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Development and Assessment of an Environmental DNA (eDNA) Assay for the Rio Grande Siren and Review of eDNA Metabarcoding Applications

Krista M. Ruppert
The University of Texas Rio Grande Valley

DEVELOPMENT AND ASSESSMENT OF AN ENVIRONMENTAL DNA (eDNA) ASSAY FOR THE RIO GRANDE SIREN AND REVIEW OF eDNA METABARCODING APPLICATIONS.

A Thesis

by

KRISTA M. RUPPERT

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COMMITTEE MEMBERS

Dr. Richard J. Kline
Chair of Committee

Dr. Md. Saydur Rahman
Committee Member

Dr. Drew R. Davis
Committee Member

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ABSTRACT

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Environmental DNA (eDNA) assays have become a major aspect of amphibian surveys in the past decade. These methods are highly sensitive, making them well-suited for monitoring rare and cryptic species. Current efforts to study the Rio Grande Siren in south Texas have been hampered due to the cryptic nature of these aquatic salamanders. Arid conditions further add to the difficulty in studying this species, as many water bodies they inhabit are ephemeral, sometimes constraining sampling efforts to a short window after heavy rain. Additionally, sirens are known to cease activity and reside underground when ponds begin to dry or as water temperatures increase. Conventional sampling efforts require extensive trap-hours to be effective, which is not always possible within the required sampling window. This study presents the development of a novel eDNA assay technique for this elusive species and compare eDNA results with simultaneous trapping at multiple sites to assess the relative effectiveness of the procedure. This methodology gives promise for future work assessing the distribution and status of the Rio Grande Siren and has potential for use on other south Texas amphibians. An expansive literature review on the subject of eDNA metabarcoding is also presented, along with a plan for implementation of this method in south Texas for community amphibian studies.

DEDICATION

To my parents, for always encouraging me to follow my dreams, even when my dreams were to dig in the mud and look for salamanders.

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CHAPTER I

INTRODUCTION

Amphibian Decline

Amphibians worldwide are in decline with some considering this increasing mortality to be a marker of the "sixth great extinction" (Wake and Vredenburg 2008). More than one third of all species of amphibians are currently threatened with extinction due to natural and human-induced factors (Wake and Vredenburg 2008), a pattern that is not predicted to change (Stuart et al. 2004). Current extinction rates for amphibians are rising rapidly and are estimated to be more than 200 times the background extinction rate, indicating the likelihood of an anthropogenic influence beyond natural processes leading to extinction (McCallum 2007). Many amphibian species have a limited geographic distribution, further increasing their susceptibility to extinction (Stuart et al. 2004). Reasons for amphibian declines are numerous and include habitat loss, disease, climate change, pollution, and human disturbance (Cushman 2006, Hof et al. 2011, Ficetola et al. 2015). As human activity continues to encroach upon natural habitat, amphibians are expected to continue to decline.

Amphibians have long been considered to be environmental indicators due to their sensitivity to environmental changes; when a harmful event occurs, they are likely to be the first to be impacted, and anthropogenic compounds can have strong effects on amphibians (Waddle 2006). This theory of amphibians as indicators is not entirely universal; some species are more

sensitive than others (Kerby et al. 2010). Other studies have found links between amphibian species and environmental factors, such as a correlation between amphibian presence and water quality (Bodinof Jachowski et al. 2016). Determining which species are sensitive to specific environmental factors can allow the environment to be observed with amphibians as indicators; however, the difficulty in studying some of the more cryptic amphibian species raises concerns about the accuracy of traditional sampling techniques such as visual encounters, call surveys, and trapping.

Salamanders

Much of the research on amphibians has been focused on Anura (frogs and toads), leaving Caudata (salamanders and newts) largely understudied. With the discovery of pathogens such as *Batrachochytrium salamandrivorans*, a fungus known to cause declines, understanding of salamander ecology is essential for conservation. Caudata is a diverse group of approximately 700 species making up 9.25% of all amphibian species (Frost 2017). Salamander effects on ecosystems range greatly among species, as life histories and habitats are highly variable (Conant and Collins 1998, Davic and Welsh 2004, Petranka 2010). Altogether, however, salamanders contribute a significant amount of biomass to their respective ecosystems, with aquatic salamanders sometimes contributing more biomass than fish in watershed headwaters (Murphy and Hall 1981, Resetarits 1997, Wilkins and Peterson 2000, Lowe and Bolger 2002), streams (Matson 1998), rivers (Petranka 2010), and ponds (Gehlbach and Kennedy 1978).

Salamanders are important predators across their ecosystems to the point of some species being described as keystone predators (Davic and Welsh 2004); specific examples include significant predation upon terrestrial invertebrates (Petranka 2010), aquatic macroinvertebrates

(Holomuzki et al. 1994, Parker 1994, Brodman et al. 2003, Petranka 2010, Keitzer and Goforth 2013), and amphibians (Calef 1973, Morin 1995, Wilbur 1997, Kurzava and Morin 1998, Walls and Williams 2001, Brodman et al. 2003). Salamanders also function as important prey items with potential long-term ecosystem stabilizing effects due to their long, slow-growing lifespans, low energy demands, and high abundance (Hairston 1988, Davic and Welsh 2004, Petranka 2010). All of these factors indicate the importance of salamanders to ecosystem function across their variable habitats and a need for further study into salamander conservation.

