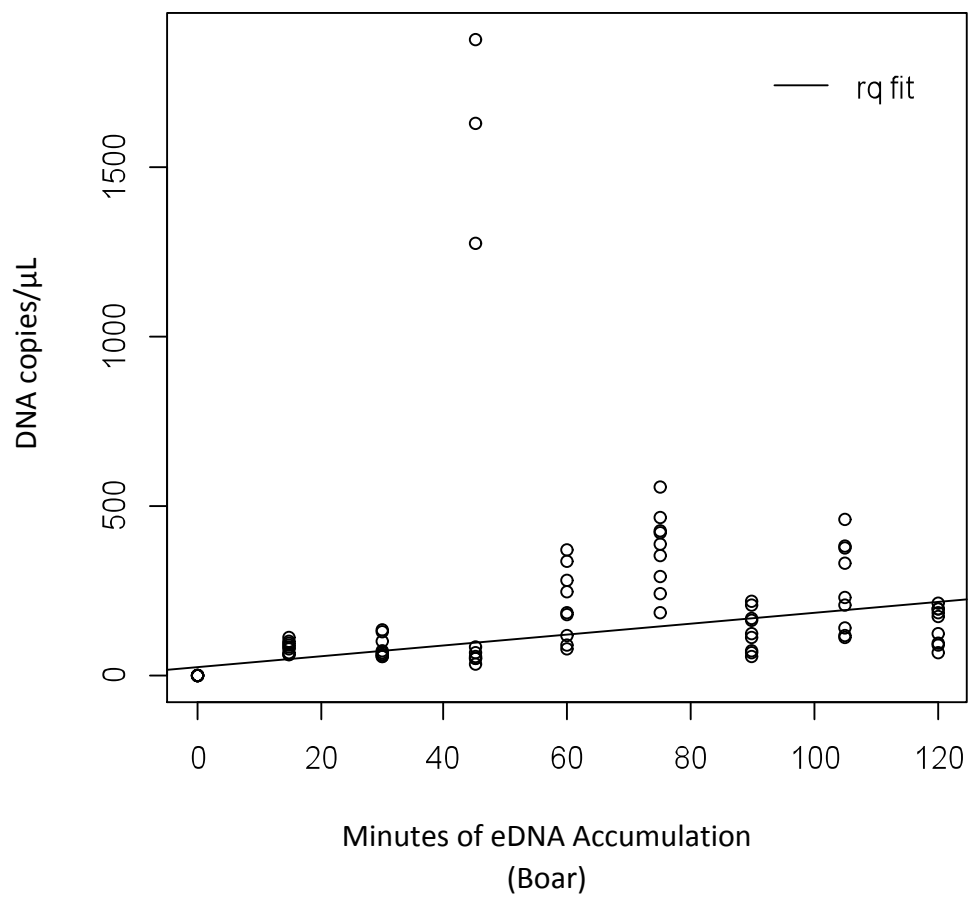


Figure 2-1: Robust regression of DNA copies/μL in response to minutes of eDNA accumulation through pig contact with water.

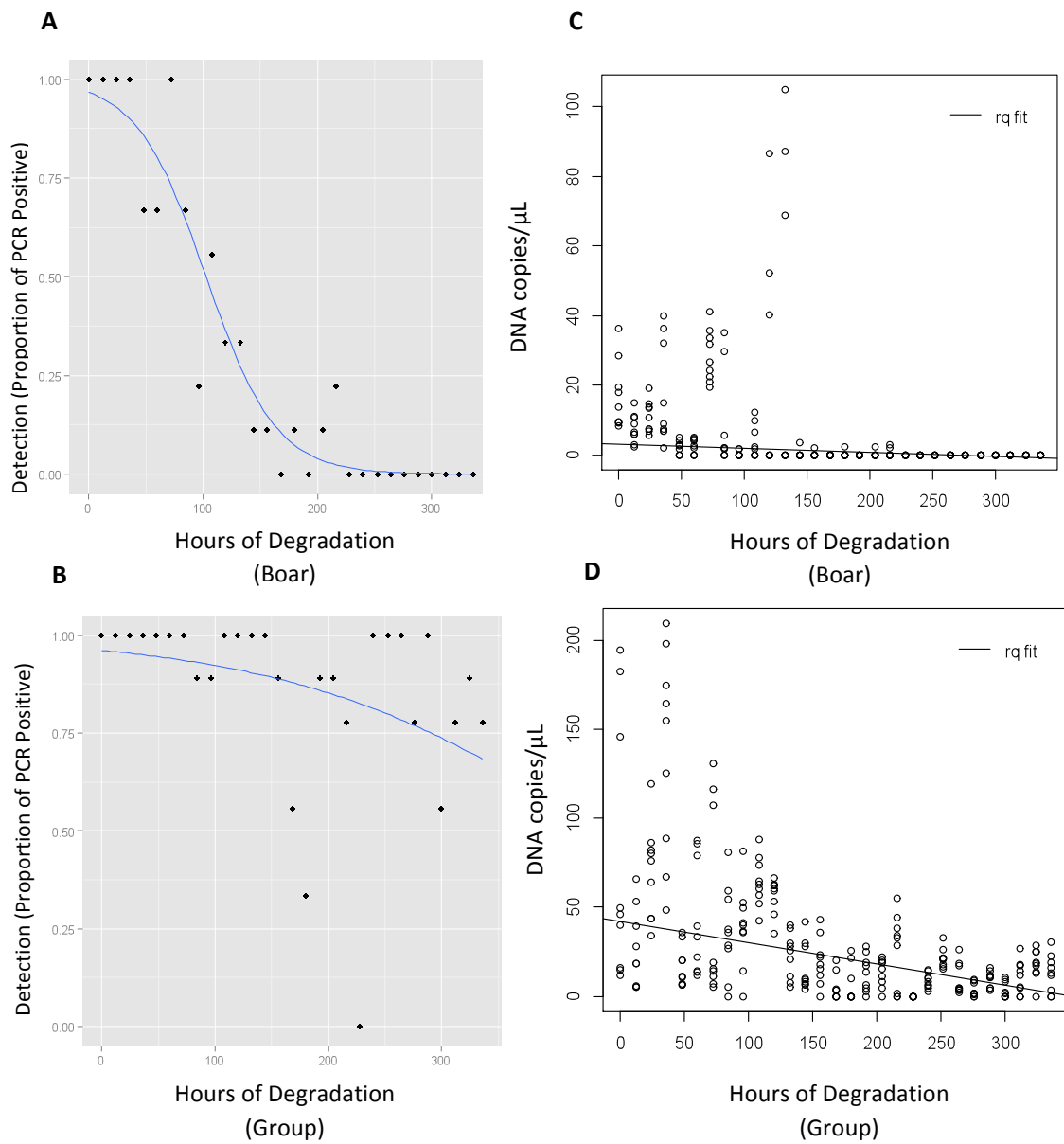


Figure 2-2: Logistic regression of detection of eDNA (proportion of PCRs positive) over the course of a two-week period (A&B) during which eDNA was allowed to degrade. Analysis of changes in DNA concentration over time using a robust regression (C & D) show the effect of degradation time on amount of eDNA in water samples.