Rio Grande Siren

The salamander genus *Siren* is currently known to encompass three species native only to the southeastern United States: Lesser Siren (*Siren intermedia*), Greater Siren (*S. lacertina*), and Reticulated Siren (*S. reticulata*). Sirens are fully aquatic, neotenic salamanders with trimodal respiration: branching external gills, well-developed lungs, and cutaneous respiration (Duke and Ultsch 1990). Their bodies are long and eel-like (Fig. 1), with small, reduced forelimbs and no hindlimbs (Petranka 2010). Sirens are omnivorous, consuming small vertebrates, invertebrates, and plant matter opportunistically (Hanlin 1978, Hill et al. 2015), and are subject to predation by birds, snakes, and large fish (Tipton et al. 2012). Sirens are most often found in shallow freshwater habitats with minimal flow, ranging from resacas (oxbow lakes) and ponds to drainage ditches. Few studies have thoroughly documented siren habitat; however, it is thought that they are generalists, opportunistically living wherever there is adequate food and standing water (Gehlbach and Kennedy 1978). It is possible that sirens are most successful in water bodies with low oxygen content as potential competitors may be unable to survive in these conditions, while their respiratory ability allows them to persist under these low dissolved

oxygen conditions (Guimond and Hutchison 1973, Duke and Ultsch 1990). Some studies suggest sirens prefer shallow water with an abundance of vegetation and mollusks; however, there have been no specific studies detailing preferred vegetation types (Gehlbach and Kennedy 1978, Schalk and Luhring 2010). A recent study in south Texas found no correlation between sympatric species, pH, conductivity, water temperature, percent cover, and vegetation presence with sirens, but did find that sirens cluster together in edge cover, or vegetation present at the shallow edges of the water body (LaFortune 2015).

Figure 1. A Rio Grande siren being released into a resaca at Sabal Palm Sanctuary, Cameron County, Texas. Photo by Erich Schlegel.

The distibution of the three recognized siren species overlap throughout the southeastern United States, making species differentiation based on location difficult (Graham et al. 2018). The greater siren occurs from Maryland through Alabama, while the lesser siren occurs throughout the east coast, north to Michigan, and south through east and south Texas (Petranka 2010). Morphological characteristics such as size and costal groove counts have traditionally been used for differentiation among species, but there is overlap in morphological characteristics as well. Reticulated sirens can be distinguished based on their distinct color pattern (Graham et al. 2018), but differentiating greater and lesser sirens can be more difficult. The greater siren is described as being 51–76 cm in length and having 36–40 costal grooves, while the lesser siren is described as being 18–68.6 cm in length and having 31–35 costal grooves (Conant and Collins 1991). Sirens in south Texas, however, are the subject of debate. They are most often described as reaching a maximum snout–vent length of 68.6 cm with 36–38 costal grooves, making their morphological characteristics similar to previously recognized species (Goin 1957). Sirens in south Texas have been described as a subspecies of the lesser siren (*Siren intermedia texana,* Fig. 1), a disjunct population of greater siren, or a different species altogether (Goin 1957, Flores-Villela and Brandon 1992, LaFortune 2015). Two published studies have used genetic tools to attempt to elucidate siren speciation, with LaFortune (2015) determining that sirens in south Texas appear to be a genetically distinct species from both *Siren intermedia* and *Siren lacertina,* and Graham et al. (2018) aligning sirens from south Texas most closely with sirens from east Texas, and more distantly from other *S. intermedia, S. lacertina,* and *S. reticulata*. Currently, Texas Parks and Wildlife Department (TPWD) lists sirens in south Texas as "South Texas Siren (large form), *Siren* sp. 1" and as state-threatened (TPWD 2018). Confusion regarding siren taxonomy and therefore the actual status of sirens in south Texas in conjunction

with the conservation concern surrounding sirens in south Texas makes the study of these salamanders imperative.

Detection of aquatic salamanders can be challenging. Cryptic species that evade detection may be incorrectly labeled as rare, and the exclusion of certain species due to lack of capture may produce inaccurate information regarding species richness (Evans et al. 2016a). Common methods for amphibian detection include visual encounter surveys (Biagas et al. 2012), call surveys (Guzy et al. 2014), funnel trapping (Buech and Egeland 2002), and dip netting (Denton and Richter 2013), but success with these methods can vary greatly depending on species, life stage, and environmental conditions (Farmer et al. 2009). Sirens can be difficult to detect via traditional means and detection is most often limited to trapping; however, dip netting and visual encounter surveys may be effective in shallow water with high siren densities (McDaniel 1969). Altogether, trap success is dependent on methodology and environmental variables; setting traps consistently even in an area known to be populated with sirens does not guarantee capture. This makes detection via trapping all the more difficult. New alternative methods for detection are needed to monitor cryptic species such as sirens.

Environmental DNA

Many amphibians are recognized as being cryptic and difficult to detect using traditional survey methods, making the development of new, more effective methods essential for their study and for the development of conservation plans (Ficetola et al. 2008, Rees et al. 2014). One method that shows promise for use in the detection and monitoring of sirens is environmental DNA (eDNA), an emerging science involving the extraction of genetic material from the environment to detect species (Jerde et al. 2011, Thomsen and Willerslev 2015, Evans et al.

2016a). eDNA allows for the study of aquatic species without needing to physically capture, view, or hear the species in the field. eDNA can be used for species monitoring by confirming species presence based on genetic material obtained from water that other methods (dip netting, funnel trapping, visual encounters, and call surveys) may miss (Spear et al. 2015, McKee et al. 2015a). Additionally, traditional methods are often time and labor intensive, as well as prone to variation based on surveyor experience, whereas eDNA surveys can reduce time in the field (Ficetola et al. 2008, Hoffmann et al. 2016). eDNA surveys are also less invasive than traditional surveys, making them ideal for reducing potential damage to sensitive wetland habitats as well as reducing physical contact with individuals, which may result in stress or injury (Brozio et al. 2017). eDNA has also found to be a more cost-effective method of detection than traditional sampling (Goldberg et al. 2011, Taberlet et al. 2012a). Though much of the genetic material extracted from water samples can be degraded, the information which can be gleaned from the fragments can still yield valuable finding as to species and subspecies variation and distributions (Renan et al. 2017).

eDNA collected from water bodies can be amplified through two different pathways. Species-specific primer sets may be developed to amplify DNA only from a single target species, or general primer sets may be used. For species-specific studies, amplification often occurs through quantitative PCR (qPCR), or through PCR followed by gel electrophoresis and Sanger sequencing. As the name suggests, using species-specific primers would only amplify a single target species, making it ideal for single-species studies. The use of general primers sets to amplify DNA followed by next-generation or high-throughput sequencing (a process called metabarcoding) has been applied to the monitoring of entire amphibian communities (Dejean et al. 2012, Thomsen et al. 2012, Ficetola et al. 2015, Lopes et al. 2017). In particular, Valentini et

al. (2016) developed a batrachia-specific primer set theoretically capable of amplifying eDNA from all salamanders, newts, frogs, and toads. This primer set has not yet been validated for south Texas species; however, the ability to identify amphibian species present from a single water sample is a promising prospect for studying community ecology. This method could give insight to overall amphibian communities as well as offer a way to monitor multiple species of concern simultaneously. Pairing these primers with tags such as those developed by Fiore-Donno et al. (2017) and analysis programs such as Geneious has the potential to streamline eDNA sampling for amphibians, making it more cost-effective and sustainable. These techniques have not yet been tested with amphibians in south Texas but show promise for monitoring communities of cryptic species. Assessing the current literature on eDNA metabarcoding and adapting procedures into a methodology applicable to south Texas is essential before metabarcoding can begin.

Study Overview

This thesis aimed to: develop an eDNA assay for the Rio Grande siren that is applicable to siren habitats in south Texas, assess the effectiveness of the assay as compared to trapping, determine the impacts of environmental variables on siren detection, review the literature on environmental DNA metabarcoding, and design a research plan for implementing eDNA metabarcoding for detection of south Texas amphibians.

This thesis is arranged in five chapters. Chapter 1 (see preceding) is a general introduction to amphibian decline, salamanders, sirens, and eDNA. This is followed by Chapter 2, which describes the development of a species-specific eDNA assay for the Rio Grande siren. Chapter 3 applies the assay to a comparison between eDNA and trapping for siren detection, as

well as assessing the environmental variables that correspond with siren detection. Chapter 4 reviews the literature on eDNA metabarcoding and applies the information therein to develop a research plan for amphibian metabarcoding in south Texas. The final chapter, Chapter 5, is an overall conclusion, briefly covering the key discoveries of this thesis.

CHAPTER II

ENVIRONMENTAL DNA ASSAY DEVELOPMENT: FIELD AND LABORATORY METHODOLOGIES FOR DETECTION OF THE RIO GRANDE SIREN

Introduction

Environmental DNA

The use of environmental DNA (eDNA) has been applied numerous times to survey amphibians, particularly those which are largely or completely aquatic (Goldberg et al. 2011, Pilliod et al. 2013, 2014, Rees et al. 2014, Spear et al. 2015, Brozio et al. 2017, Takahashi et al. 2018, Eiler et al. 2018, Wineland et al. 2019). The development of an eDNA assay involves several parts: primers must be designed to be specific to the species without creating dimers, PCR conditions must be optimized for amplification of small amounts of DNA, sampling procedures must be tested to eliminate contamination, preservation and extraction procedures must be assessed, and field tests must be completed to ensure the entire process is functioning adequately (Goldberg et al. 2016). In the case of amphibians, most eDNA collection methods involve filtering water through small-pore specialized scientific filters, which are then preserved and later treated to extract DNA (Goldberg et al. 2016). The exact methods vary across studies, with variation attributed to environment, species, and researcher preference. Additionally, it is important to note that eDNA is still a developing field, and as such, further standardization can be expected in the future (Goldberg et al. 2016).

Different environments hold unique challenges for eDNA studies, as each water body has its own set of characteristics that must be accounted for. Some methods of eDNA retrieval are ethanol precipitation of DNA directly from a water sample, centrifugation, or filtration of water to catch DNA (Tsuji et al. 2019). Filtration is the most common method of obtaining eDNA, with 78% of studies using this method, but there are numerous factors within this category that may contribute to success (Tsuji et al. 2019). Most notably, pore size and filter material may have an impact on eDNA collection (Tsuji et al. 2019). These factors must be assessed for each study to determine the optimal eDNA collection method. Preservation and extraction of eDNA from filters also varies throughout studies; most studies place filters in ethanol and extract DNA using a commercial kit, however the suitability of methods varies based on filter type and environment (Tsuji et al. 2019).

Following eDNA collection and extraction, samples can be amplified with polymerase chain reaction (PCR) using species-specific primers. Depending on the specificity and efficiency of these primer sets, products may be visualized on a gel, quantified via quantitative PCR (qPCR), purified and sequenced, or run through nested PCR to improve detection capabilities.

eDNA Sample Collection

The first step of obtaining an aquatic eDNA sample is to collect water. While this step seems straightforward, it varies considerably between studies. Many studies have used single-use collection bags such as Whirl-Paks for water collection, while others use pitchers, Nalgene bottles, or buckets (Deiner et al. 2015, Eichmiller et al. 2016b, Goldberg et al. 2016, Spens et al. 2017). Decisions regarding this step are dependent on the methods of processing; if eDNA is filtered on site in the field, buckets or pitchers may be preferable, while bottles or bags would be

ideal for samples that are taken to be filtered at a secondary location. For samples not filtered in the field, ice is necessary for transport to reduce DNA degradation, making filtering in the field seem ideal; however, that requires the transport of all filtration materials to the field and may increase overall time invested in the samples (Goldberg et al. 2016).

Actual methods of filtration, and thus the difficulty in transport, also vary across studies. The ideal filtration device would be portable, powerful, fast, and simple, but often studies are only able to fulfill some of these criteria. Manual hand-driven vacuum pumps are portable and simple but fatigue is common when filtering large amounts or turbid water, and the time and power required may be longer than desired (Laramie et al. 2015b). Battery-powered peristaltic pumps are powerful and fast but are heavy and may be difficult to carry to remote sites (Laramie et al. 2015b). Lab-based filtration is powerful and fast, sometimes allowing for the filtration of multiple samples simultaneously, but requires the transport of samples on ice, something which is not always possible, particularly in remote regions or areas where the air temperature is especially high (Robson et al. 2016). Each method of filtration has advantages and disadvantages that must be evaluated for each study and can be improved by creative solutions to unique problems.