Table 2-2: Candidate models for detection of DNA over degradation time ranked by AIC. The best supported model includes all variables and an interaction between group and hours of degradation. The AIC values, number of parameters (K) in each model, and the log likelihood are reported for each candidate model.

<b>Model</b>	<b>AIC</b>	<b>K</b>	<b>LogLikelihood</b>
glm(Detection~Hour+Group+Group*Hour)	33.02	4	-12.50807
glm(Detection~Hour+ Group)	39.044	3	-16.52215
glm(Detection~Hour)	71.344	2	-33.67223

Table 2-3: Generalized logistic model evaluating the effect of group and hour of degradation on detectability of eDNA after Boar and Group data are combined. Variable estimates derived from most supported model: `glm(Detection~Hour+Group+Group*Hour)`.

Coefficients	Estimate	P value
Intercept	3.35837	0.02406 *
Hour	-0.03277	0.00961 **
Group	-0.16191	0.93717
Hour:Group	0.02554	0.06844

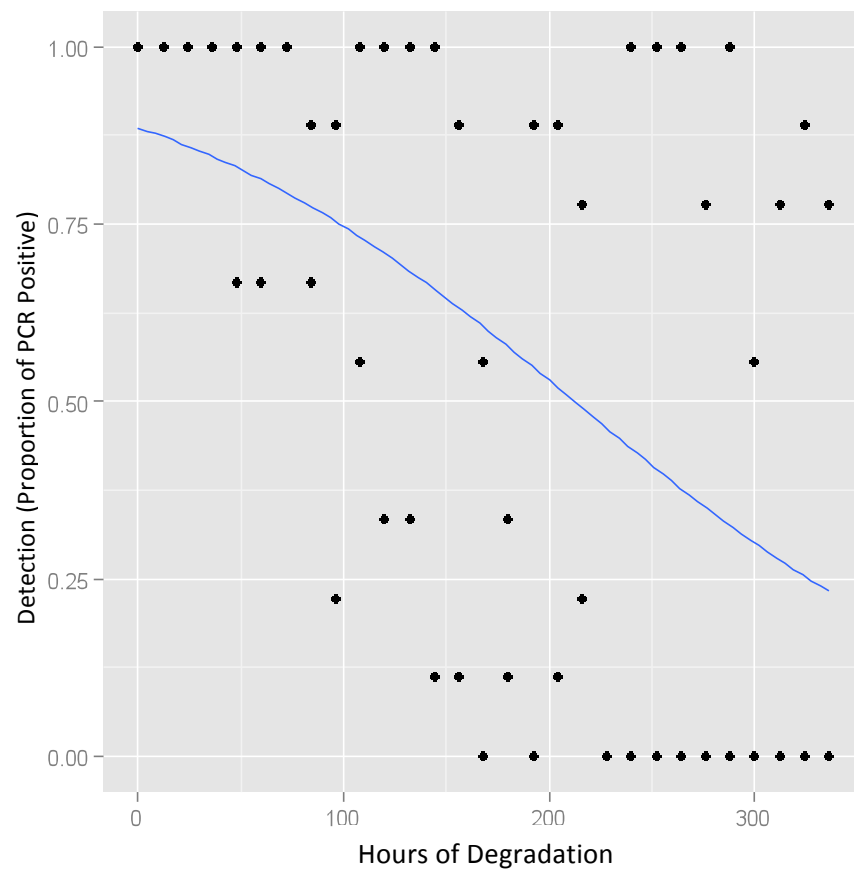


Figure 2-3: Combined logistic regression of pig group and single pig eDNA degradation data used to generate predictive model

## Chapter 3: No filters, no fridges: A method for preservation of environmental DNA

### Summary

Advancements in the detection of environmental DNA (eDNA) for detecting species of interest will likely allow for expanded use in the field. One obstacle that continues to hinder applications in the field is the requirement of a cold chain of storage for water samples containing eDNA. While eDNA has been successfully preserved using Longmire's lysis buffer applied to filters, it has yet to be tried with water samples collected for eDNA detection of an invasive species. I tested the utility of Longmire's solution as an additive to water samples for preservation of eDNA. Environmental DNA was effectively preserved in 15 mL water samples with Longmire's solution added; eDNA detection was comparable to freezing the samples at -80°C and occurred out to 56 days at the highest concentration (5 mL Longmire's solution: 15 mL sample water). Medium and low concentrations of Longmire's solution added to 15 mL of sample water generally preserved eDNA out to 56 days but not as well as did freezing or application of the highest concentration of Longmire's lysis buffer. I conclude that Longmire's lysis buffer is a viable alternative to cold chain storage that can simplify the collection of eDNA by eliminating the need for filtering and allow more time for sample collection, which could translate to an increase in the chances of detecting a rare or elusive species.

### Introduction

Analysis of environmental DNA (eDNA), or DNA of a target species captured noninvasively from samples such as soil or water, is a novel method of detecting species of

interest in the environment (Ficetola et al. 2008, Lodge et al. 2012, Taberlet et al. 2012, Sutherland et al. 2013). Collection of DNA from water has been successfully used to detect a variety of species from marine and freshwater systems (Ficetola et al. 2008, Goldberg et al. 2011, Jerde et al. 2011, Thomsen et al. 2012b). Methods to capture eDNA from water begins by filtering water samples at the collection site (Goldberg et al. 2011, Jerde et al. 2011) or collecting a water sample and concentrating the eDNA it contains using laboratory methods (chemical and physical) prior to extraction (Ficetola et al. 2008, Piaggio et al. 2014). The preservation of eDNA in water samples requires cold storage (Mahon et al. 2010, Takahara et al. 2012, Pilliod et al. 2014) or the addition of a preservative for transportation of filters from the field to the lab. Requiring field personnel to filter and/or manage a continuous cold chain can be expensive, challenging, and time-consuming. Further, freezing and thawing samples prior to analysis reduces DNA viability and thus detection (Takahara et al. 2015). Longmire's lysis buffer (Renshaw et al. 2015) and ethanol (Goldberg et al. 2013, Pilliod et al. 2013a) have both been shown to be effective for storage of water filters containing eDNA in the absence of a cold chain.

Longmire's solution is a lysis buffer that neutralizes cellular components of a sample allowing the DNA to become soluble (Longmire et al. 1997) and to accumulate in the buffer solution over time (Kilpatrick 2002). Longmire's solution (100mM Tris, 100mM EDTA, 10mM NaCl, 0.5% SDS, 0.2% sodium azide) was originally intended for preservation of tissues for museum collections because such samples are often collected under field conditions without the benefit of refrigeration (Longmire et al. 1997). Blood samples can be stored in this solution for several years prior to DNA isolation (Longmire et al. 1991). Longmire's solution effectively



preserved DNA in brain and tail tissue samples from rats for up to ten months (Camacho-Sanchez et al. 2013) and liver tissue samples from mice for up to six months (Kilpatrick 2002). More recently, Longmire's solution effectively preserved eDNA captured on water filters (Renshaw et al. 2015) for up to 150 days (Wegleitner et al. 2015) without need for a cold chain. Many forms of lysis buffer have proven effective for noncryogenic preservation of blood and tissue samples in the field (Cockburn and Seawright 1988, Seutin et al. 1991, Dawson et al. 1998). Nonetheless, the use of Longmire's solution as a preservative of eDNA samples from unfiltered water exposed to natural conditions is untested. Here, I present tests of Longmire's solution for preservation of eDNA from unfiltered water samples. Eliminating the need for time-consuming eDNA capture in the field (filtering) and costly cold chain storage for collecting and transporting water could reduce the time and effort required to collect eDNA samples.