More variable and arguably more important than the method of filtration are the filters themselves. Filter pore size and material both have the potential to impact results of eDNA studies (Turner et al. 2014). In south Texas, many of water bodies are highly turbid, making filtration of large amounts of water through small pore filters difficult. Previous research suggests that filtering large amounts of water through large-pore filters is more effective at retrieving eDNA than small amounts of water through small-pore filters; however, the use of large-pore filters (>10 μm) for eDNA capture in highly turbid waters has not been tested (Turner

et al. 2014, Minamoto et al. 2016). Filter pore sizes for eDNA studies typically range from 0.45-3 μm, but even the largest of these is not able to consistently filter 1 L of water from siren habitat (KMR, unpubl. data). Robson et al. (2016) tested polycarbonate filters up to 20 μm for use in turbid, tropical water, but determined that 4 L would be necessary for detection based on work by Turner et al. (2014). Siren habitat in south Texas does not necessarily allow the filtering of this much water before becoming clogged. Given small fragments of DNA, a small pore filter would prevent more fragments from passing through, thus catching more DNA as was found by Liang and Keeley (2013) and Eichmiller et al. (2016), but a large amount of water would give a greater sample volume, which has been found to increase the probability of detection (Lopes et al. 2017). It is also important to consider inhibition of PCR from eDNA samples; sediment presence has been shown to reduce eDNA detection, particularly when it contains humic substances, algae, or siliceous particles (Stoeckle et al. 2017). Assessment of the utility of large-pore filters is needed to determine the applicability of eDNA surveys to highly turbid environments.

Filter materials have also been found to play a role in eDNA retrieval. Cellulose, polycarbonate, and glass fiber are some of the more commonly used filter materials, with the added option of disposable filter capsules (Spens et al. 2017). Not all filter types perform equally, however; comparisons between glass and polycarbonate filters have found glass to be consistently more effective in retrieving eDNA (Eichmiller et al. 2016b, Minamoto et al. 2016), and cellulose filters have been found to outperform both polycarbonate and glass filters (Liang and Keeley 2013, Hinlo et al. 2017, Majaneva et al. 2018). Notably, these filter materials have not been tested in highly turbid siren habitat or in large-pore sizes; in fact, not all filter materials

are available in larger pore sizes. Of the materials discussed herein, non-capsule cellulose filters are affordable, accessible, and effective (Turner et al. 2014).

Preservation of eDNA samples is usually accomplished by submerging the filter in ethanol or lysis buffer, or immediately freezing the filter at -20°C. Hinlo et al. (2017) did not find significant differences in eDNA retrieval between freezing and ethanol preservation, but Majaneva et al. (2018) found ethanol to be a poor preservative in comparison to freezing and buffer preservations. Lysis buffers such as Longmire's or CTAB have the benefit of preserving samples for longer periods of time at room temperature (Renshaw et al. 2015). One promising buffer that has not yet been explored for eDNA is DNAzol (DN127, Molecular Research Center, Cincinnati, OH, USA), a DNA isolation reagent (Chomczynski et al. 1997). Placing filters directly into DNAzol has the potential to preserve the DNA for at least one year at room temperature, and as such the efficiency of DNAzol in preserving eDNA needs to be assessed.

Perhaps most important to eDNA studies is the use of sterile technique. Washing equipment with 50% chlorine bleach (NaOCl) is suggested by Goldberg et al. (2016) to remove all extraneous DNA from materials and may be followed by sodium thiosulfate to deactivate the bleach, or thorough rinsing with clean water (Goldberg et al. 2016, Agersnap et al. 2017). Negative controls at each stage of the process from field to lab should be used to identify contamination, and gloves should be worn throughout the field collection process (Goldberg et al. 2016). The importance of sterile technique carries over to lab work; clean labs specifically for eDNA or ancient DNA are necessary along with frequent washing of all equipment (Goldberg et al. 2016).

eDNA Laboratory Analyses

Extraction of DNA from the filters varies across survey methods as well. Commercially available kits are by-far the most popular method of extraction, but different kits have different applicability to different filter types (Goldberg et al. 2016). These kits have the advantage of being easy and safe to use (Hinlo et al. 2017). An alternative is the use of phase-separation and precipitation methods, such as using a phenol-chloroform extraction, which has been found to yield more DNA than kit-based methods (Renshaw et al. 2015, Deiner et al. 2015). While more effective, phenol and chloroform are volatile and hazardous, requiring the use of a fume hood to prevent inhalation (Deiner et al. 2015). As such studies must assess the costs and benefits between methods to ascertain the best extraction method for the study system. Regardless, extracted DNA should be stored at -20°C or below to prevent degradation (Goldberg et al. 2016).

Primers for eDNA barcoding can be designed in multiple ways based on personal preference, but the software Primer3 is a popular freely available program for primer design (Rozen and Skaletsky 1999). In designing primers to detect eDNA, it is essential that primers are specific to the desired species and short enough for amplification of degraded fragments (Bohmann et al. 2014). Many species genomes are readily available for access via GenBank, making design and testing straightforward; published genomes can be used for primer development, and then primers can be tested via BLAST in order to assess specificity (Tsuji et al. 2019). Unfortunately, this process is complicated when studying species whose genomes have yet to be determined. In the case of sirens, several genomes have been published on GenBank, allowing for primer development. Primer specificity should also be tested with tissue DNA samples from the lab upon finalization of a protocol, particularly when sympatric species genomes are not available. The mitochondrial cytochrome oxidase 1 region is frequently chosen

for eDNA studies due to its variation between species and minimal intraspecific variation, as well as its high coverage in genetic databases and the abundance of mitochondrial DNA copies per cell (Rees et al. 2014, Tsuji et al. 2019). Commercial master mixes often have suggestions regarding reagent volumes and times for each PCR step, while temperature is dependent on the primers themselves; however, PCR protocols should be optimized via testing with tissue and eDNA samples to ensure consistent replication even at low DNA concentrations as is common in eDNA samples. Nested PCR is a possible solution for low-yield samples, where a sample is run through PCR once, then the product is purified and amplified using an internal primer set resulting in a shorter product size (Jackson et al. 2017, Stoeckle et al. 2018).