Feral pigs are a destructive, invasive species in North America that have widespread negative impacts on ecosystems (United States Department of Agriculture, Chavarria et al. 2007, West et al. 2009). Management of this species can be challenging when abundance is low, either at the tail end of an eradication effort or in the beginning stages of an invasion process. Successful management of feral pigs requires detection and elimination of individuals before they increase in numbers and spread into new areas (Saunders and Bryant 1988, Choquenot et al. 1996, West et al. 2009). Feral pigs spend time drinking or wallowing in water (Jay et al. 2007, West et al. 2009) to thermoregulate and to provide relief from insects and parasites (Graves 1984, Heinken et al. 2006, Campbell and Long 2009). I developed an assay that effectively captures eDNA shed by pigs in turbid waters (Chapter 1). Application of this assay for surveillance of feral pigs requires sampling from turbid waters (i.e., wallows) under

often unfavorable field conditions. Collection of these types of samples needs to be intensive to reach sufficiently large sample sizes needed for detection of feral pigs when abundance is low (Hayes et al. 2005, Darling and Mahon 2011, Thomsen et al. 2012b). Any efficiencies realized in the field, such as eliminating the need to filter each sample or cold chain storage, will reduce the burden on sampling efforts and increase the efficiency of detection surveys. My goal was to test the effectiveness of Longmire's solution for preserving unfiltered water samples containing eDNA. Further, I wanted to assess the appropriate volume of Longmire's solution to add to a 15 mL water sample known to contain feral pig eDNA and to determine the optimal concentration for robust preservation.

## **Methods**

Laboratory work was completed at the USDA-APHIS National Wildlife Research Center (NWRC) in Fort Collins, Colorado, USA. DNA extractions were performed in a lab dedicated to non-invasive and eDNA samples. All PCR and post-PCR procedures were completed in separate rooms. Equipment, benchtops, pipettors, and fume hoods were cleaned with a 10% bleach solution before and after all procedures.

Water was collected from a 94.6 L tub that served as the water source for a single feral swine sow in captivity at the USDA-APHIS/Colorado State University Wildlife Research Facility. Water was collected on June 29, 2015 by submerging a single sterilized 2 L Nalgene bottle and filling it to 1 L.

The 1 L water sample was first mixed using a magnetic stir bar on a stir plate and then subsampled into sixty 50 mL centrifuge tubes in volumes of 15 mL each. Subsamples were

numbered in order of collection and then randomly assigned to one of five treatment groups using a random number generator. Treatment groups included a positive control where twelve samples were stored at -80°C (this is an effective method for preserving DNA (Dessauer et al. 1996)), a high concentration of Longmire's solution to sample water (1:3; 5 mL Longmire's: 15 mL sample water), a medium concentration (1:6; 2.5 mL Longmire's: 15 mL sample water), a low concentration (1:15, 1 mL Longmire's: 15 mL sample water), and a no treatment control of 15 mL sample water without lysis buffer or cold storage. Comparison of these groups of 12 samples each allowed me to test whether varying amounts of Longmire's solution affected the preservation of eDNA across the duration of the trial period (56 days). The no treatment control and Longmire's solution groups were stored outside in a covered, but not enclosed, area that was exposed to the sun from the West. The tubes were placed upright in a shallow Styrofoam rack that did not completely block incident UV.

One half ( $n = 6$ ) of each treatment group was extracted after 28 days and the second half was extracted after 56 days during which eDNA degradation was allowed to occur. During the first 28 days, the treatment groups (excluding the positive control group) were exposed to air temperatures ranging from 12.6°C to 33.7°C. During the second 28 days, air temperatures ranged from 7.3°C to 34.2°C as reported at the Fort Collins Weather Station.

DNA was concentrated from the samples via centrifugation (9000g, 15 mins, room temperature (Caldwell et al. 2007)). The supernatant was decanted and the DNA pellet was extracted using the DNeasy *mericon* Food Kit using the 200 mg manufacturer's protocol (Qiagen, Hilden, Germany). Finally, the elution was cleaned with Zymo IRT columns (additional

details in Chapter 1). I included a negative control in each set of extractions to monitor for contamination.

Primers and probe for quantitative PCR (qPCR) were used from another study that established best practices for feral swine eDNA capture from turbid water (Chapter 1). The qPCR recipe and thermocycling program used are also reported in Chapter 1. Each PCR set included a “no template” negative control including only PCR reagents to monitor for contamination. Each extracted water sample was run in triplicate via qPCR. A water sample was considered “positive” if all three qPCR replicates were positive. My cycle threshold (Ct) cutoff value was 40 cycles for a positive detection.

I used a Fisher’s exact test to compare the number of samples in which feral pig eDNA was detected (classified as “positive”) between those samples with any lysis buffer treatment and no treatment. To determine the optimal treatment for preservation, I also compared the performance of each concentration of lysis buffer across both time points (28 days and 56 days) using Fisher’s exact tests. Statistical analyses were conducted in R x 64 3.1.2.

## **Results**

All positive control (frozen) samples had perfect detection across all qPCR replicates for the duration of the experiment (56 days). After 28 days and 56 days, the qPCR results demonstrated that all volumes of the Longmire’s solution preserved eDNA in my samples significantly better than the “no treatment” group over both time points (Fisher’s Exact test, 28 days:  $p < 0.00001$ , 56 days:  $p < 0.01$ , Figure 3-1). The “no treatment” control group produced only 2 of 3 qPCR positives for a single sample out of the total 6 samples taken at 28 days.

Because the threshold for a positive detection was complete detection (3 of 3 qPCR results with Ct below 40 cycles), no samples from this treatment group were considered positive. The number of positives did not differ significantly among lysis treatment groups after 28 days (Fisher's Exact test,  $p = 1.0$ ); but after 56 days, results across treatments varied (Figure 3-1), although these differences were not statistically significant (Fisher's Exact test,  $p = 0.08$ ). The highest ratio of Longmire's solution to water sample had 100% detection (all positive qPCRs) across both time points. However, detection of eDNA in the medium and low ratios of Longmire's lysis buffer declined with fewer qPCR positives after 56 days, suggesting that degradation of DNA had occurred (Fisher's Exact test; Medium treatment:  $p = 1.0$ , Low treatment:  $p = 0.06$ ).

## **Discussion**

The results demonstrate that Longmire's lysis buffer can serve as a viable method for preserving eDNA in unfiltered water samples. After 28 days, all levels of Longmire's solution preserved eDNA as reflected by positive detections at this time point. However, only the highest level (1 part Longmire's:3 parts water) preserved all samples out to 56 days, thus I recommend using this concentration of Longmire's solution for effective preservation of eDNA at water volumes up to 15mL. Detection declined for the medium and low treatment groups between 28 and 56 days of exposure (Figure 3-1). In addition, threshold cycles in the qPCR assay were lower for the higher Longmire's concentration treatment than for the medium and low concentration treatments, suggesting that more intact eDNA was present and was amplified more readily in the highest treatment group.

## Conclusion

As a developing field, advancements in eDNA collection and sample processing are important. Recent reviews and studies have provided optimized methods of eDNA capture from various systems (Deiner et al. 2015, Goldberg et al. 2015). Longmire's lysis buffer effectively preserves eDNA on filters (Renshaw et al. 2015) without a cold chain and now, based on this study, I know that it is effective in preserving eDNA in unfiltered water samples. Eliminating cold storage of eDNA samples allows for a more efficient method of sample collection that can be used for species detection in field settings associated with monitoring or management activities. For many studies this approach will simplify the collection of eDNA and allow more time for sample collection which could mean increasing the chances of detection of a rare or elusive species (Renshaw et al. 2015)

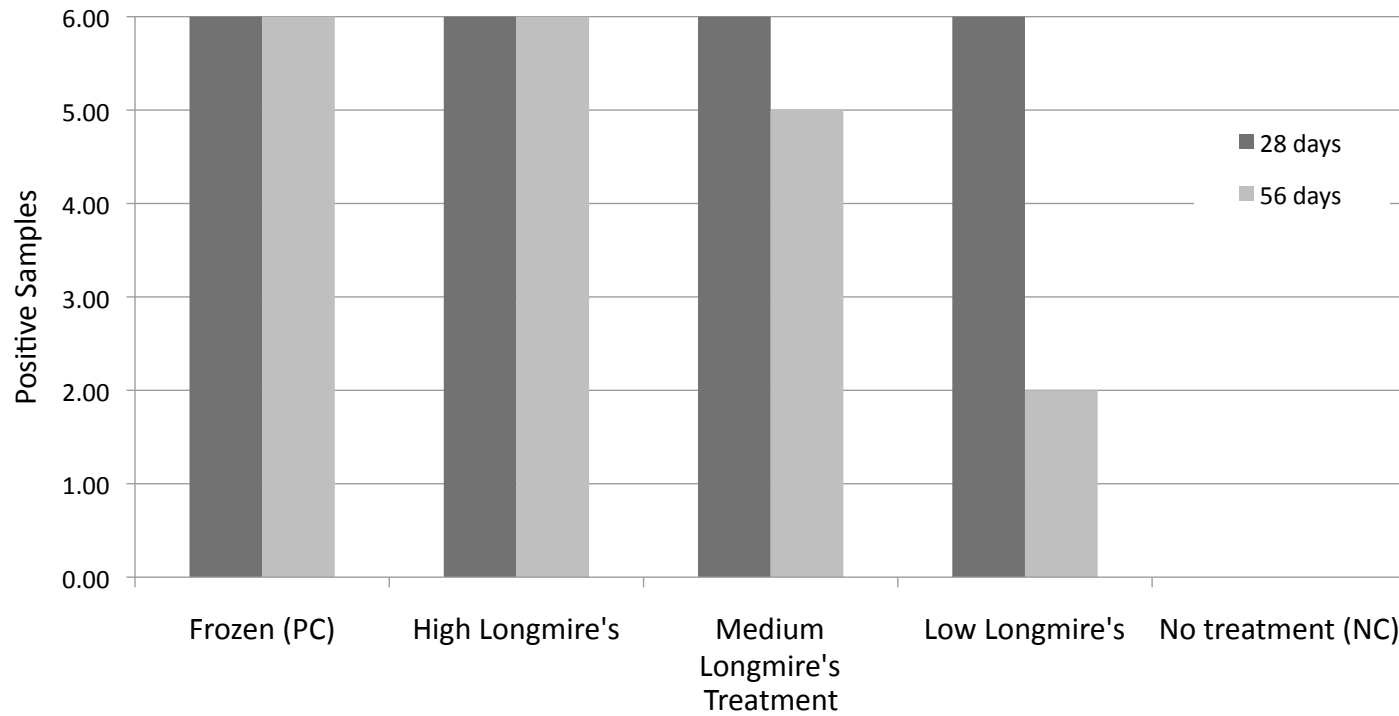


Figure 3-1: Performance of an eDNA detection assay on water samples stored with Longmire's lysis buffer compared to frozen positive control and no treatment negative controls. Number of positive samples (where 3 of 3 qPCR replicates per treatment were required for a sample to be considered "positive") per treatment at 28 days (black) and 56 days (gray) are shown.

## References

- Adkins, R. N., and L. A. Harveson. 2007. Demographic and spatial characteristics of feral hogs in the Chihuahuan Desert, Texas. *Human-Wildlife Conflicts* **1**:152-160.
- Albers, C. N., A. Jensen, J. Bælum, and C. S. Jacobsen. 2013. Inhibition of DNA Polymerases Used in Q-PCR by Structurally Different Soil-Derived Humic Substances. *Geomicrobiology Journal* **30**:675-681.
- Amberg, J. J., S. G. McCalla, L. Miller, P. Sorensen, and M. P. Gaikowski. 2013. Detection of environmental DNA of Bigheaded Carps in samples collected from selected locations in the St. Croix River and in the Mississippi River.
- Barnes, M. A., C. R. Turner, C. L. Jerde, M. A. Renshaw, W. L. Chadderton, and D. M. Lodge. 2014. Environmental Conditions Influence eDNA Persistence in Aquatic Systems. *Environ Sci Technol* **48**:1819-1827.
- Barrett, R. H., and D. S. Pine. 1980. History and status of wild pigs, *Sus scrofa*, in San Benita County, California. *California Fish and Game* **67**:105-117.
- Beja-Pereira, A., R. Oliveira, P. C. Alves, M. K. Schwartz, and G. Luikart. 2009. Advancing ecological understandings through technological transformations in noninvasive genetics. *Molecular Ecology Resources* **9**:1279-1301.
- Benson, D. A., M. Cavanaugh, K. Clark, I. Karsch-Mizrachi, D. J. Lipman, J. Ostell, and E. W. Sayers. 2013. GenBank. *Nucleic Acids Research* **41**:D36-D42.
- Bieber, C., and T. Ruf. 2005. Population dynamics in wild boar *Sus scrofa*: ecology, elasticity of growth rate and implications for the management of pulsed resource consumers. *Journal of Applied Ecology* **42**:1203-1213.
- Blackburn, T. M., P. Cassey, and J. L. Lockwood. 2009. The role of species traits in the establishment success of exotic birds. *Global Change Biology* **15**:2852-2860.
- Bohmann, K., A. Evans, M. T. P. Gilbert, G. R. Carvalho, S. Creer, M. Knapp, D. W. Yu, and M. de Bruyn. Environmental DNA for wildlife biology and biodiversity monitoring. *Trends in Ecology & Evolution* **29**:358-367.
- Caldwell, J. M., M. E. Raley, and J. F. Levine. 2007. Mitochondrial multiplex real-time PCR as a source tracking method in fecal-contaminated effluents. *Environ Sci Technol* **41**:3277-3283.
- Camacho-Sanchez, M., P. Burraco, I. Gomez-Mestre, and J. A. Leonard. 2013. Preservation of RNA and DNA from mammal samples under field conditions. *Molecular Ecology Resources* **13**:663-673.
- Campbell, T. A., and D. B. Long. 2009. Feral swine damage and damage management in forested ecosystems. *Forest Ecology and Management* **257**:2319-2326.
- Chavarria, P. M., R. R. Lopez, G. Bowser, and N. J. Silvy. 2007. A landscape-level survey of feral hog impacts to natural resources of the Big Thicket National Preserve.
- Choquenot, D., J. Mclroy, and T. Korn. 1996. Managing vertebrate pests: Feral pigs. Bureau of Resource Sciences, Canberra Australia.