With species-specific primers, running PCR through gel electrophoresis can give a general idea of species presence based on the expected and observed band sizes, but species presence should be confirmed by sequencing (Evans and Lamberti 2018). Sequencing can be accomplished using Sanger sequencing or next-generation sequencing, both of which read the DNA sequence to give a string of nucleotides which can then be compared to known species genomes on GenBank or other genetic libraries to determine the species of origin. Sanger sequencing produces only one forward and reverse read and is limited by fragment size, while next-generation sequencing can give thousands of reads from a single sample and is capable of sequencing entire genomes (Shokralla et al. 2012, Valentini et al. 2016).

Objectives and Hypotheses

The objective of this study was to create and validate an eDNA assay for the Rio Grande siren. This included determining optimal methods of water collection and filtration, DNA extraction and amplification, and confirmation of siren DNA detection or nondetection from

field-based samples. Due to genetic similarity, it was hypothesized that the assay would be applicable for lesser sirens from multiple geographic areas, while remaining specific enough to amplify only siren DNA. DNAzol was hypothesized to be a valid storage and extraction buffer for eDNA samples. Additionally, nested primers were expected to be an adequate solution for low yield samples. This study sets the groundwork for further, in-depth eDNA surveys for sirens across Texas, and develops methods that can be applied to other species-specific or generalized eDNA studies in similar environments to the Rio Grande Valley.

Materials and Methods

eDNA Collection

The filtration device designed for this project consisted of a 47mm filter cup (XX1104700, MilliporeSigma, Darmstadt, Germany) on a PVC arm that could be inserted into a hand-powered automotive fluid evacuator (MV7400, Mityvac, St. Louis, MO, USA). Other filtration methods were tested, and this was found to be the most effective for filtering water quickly in the field without requiring carrying heavy equipment (Fig.2). Methodology to prevent contamination in the field included washing equipment with 50% chlorine bleach solution between sites followed by a 0.1 M sodium thiosulfate wash and distilled water rinse, filtering distilled water after cleaning as a blank to determine if field samples may be contaminated, wearing fresh gloves for each site, and taking care not to stir up sediment while obtaining water, as eDNA from sediment may persist longer than aquatic eDNA (Goldberg et al. 2016). Water was obtained in the field using a pitcher attached to an extendable telescoping pole, which was similarly washed between sites. All eDNA samples were taken in triplicate. The filter tested in this project was a Whatman Grade 4 cellulose filter (1004047, GE Healthcare, Chicago, IL,

USA) with an estimated pore size of 25 µm. After filtration, filters were promptly placed in 2-mL microcentrifuge tubes pre-filled with 700 µL of DNAzol (DN127, Molecular Research Center, Cincinnati, OH, USA).

Figure 2. Water filtration device in use in the field. Photo by Erich Schlegel.

DNA Extraction

The filter DNA extraction protocol was adapted from the DNAzol manual. Filters sat in DNAzol for at least 3 d at room temperature before extraction procedures. The sample was then heated at 55°C for 30 min, vortexed, and centrifuged for 1 min at 5000 *g*. The filter was then removed from the tube and squeezed using clean forceps to collect all DNAzol in the tube. 500

µL of chloroform was added, vortexed, let to stand 1 min, and centrifuged 2 min at 12,000 *g*. The supernatant was extracted into a clean 1.5-mL microcentrifuge tube. 500 µL of absolute ethanol was added, inverted until mixed, and centrifuged for 10 min at 16,000 *g* to pellet the DNA. The supernatant was discarded, and the pellet was washed with 500 µL of 95% EtOH, followed by a second wash in 500 µL of 75% EtOH. The pellet was then air-dried for at least 30 minutes before being dissolved in 22µL of a dilute TE buffer solution (3mM Tris-Cl, 0.3mM EDTA, pH 8.0) at 55°C.

All extracted samples were quantified for total DNA concentration using a Qubit 3.0 Fluorometer (Q33216, ThermoFisher Scientific, Waltham, MA, USA) with a Qubit dsDNA high sensitivity assay kit (Q32851, ThermoFisher Scientific, Waltham, MA, USA) and stored at -20°C. All eDNA extractions took place in a separate clean lab from the PCR and tissue processing lab to help prevent contamination of samples (Goldberg et al. 2016). Benchtops and micropipettors were cleaned before extractions, and only sterile filter pipette tips were used.

PCR Protocols

Primers were designed based on previous research into the genome of the Rio Grande siren using sequences (GenBank accession numbers: KU871392, KU904482, KU904483, KU904484, KU904485, KU904486, KU904487, KU904489) obtained via Illumina sequencing and analyzed using Geneious (LaFortune 2015, Bare 2018). Primer design was completed using the software Primer3 (Rozen and Skaletsky 1999), with the goal of developing primer sets specific to siren with similar melting temperatures and minimal dimer formation. As the primer sets were intended for use with eDNA, they also needed to amplify a relatively small region as eDNA is often degraded. The cytochrome oxidase 1 (CO1) region is a region frequently used for

eDNA research due to its high specificity (Tsuji et al. 2019); primer development was focused on this region. While the target for the primer design was for the Rio Grande siren, amplification of other lesser siren from across the state was not intentionally avoided. Primers were tested for specificity in GenBank before being ordered from Eurofins Genomics (Louisville, KY, USA).

The final primer set amplified 183 bp in the CO1 region, while the nested primer set amplified 178 bp within the original primer set. Primers with melting temperatures and full amplified sequences can be found in Table 1.

Table 1. Sequences and melting temperatures for primers, probe, and nested primers, and full amplified sequences for each primer set. Underlined sections of dull and nested sequences indicate primers.