- Cockburn, A. F., and J. A. Seawright. 1988. Techniques for mitochondrial and ribosomal DNA analysis of anopheline mosquitoes. *J Am Mosq Control Assoc* **4**:261-265.
- Coyne, K. J., D. A. Hutchins, C. E. Hare, and S. C. Cary. 2001. Assessing temporal and spatial variability in *Pfiesteria piscicida* distributions using molecular probing techniques. *Aquatic Microbial Ecology* **24**:275-285.
- Darling, J. A., and A. R. Mahon. 2011. From molecules to management: adopting DNA-based methods for monitoring biological invasions in aquatic environments. *Environ Res* **111**:978-988.
- Dawson, M. N., K. A. Raskoff, and D. K. Jacobs. 1998. Field preservation of marine invertebrate tissue for DNA analyses. *Mol Mar Biol Biotechnol* **7**:145-152.
- Debroas, D., J. F. Humbert, F. Enault, G. Bronner, M. Faubladier, and E. Cornillot. 2009. Metagenomic approach studying the taxonomic and functional diversity of the bacterial community in a mesotrophic lake (Lac du Bourget--France). *Environ Microbiol* **11**:2412-2424.
- Deiner, K., J.-C. Walser, E. Mächler, and F. Altermatt. 2015. Choice of capture and extraction methods affect detection of freshwater biodiversity from environmental DNA. *Biological Conservation* **183**:53-63.
- Dejean, T., A. Valentini, A. Duparc, S. Pellier-Cuit, F. Pompanon, P. Taberlet, and C. Miaud. 2011. Persistence of Environmental DNA in Freshwater Ecosystems. *PLoS ONE* **6**:e23398.
- Dessauer, H., C. Cole, and M. Hafner. 1996. *Molecular Systematics*. Second edition. Sinauer Associates, Sunderland, MA.
- Duncan, R. P., T. M. Blackburn, and C. J. Veltman. 1999. Determinants of geographical range sizes: a test using New Zealand birds. *Journal of Animal Ecology* **68**:963-975.
- Farrington, H. L., C. E. Edwards, X. Guan, M. R. Carr, K. Baerwaldt, and R. F. Lance. 2015. Mitochondrial genome sequencing and development of genetic markers for the detection of DNA of invasive bighead and silver carp (*Hypophthalmichthys nobilis* and *H. molitrix*) in environmental water samples from the United States. *PLoS ONE* **10**:e0117803.
- Ficetola, G. F., C. Miaud, F. Pompanon, and P. Taberlet. 2008. Species detection using environmental DNA from water samples. *Biol Lett* **4**:423-425.
- Fogarty, E. 2007. National distribution and stakeholder attitudes toward feral pigs. Mississippi State University, Starkville, Mississippi, USA.
- Folloni, S., D.-M. Kagkli, B. Rajcevic, N. C. C. GuimarÃEs, B. Van Droogenbroeck, F. H. Valicente, G. U. Y. Van den Eede, and M. Van den Bulcke. 2012. Detection of airborne genetically modified maize pollen by real-time PCR. *Molecular Ecology Resources* **12**:810-821.
- Foote, A. D., P. F. Thomsen, S. Sveegaard, M. Wahlberg, J. Kielgast, L. A. Kyhn, A. B. Salling, A. Galatius, L. Orlando, and M. T. P. Gilbert. 2012. Investigating the Potential Use of Environmental DNA (eDNA) for Genetic Monitoring of Marine Mammals. *PLoS ONE* **7**:e41781.
- Furlan, E. M., D. Gleeson, C. M. Hardy, and R. P. Duncan. 2015. A framework for estimating the sensitivity of eDNA surveys. *Molecular Ecology Resources*:n/a-n/a.
- Garber, R. C., and O. C. Yoder. 1983. Isolation of DNA from filamentous fungi and separation into nuclear, mitochondrial, ribosomal, and plasmid components. *Analytical Biochemistry* **135**:416-422.

- Gaston, W. D. 2008. Feral pig (*Sus scrofa*) survival, home range, and habitat use at Lowndes County Wildlife Management Area, Alabama. Auburn University, Auburn, Alabama, USA.
- Goldberg, C. S., D. S. Pilliod, R. S. Arkle, and L. P. Waits. 2011. Molecular Detection of Vertebrates in Stream Water: A Demonstration Using Rocky Mountain Tailed Frogs and Idaho Giant Salamanders. *PLoS ONE* **6**:e22746.
- Goldberg, C. S., A. Sepulveda, A. Ray, J. Baumgardt, and L. P. Waits. 2013. Environmental DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*). *Freshwater Science* **32**:792-800.
- Goldberg, C. S., K. M. Strickler, and D. S. Pilliod. 2015. Moving environmental DNA methods from concept to practice for monitoring aquatic macroorganisms. *Biological Conservation* **183**:1-3.
- Graves, H. 1984. Behavior and Ecology of Wild and Feral Swine (*Sus Scrofa*). *Journal of Animal Science*:482.
- Hall, J. S., R. B. Minnis, T. A. Campbell, S. Barras, R. W. DeYoung, K. Pabilonia, M. L. Avery, H. Sullivan, L. Clark, and R. G. McLean. 2008. Influenza exposure in United States feral swine populations. *Journal of Wildlife Diseases* **44**:362-368.
- Hampton, J., P. B. S. Spencer, A. D. Elliot, and R. C. A. Thompson. 2006. Prevalence of Zoonotic Pathogens from Feral Pigs in Major Public Drinking Water Catchments in Western Australia. *EcoHealth* **3**:103-108.
- Hayes, K. R., R. Cannon, K. Neil, and G. Inglis. 2005. Sensitivity and cost considerations for the detection and eradication of marine pests in ports. *Mar Pollut Bull* **50**:823-834.
- Heinken, T., M. Schmidt, G. v. Oheimb, W.-U. Kriebitzsch, and H. Ellenberg. 2006. Soil seed banks near rubbing trees indicate dispersal of plant species into forests by wild boar.
- Hess, S. C., J. J. Jeffrey, L. W. Pratt, and D. L. Ball. 2010. Effects of Ungulate Management on Vegetation at Hakalau Forest National Wildlife Refuge, Hawai'i Island. *Pacific Conservation Biology* **16**:144-150.
- Hone, J. 1983. A Short-Term Evaluation of Feral Pig Eradication at Willandra in Western New South Wales. *Wildlife Research* **10**:269-275.
- Hone, J. 2002. Feral pigs in Namadgi National Park, Australia: dynamics, impacts and management. *Biological Conservation* **105**:231-242.
- Hunter, M. E., S. J. Oyler-McCance, R. M. Dorazio, J. A. Fike, B. J. Smith, C. T. Hunter, R. N. Reed, and K. M. Hart. 2015. Environmental DNA (eDNA) Sampling Improves Occurrence and Detection Estimates of Invasive Burmese Pythons. *PLoS ONE* **10**:e0121655.
- Jay, M. T., M. Cooley, D. Carychao, G. W. Wiscomb, R. A. Sweitzer, L. Crawford-Miksza, J. A. Farrar, D. K. Lau, J. O'Connell, A. Millington, R. V. Asmundson, E. R. Atwill, and R. E. Mandrell. 2007. *Escherichia coli* O157:H7 in feral swine near spinach fields and cattle, central California coast. *Emerg Infect Dis* **13**:1908-1911.
- Jerde, C. L., A. R. Mahon, W. L. Chadderton, and D. M. Lodge. 2011. "Sight-unseen" detection of rare aquatic species using environmental DNA. *Conservation Letters* **4**:150-157.
- Jeschke, J. M., and D. L. Strayer. 2005. Invasion success of vertebrates in Europe and North America. *Proceedings of the National Academy of Sciences of the United States of America* **102**:7198-7202.
- Jeschke, J. M., and D. L. Strayer. 2006. Determinants of vertebrate invasion success in Europe and North America. *Global Change Biology* **12**:1608-1619.

- Jones, C. G., J. H. Lawton, and M. Shachak. 1997. Positive and negative effects of organisms as physical ecosystem engineers. *Ecology* **78**:1946-1957.
- Kelly, R. P., J. A. Port, K. M. Yamahara, and L. B. Crowder. 2014. Using Environmental DNA to Census Marine Fishes in a Large Mesocosm. *PLoS ONE* **9**:e86175.
- Kida, H., T. Ito, J. Yasuda, Y. Shimizu, C. Itakura, K. F. Shortridge, Y. Kawaoka, and R. G. Webster. 1994. Potential for transmission of avian influenza viruses to pigs. *Journal of General Virology* **75**:2183-2188.
- Kilpatrick, C. W. 2002. Noncryogenic preservation of mammalian tissues for DNA extraction: an assessment of storage methods. *Biochem Genet* **40**:53-62.
- Klymus, K. E., C. A. Richter, D. C. Chapman, and C. Paukert. 2015. Quantification of eDNA shedding rates from invasive bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix*. *Biological Conservation* **183**:77-84.
- Lance, R. 2016. Standard curve and copy number.
- Larson, G., K. Dobney, U. Albarella, M. Fang, E. Matisoo-Smith, J. Robins, S. Lowden, H. Finlayson, T. Brand, E. Willerslev, P. Rowley-Conwy, L. Andersson, and A. Cooper. 2005. Worldwide Phylogeography of Wild Boar Reveals Multiple Centers of Pig Domestication. *Science* **307**:1618-1621.
- Lodge, D. M., C. R. Turner, C. L. Jerde, M. A. Barnes, L. Chadderton, S. P. Egan, J. L. Feder, A. R. Mahon, and M. E. Pfrender. 2012. Conservation in a cup of water: estimating biodiversity and population abundance from environmental DNA. *Mol Ecol* **21**:2555-2558.
- Longmire, J. L., R. E. Ambrose, N. C. Brown, T. J. Cade, T. L. Maechtle, W. S. Seegar, F. P. Ward, and C. M. White. 1991. Use of Sex-Linked Minisatellite Fragments to Investigate Genetic Differentiation and Migration of North American Populations of the Peregrine Falcon (*Falco peregrinus*). Pages 217-229 in T. Burke, G. Dolf, A. Jeffreys, and R. Wolff, editors. *DNA Fingerprinting: Approaches and Applications*. Birkhäuser Basel.
- Longmire, J. L., M. Maltbie, R. J. Baker, and T. T. U. Museum. 1997. Use of "Lysis Buffer" in DNA Isolation and Its Implication for Museum Collections. Museum of Texas Tech University.
- Lynes, B. C., and S. D. Campbell. 2000. Germination and Viability of Mesquite (*Prosopis pallida*) Seed Following Ingestion and Excretion by Feral Pigs (*Sus scrofa*). *Tropical Grasslands* **34**:125-128.
- Mack, R. N. 1995. Understanding the processes of weed invasions: the influence of environmental stochasticity. Pages 65–74 in C. Stirton, editor. *Weeds in a changing world*, Brighton, UK.
- Mack, R. N., D. Simberloff, W. M. Lonsdale, H. Evans, M. Clout, and F. A. Bazzaz. 2000. Biotic Invasions: Causes, Epidemiology, Global Consequences, and Control. *Ecological Applications* **10**:689-710.
- MacKenzie, D. I., J. D. Nichols, J. A. Royle, K. H. Pollock, L. L. Bailey, and J. E. Hines. 2006. *Occupancy Estimation and Modeling : Inferring Patterns and Dynamics of Species Occurrence*. Elsevier/Academic Press, Burlington, MA.
- Mahon, A., A. Rohly, M. Budny, E. Elgin, C. Jerde, W. Chadderton, and D. Lodge. 2010. *Environmental DNA Monitoring and Surveillance: Standard Operation Procedures*. , Vicksburg, Mississippi.