Name	Sequence 5'-3'	Melt Temp. (°C)
CO1B-FW	ACGCTATTCCGATTATCCAG	55.2
CO1B-RV	GACATCCGTGAAGTCATTC	54.5
PROBE4	[6~FAM]CAGCTAAACGAGAAGTTAAGTCCACTGAACTA[BHQ1A~Q]	65.6
CO1B-FWnest	CGACGCTATTCTGATTATCCAG	58.4
CO1B-RVnest	CGTGAAGTCATTCTACATTAGTTG	57.6
FULL SEQUENCE	GACGCTATTCTGATTATCCAGATGCATATACGCTATGAAATTCCATCTCATCAATTGGATCCTTAATCTCATTAGTAGCAGTTATTATAATAATATTTATCATTTGAGAAGCCTTCTCAGCTAAACGAGAAGTTAAGTCCACTGAACTAACTTCAACTAATGTAGAATGACTTCACGGATGTC	N/A
NESTED SEQUENCE	CGACGCTATTCTGATTATCCAGATGCATATACGCTATGAAATTCCATCTCATCAATTGGATCCTTAATCTCATTAGTAGCAGTTATTATAATAATATTTATCATTTGAGAAGCCTTCTCAGCTAAACGAGAAGTTAAGTCCACTGAACTAACTTCAACTAATGTAGAATGACTTCACG	N/A

Primer concentrations and PCR conditions were optimized with extracted siren tissue DNA. To validate the primer pairs with water samples, water from an aquarium containing two sirens was filtered and extracted following the eDNA protocol. An additional positive control consisted of pond water spiked with this aquarium water at a concentration of 5%. Finally, primers were tested with samples from water bodies were sirens had been repeatedly captured as well as water bodies where sirens were not expected (LaFortune 2015). Primers were also tested against tissue samples from sympatric amphibians to ensure specificity to sirens.

To decrease the detection threshold and to overcome potential inhibitory substances in the DNA extract, forward and reverse nested primers were developed and optimized using the same methods described above. Sanger sequencing of samples was performed by Eurofins Genomics.

```
CLUSTAL W (1.83) multiple sequence alignment

AmplifiedRegion       ACGCTATTCCGATTATCCAGATGCATATACGCTATGAAATTCCATCTCAT
texana_KU904486       ACGCTATTCTGATTATCCAGATGCATATACGCTATGAAATTCCATCTCAT
intermedia_MH888024   ACGCTATTCTGATTACCCAGATGCATATACGCTATGAAATTCCATCTCAT
reticulata_MH888031   ACGATACTCAGACTACCCAGATGCATACACCTCTGAAATTCTGTTTCAT
                      *** ** ** ** ** ********** ** ** ********  * ****

AmplifiedRegion       CAATTGGATCCTTAATCTCATTAGTAGCAGTTATTATAATAATATTTATC
texana_KU904486       CAATTGGATCCTTAATCTCATTAGTAGCAGTTATTATAATAATATTTATC
intermedia_MH888024   CAATTGGATCCTTAATCTCATTAGTAGCAGTTATTATAATAATATTTATC
reticulata_MH888031   CAATCGGATCACTAATTTCATTAGTAGCAGTTATTATAATAATATTTATT
                      **** *****  **** ********************************

AmplifiedRegion       ATTTGAGAAGCCTTCTCAGCTAAACGAGAAGTTAAGTCCACTGAACTAAC
texana_KU904486       ATTTGAGAAGCCTTCTCAGCTAAACGAGAAGTTAAGTCCACTGAACTAAC
intermedia_MH888024   ATTTGAGAAGCCTTCTCAGCTAAGCGAGAAGTCAAGTCCACCGAACTAAC
reticulata_MH888031   ATTTGAGAAGCCTTCTCAGCCAAACGAGAAGTGAAATCTACTGAACTTAC
                      ******************** ** ******** ** ** ** ***** **

AmplifiedRegion       TTCAACTAATGTAGAATGACTTCACGGATGTC
texana_KU904486       TTCAACTAATGTAGAATGACTTCACGGATGTC
intermedia_MH888024   TTCAACTAATGTAGAGTGACTTCATGGATGCC
reticulata_MH888031   ATCAACTAAAGTAGAATGACTTCACGGATGCC
                       ******** ***** ******** ***** *
```

Figure 3. Alignment of the amplified region with published sequences from Siren intermedia texana, S. intermedia, *and* S. reticulata*. Original primers are underlined, with mismatches identified in red text. One base pair in the forward primer was intentionally changed from T to C to prevent dimer formation. Percent overlap with the amplified region is 99.45% for* S. i. texana*, 95.63% for* S. intermedia*, and 86.89% for* S. reticulata*.*

A probe-based qPCR protocol and standard curve were also developed for future uses in quantification. The standard curve was generated via synthetic DNA of the target segment ordered from Eurofins Genomics. The synthetic DNA was quantified using a Qubit with a high sensitive kit and diluted serially. Dilutions were also quantified using a Qubit with a high sensitivity kit to ensure evenness of the standard curve.

Results

Both the CO1B and CO1Bnest primer sets (Table 1) were tested against sirens collected from Cameron County (*S. i. texana*), Kinney County (*S. i. texana*), and Harris County (*S. i. nettingi*) to confirm effectiveness for sirens across the state, as well as tissue of sirens from Arkansas, Mississippi, and Oklahoma (Appendix 1) to further test breadth of applicability (Fig. 4). The primer sets were not found to amplify DNA from any other species in GenBank, and when tested with tissue samples from sympatric amphibian species, no amplification occurred.

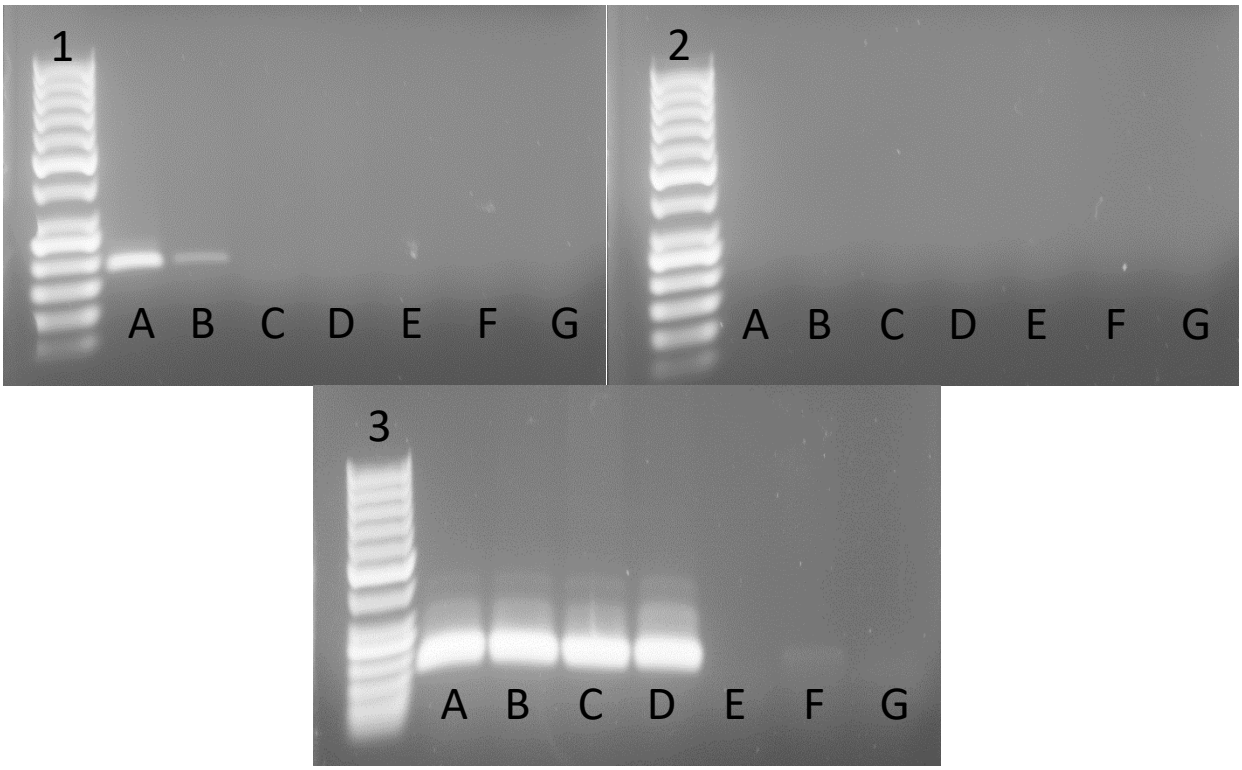

Figure 4. Photos of gels from the primer set tests with sympatric amphibian species. Species tested are Gel 1: A) Cameron County siren, B) Harris County siren, C) Rana catesbeiana *(American Bullfrog), D)* Rana berlandieri *(Rio Grande Leopard Frog), E)* Hypopachus variolosus *(Sheep Frog), F)* Gastrophryne olivacea *(Western Narrow-Mouthed Toad), and G) negative control (NTC). Gel 2: A)* Anaxyrus speciosus *(Texas Toad), B)* Incilius nebulifer *(Gulf Coast Toad), C)* Incilius horribilis *(Cane Toad), D)* Scaphiopus couchii *(Couch's Spadefoot Toad), E)* Ambystoma tigrinum *(Tiger Salamander), F)* Notopthalmus meridionalis *(Black-Spotted Newt), and G) NTC. Gel 3: A) Kinney County siren, B) Arkansas siren, C) Mississippi siren, D) Oklahoma siren, E)* Rhinophrynus dorsalis *(Mexican Burrowing Toad), F)* Leptodactylis fragilis *(White-Lipped Frog), and G)* Smilisca baudinii *(Mexican Treefrog).*

For PCR, 25µL reactions were used consisting of 12.5 µL master mix (GoTaq G2 HotStart MasterMix), 5.5 µL H_2O, 1 µL 2 µM CO1B-FW, 1 µL 2 µM CO1B-RV, and 5 µL sample. For nested PCR, the protocol was the same with the following changes to reagents: CO1B-FWnest and CO1B-RVnest were used at 5 µM, and the sample input was 5 µL of purified product from the first round of PCR. For qPCR, Luna Universal Probe-Based MasterMix was used. Reagent amounts per reaction were 12.5 µL master mix, 9 µL H_2O, 1 µL 2 µM CO1B-FW, 1 µL 2 µM CO1B-RV, 0.5 µL Probe-4, and 1 µL sample for the standard curve. The amplification protocol was identical to that used in PCR; however, number of cycles was increased to 50 (Table 2). A standard curve was developed for use with qPCR (Fig. 5).

Table 2. PCR protocol for amplifying siren eDNA.

Step	Temperature	Time	
Hot Start	95°C	∞	
Initial Denaturing	95°C	30 s	
Denaturing	95°C	15 s	
Annealing	57°C	30 s	40x
Extension	72°C	15 s	
Cooling	4°C	10 min	