- Martellini, A., P. Payment, and R. Villemur. 2005. Use of eukaryotic mitochondrial DNA to differentiate human, bovine, porcine and ovine sources in fecally contaminated surface water. *Water Research* **39**:541-548.
- Matheson, C. D., C. Gurney, N. Esau, and R. Lehto. 2010. Assessing PCR Inhibition from Humic Substances. *The Open Enzyme Inhibition Journal* **3**:38-45.
- Mayer, J., and L. Brisbin. 1991. *Wild pigs of the United States: their history, morphology and current status*. University of Georgia Press, Athens, Georgia, USA.
- McCann, B. E., M. J. Malek, R. A. Newman, B. S. Schmit, S. R. Swafford, R. A. Sweitzer, and R. B. Simmons. 2014. Mitochondrial diversity supports multiple origins for invasive pigs. *The Journal of Wildlife Management* **78**:202-213.
- McKee, A. M., S. F. Spear, and T. W. Pierson. 2015. The effect of dilution and the use of a post-extraction nucleic acid purification column on the accuracy, precision, and inhibition of environmental DNA samples. *Biological Conservation* **183**:70-76.
- Mountfort, D., and B. Hayden. 2006. Techniques for detection and enumeration of marine biosecurity risk species and compliance testing.
- Nathan, L. M., M. Simmons, B. J. Wegleitner, C. L. Jerde, and A. R. Mahon. 2014. Quantifying Environmental DNA Signals for Aquatic Invasive Species Across Multiple Detection Platforms. *Environ Sci Technol* **48**:12800-12806.
- Nielsen, K., L. Calamai, and G. Pietramellara. 2006. Stabilization of Extracellular DNA and Proteins by Transient Binding to Various Soil Components. Pages 141-157 in P. Nannipieri and K. Smalla, editors. *Nucleic Acids and Proteins in Soil*. Springer Berlin Heidelberg.
- Nielsen, K. M., P. J. Johnsen, D. Bensasson, and D. Daffonchio. 2007. Release and persistence of extracellular DNA in the environment. *Environ Biosafety Res* **6**:37-53.
- Nuwer, R. 2014. The last places on Earth with no invasive species. British Broadcasting Corporation.
- Oh, S., A. Caro-Quintero, D. Tsementzi, N. DeLeon-Rodriguez, C. Luo, R. Poretsky, and K. T. Konstantinidis. 2011. Metagenomic insights into the evolution, function, and complexity of the planktonic microbial community of Lake Lanier, a temperate freshwater ecosystem. *Appl Environ Microbiol* **77**:6000-6011.
- Pelt-Verkuil, E. v., A. v. Belkum, and J. P. Hays. 2008. *Principles and Technical Aspects of PCR Amplification*. Springer Netherlands.
- Perez-Mendez, A., J. C. Chandler, B. Bisha, and L. D. Goodridge. 2014. Concentration of enteric viruses from tap water using an anion exchange resin-based method. *J Virol Methods* **206**:95-98.
- Piaggio, A. J., R. M. Engeman, M. W. Hopken, J. S. Humphrey, K. L. Keacher, W. E. Bruce, and M. L. Avery. 2014. Detecting an elusive invasive species: a diagnostic PCR to detect Burmese python in Florida waters and an assessment of persistence of environmental DNA. *Mol Ecol Resour* **14**:374-380.
- Pietramellara, G., J. Ascher, F. Borgogni, M. T. Ceccherini, G. Guerri, and P. Nannipieri. 2008. Extracellular DNA in soil and sediment: fate and ecological relevance. *Biology and Fertility of Soils* **45**:219-235.
- Pilliod, D., C. Goldberg, M. Laramie, and L. Waits. 2013a. Application of Environmental DNA for Invasive Monitoring of Aquatic Species. Page 4 in U. S. G. Survey, editor. website.

- Pilliod, D. S., C. S. Goldberg, R. S. Arkle, and L. P. Waits. 2013b. Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. *Canadian Journal of Fisheries and Aquatic Sciences* **70**:1123-1130.
- Pilliod, D. S., C. S. Goldberg, R. S. Arkle, and L. P. Waits. 2014. Factors influencing detection of eDNA from a stream-dwelling amphibian. *Molecular Ecology Resources* **14**:109-116.
- Pimentel, D., R. Zuniga, and D. Morrison. 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics* **52**:273-288.
- Raut, A. D., B. P. Kapadnis, R. Shashidhar, J. R. Bandekar, P. Vaishampayan, and Y. S. Shouche. 2007. Nonspecific PCR Amplification of the 16S rRNA Gene Segment in Different Bacteria by Use of Primers Specific for *Campylobacter*, *Arcobacter*, and *Helicobacter* spp. *Journal of Clinical Microbiology* **45**:1376-1377.
- Rees, H. C., B. C. Maddison, D. J. Middleditch, J. R. M. Patmore, and K. C. Gough. 2014. REVIEW: The detection of aquatic animal species using environmental DNA – a review of eDNA as a survey tool in ecology. *Journal of Applied Ecology* **51**:1450-1459.
- Renshaw, M. A., B. P. Olds, C. L. Jerde, M. M. McVeigh, and D. M. Lodge. 2015. The room temperature preservation of filtered environmental DNA samples and assimilation into a phenol–chloroform–isoamyl alcohol DNA extraction. *Molecular Ecology Resources* **15**:168-176.
- Rodgers, T., and K. Mock. 2015. Drinking water as a source of environmental DNA for the detection of terrestrial wildlife species. *Conservation Genetics Resources* **7**:693-696.
- S.C.W.D. 2014. National Feral Swine Mapping System. College of Veterinary Medicine, The University of Georgia.
- Sakai, A. K., F. W. Allendorf, J. S. Holt, D. M. Lodge, J. Molofsky, K. A. With, S. Baughman, R. J. Cabin, J. E. Cohen, N. C. Ellstrand, D. E. McCauley, P. O'Neil, I. M. Parker, J. N. Thompson, and S. G. Weller. 2001. The Population Biology of Invasive Species. *Annual Review of Ecology and Systematics* **32**:305-332.
- Saunders, G., and H. Bryant. 1988. The Evaluation of a Feral Pig Eradication Program During a Simulated Exotic Disease Outbreak. *Wildlife Research* **15**:73-81.
- Schley, L., and T. J. Roper. 2003. Diet of wild boar *Sus scrofa* in Western Europe, with particular reference to consumption of agricultural crops. *Mammal Review* **33**:43-56.
- Schmelzle, M. C., and A. P. Kinziger. 2016. Using occupancy modelling to compare environmental DNA to traditional field methods for regional-scale monitoring of an endangered aquatic species. *Molecular Ecology Resources*:n/a-n/a.
- Schmidt, B. R., M. Kéry, S. Ursenbacher, O. J. Hyman, and J. P. Collins. 2013. Site occupancy models in the analysis of environmental DNA presence/absence surveys: a case study of an emerging amphibian pathogen. *Methods in Ecology and Evolution* **4**:646-653.
- Seutin, G., B. N. White, and P. T. Boag. 1991. Preservation of avian blood and tissue samples for DNA analyses. *Canadian Journal of Zoology* **69**:82-90.
- Singer, F. J., D. K. Otto, A. R. Tipton, and C. P. Hable. 1981. Home ranges, movements, and habitat use of European wild boar in Tennessee. *Journal of Wildlife Management* **45**:343-353.
- Sodeikat, G., and K. Pohlmeier. 2003. Escape movements of family groups of wild boar *Sus scrofa* influenced by drive hunts in Lower Saxony, Germany. Pages 43-49.

- Springer, M. D. 1977. Ecological and economic aspects of wild hogs in Texas. *in* G. W. Wood, editor. Research and management of wild hog populations: Belle Baruch Forest Science Institute of Clemson University, Georgetown, South Carolina, USA.
- Station, F. C. W. <http://climate.colostate.edu/~autowx/>.
- Sutherland, W. J., S. Bardsley, M. Clout, M. H. Depledge, L. V. Dicks, L. Fellman, E. Fleishman, D. W. Gibbons, B. Keim, F. Lickorish, C. Margerison, K. A. Monk, K. Norris, L. S. Peck, S. V. Prior, J. P. W. Scharlemann, M. D. Spalding, and A. R. Watkinson. 2013. A horizon scan of global conservation issues for 2013. *Trends in Ecology & Evolution* **28**:16-22.
- Sutton, J. T., B. C. Robertson, and I. G. Jamieson. 2011. Dye shift: a neglected source of genotyping error in molecular ecology. *Molecular Ecology Resources* **11**:514-520.
- Sweeney, J. R., J. M. Sweeney, and S. W. Sweeney. 2003. Feral hog. *in* B. C. T. G. A. Feldhamer, J. A. Chapman, editor. Wild mammals of North America. Johns Hopkins University Press, Baltimore, Maryland, USA.
- Taberlet, P., E. Coissac, M. Hajibabaei, and L. H. Rieseberg. 2012. Environmental DNA. *Mol Ecol* **21**:1789-1793.
- Taberlet, P., S. Griffin, B. Goossens, S. Questiau, V. Manceau, N. Escaravage, L. P. Waits, and J. Bouvet. 1996. Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research* **24**:3189-3194.
- Takahara, T., T. Minamoto, and H. Doi. 2015. Effects of sample processing on the detection rate of environmental DNA from the Common Carp (*Cyprinus carpio*). *Biological Conservation* **183**:64-69.
- Takahara, T., T. Minamoto, H. Yamanaka, H. Doi, and Z. Kawabata. 2012. Estimation of fish biomass using environmental DNA. *PLoS ONE* **7**:e35868.
- Taylor, R. B. 1993. Feral hogs: history and distribution in Texas. Pages 17-27 *in* J. F. C. C.W. Hanselka, editor. Feral swine: a compendium for resource managers. , Agric. Ext. Service, College Station, Texas.
- Taylor, R. B., E. C. Hellgren, T. M. Gabor, and L. M. Ilse. 1998. Reproduction of Feral Pigs in Southern Texas. *Journal of Mammalogy* **79**:1325-1331.
- Technologies, I. D. 2016. Dye shift.
- Thomsen, P. F., J. Kielgast, L. L. Iversen, P. R. Møller, M. Rasmussen, and E. Willerslev. 2012a. Detection of a Diverse Marine Fish Fauna Using Environmental DNA from Seawater Samples. *PLoS ONE* **7**:e41732.
- Thomsen, P. F., J. Kielgast, L. L. Iversen, C. Wiuf, M. Rasmussen, M. T. Gilbert, L. Orlando, and E. Willerslev. 2012b. Monitoring endangered freshwater biodiversity using environmental DNA. *Mol Ecol* **21**:2565-2573.
- Towne, C. W., and E. N. Wentworth. 1950. Pigs: From Cave to Corn Belt. University of Oklahoma Press; 1st edition (1950).
- Tréguier, A., J.-M. Paillisson, T. Dejean, A. Valentini, M. A. Schlaepfer, and J.-M. Roussel. 2014. Environmental DNA surveillance for invertebrate species: advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds. *Journal of Applied Ecology* **51**:871-879.
- Tsai, Y. L., and B. H. Olson. 1992. Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. *Applied and Environmental Microbiology* **58**:2292-2295.

- Turner, C. R., D. J. Miller, K. J. Coyne, and J. Corush. 2014. Improved Methods for Capture, Extraction, and Quantitative Assay of Environmental DNA from Asian Bigheaded Carp. *PLoS ONE* **9**:e114329.
- United States Department of Agriculture, U. Budget Summary and Annual Performance Plan FY; 2014.
- Valiere, N., and P. Taberlet. 2000. Urine collected in the field as a source of DNA for species and individual identification. *Mol Ecol* **9**:2150-2152.
- Waithman, J. D., R. A. Sweitzer, D. V. Vure, J. D. Drew, A. J. Brinkhaus, I. A. Gardner, and W. M. Boyce. 1999. Range expansion, population sizes, and management of wild pigs in California. *Journal of Wildlife Management* **63**:298-308.
- Wegleitner, B., C. Jerde, A. Tucker, W. L. Chadderton, and A. Mahon. 2015. Long duration, room temperature preservation of filtered eDNA samples. *Conservation Genetics Resources*:1-3.
- West, B., A. Cooper, and J. Armstrong. 2009. Managing wild pigs: A technical guide. . The Berryman Institute, Starkville, MS and Logan, UT.
- Wetzel, R. G. 1992. Gradient-dominated ecosystems: sources and regulatory functions of dissolved organic matter in freshwater ecosystems. *Hydrobiologia* **229**:181-198.
- White, G. C., and K. P. Burnham. 1999. Program Mark: Survival estimation from populations of marked animals. *Bird Study* 46 Supplement:120-138.
- Wilcox, T. M., K. S. McKelvey, M. K. Young, S. F. Jane, W. H. Lowe, A. R. Whiteley, and M. K. Schwartz. 2013. Robust Detection of Rare Species Using Environmental DNA: The Importance of Primer Specificity. *PLoS ONE* **8**:e59520.
- Wood, G. W., and R. H. Barrett. 1979. Status of the wild pig in the United States. *Wildlife Society Bulletin* **36**:237-246.
- Wu, N., C. Abril, A. Thomann, E. Grosclaude, M. G. Doherr, P. Boujon, and M.-P. Ryser-Degiorgis. 2012. Risk factors for contacts between wild boar and outdoor pigs in Switzerland and investigations on potential *Brucella suis* spill-over. *BMC Veterinary Research* **8**:1-12.
- Wyckoff, A. C., E. H. Scott, A. C. Tyler, G. H. David, and C. V. Kurt. 2012. Movement and Habitat Use of Feral Swine Near Domestic Swine Facilities. *Wildlife Society Bulletin (2011-)* **36**:130-138.

## Appendix A

This data is a record of observations during the eDNA accumulation study involving a single boar interacting with a wallow and a single pig interacting with a waterer.

Key:

\* = Sample collected

Triplicates of each sample in 60 ml bottles

### **Boar Wallow Sampling**

July 9, 2014

Filled tub at 7:15AM

94.6 L at a time

~852 L

71.8°F at 8:10AM

8:10 AM \*

Time zero sample collected

8:10AM Boar in pen

8:19 AM walked through tub

8:21 AM walked through tub

8:25 AM \*

8:30 AM

Standing in water/rooting under water

8:40 AM \*

No movement in tub

8:55 AM \*

8:57 AM

Drinking/walking in tub



9:03 AM

Drinking/walking through tub

9:06 AM

Drinking/wallowing in tub

9:10 AM \*

9:12 AM

Boar in tub

9:15 AM

Boar in tub

9:17 AM

Walk through tub

9:19 AM

Walk through tub

9:25 AM \*

9:29 AM

Boar in tub

9:40 AM \*

9:45 AM

Laying in tub

9:55 AM \*

10:00 AM

Wallowing

10:10 AM \*

### **Waterer Sampling**

Mississippi Field Station

8:53 AM\*

8:57 AM

Pig in pen

Pig drank for a few seconds

9:04 AM

Pig drank

9:07 AM

Pig drank

9:12 AM

Pig drank immediately before sample collection

9:13 AM \*

9:22 AM

Pig drank for a few seconds

9:27 AM \*

9:42 AM \*

9:43 AM

Pig drank for 3-4 seconds

9:57 AM\*

9:58 AM

Stuck nose in for a second

10:12 AM\*

10:27 AM\*

10:30 AM

Pig stuck nose in for a second

10:42 AM\*

10:57 AM\*