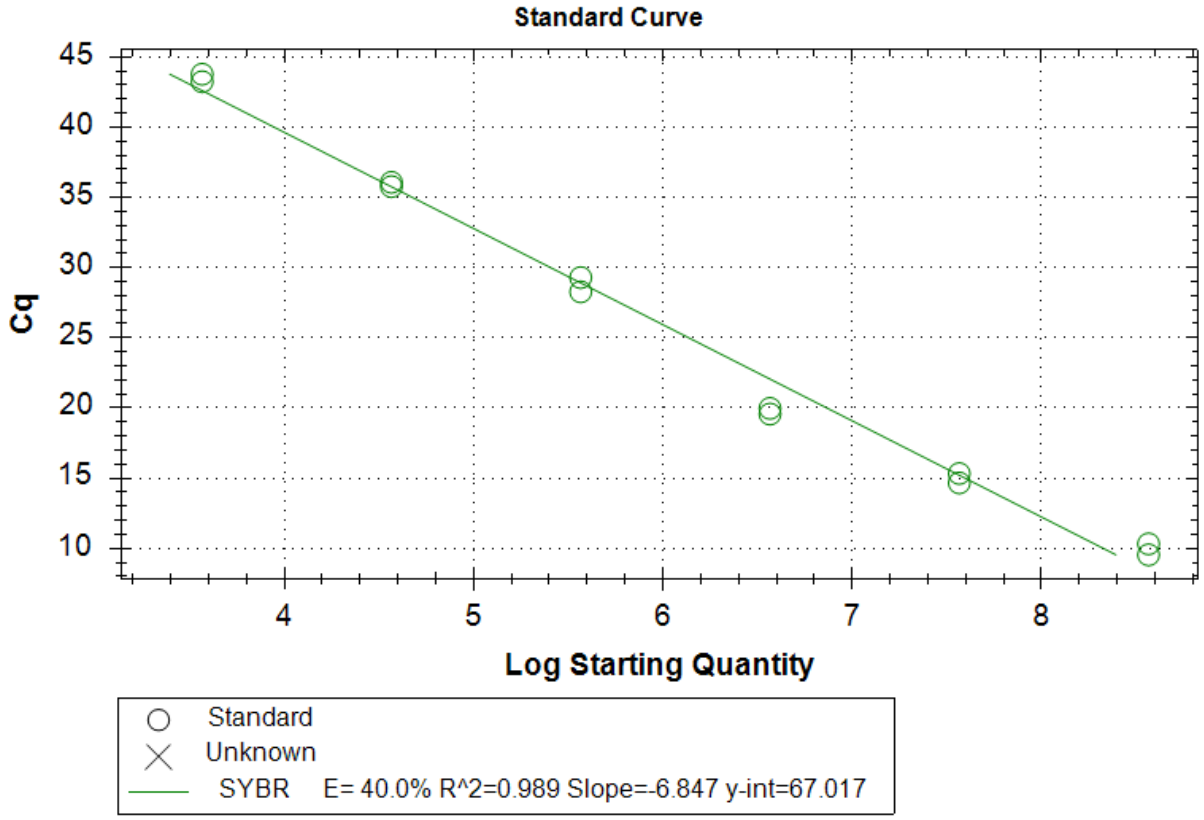

Figure 5. Standard curve generated via synthetic DNA in qPCR. The r^2 value of 0.989 indicates goodness of fit.

eDNA and Sequencing Results

PCR products from extracted aquarium water and siren tissue were run on 1% agarose EtBr gel electrophoresis, resulting in similar high fluorescent intensities when viewed under a UV transilluminator. An extracted eDNA sample from a pond known to be occupied by sirens also resulted in a high intensity band of the expected size. eDNA samples run in triplicate from a pond where sirens were not expected did not yield any detectable product, indicating that siren DNA was not present in the sample. For most samples, a single round of PCR was not adequate to produce enough copies of the target region for Sanger sequencing; this was likely due to

inhibition, which was overcome by using a nested PCR procedure. When samples were run through nested PCR and sequenced, sequences for the siren tank as well as the known pond could be identified as siren DNA based on an NCBI BLAST search. eDNA samples from ponds not expected to contain sirens did not yield a detectable product in the six samples tested.

Discussion

Siren DNA Amplification

The described protocol with initial and nested primers was able to successfully amplify and provide matching sequences to the GenBank database for sirens from numerous locations; as such, the primer set and PCR protocol can be classified as valid for amplifying siren DNA. The specificity of the primers was confirmed by testing them against other sympatric amphibian species from south Texas as well as water samples from areas where sirens were not expected; as none of these amplified, the primers can be further classified as appropriate for specific detection of sirens via eDNA.

The assay developed herein opens new avenues for siren detection and monitoring. As sirens are cryptic species that are difficult to detect through conventional means, eDNA offers an exciting avenue to assist in siren studies. While the target area of this study was the south Texas habitat of the Rio Grande siren, the successful amplification of siren DNA from other regions introduces the prospect of widespread siren monitoring via eDNA. Altogether, the development of this assay can be considered successful and applicable for the desired purposes and can function as another tool in the toolbox of amphibian research and conservation.

The successful development of a qPCR assay and standard curve allows for future studies in quantification. While it is not yet possible to quantify species abundance from eDNA alone,

relative abundances from repeat comparable sampling may be possible; with current technology, any attempts at even relative quantification should be interpreted skeptically (Jane et al. 2015, Kamoroff and Goldberg 2018, Kakuda et al. 2019). Additionally, a specific qPCR assay can eliminate the need for gels, nested PCR, and Sanger sequencing for identification. As probe-based qPCR is frequently used for eDNA preferentially over gel assays, this also enables projects studying sirens via eDNA to better fit with the known recommendations for eDNA for amphibians (Goldberg et al. 2016).

eDNA detection has the added benefit of potentially being more cost-effective than traditional surveys, particularly for elusive or cryptic species such as sirens (Biggs et al. 2015, Sigsgaard et al. 2015, Davy et al. 2015). While the exact cost is variable between studies, once an assay has been developed the cost may be less than for traditional surveys, particularly in labs already equipped with the necessary machinery (Goldberg et al. 2011, Biggs et al. 2015, Smart et al. 2016). For labs not equipped with PCR machines or other equipment, and without a developed assay for the species in question, the upfront cost may be prohibitive; however, options in outsourcing multiplexing, metabarcoding, and next-generation sequencing are predicted to become more affordable and accessible over time (Bohmann et al. 2014, Davy et al. 2015, Creer et al. 2016, Borrell et al. 2017, Deiner et al. 2017). For elusive species, the cost of reagents for running eDNA samples may be preferable to the cost of employing workers to set and check traps over long periods of time (Huver et al. 2015, Biggs et al. 2015, Sigsgaard et al. 2015, Davy et al. 2015, Smart et al. 2016).

Nested PCR proved to be an effective method in improving detection of siren eDNA via Sanger sequencing, possibly by elimination of inhibitors in the second round of PCR (Erume et al. 2001). Additionally, by running two rounds of PCR, the specificity of amplification may be

increased as two separate sets of primers are used (Erume et al. 2001, Jackson et al. 2017). Most noticeably, nested PCR seems to offset issues of low copy numbers that are often problematic in eDNA studies (Wilcox et al. 2013).

Future Directions

While the development of this assay is the first step towards a fully validated method to detect sirens via eDNA, further research is needed. In particular, the presence of inhibitors in natural environments may not be fully removed through the filter extraction protocol (Jane et al. 2015, McKee et al. 2015b, Harper et al. 2019). As such, the protocol could benefit from further optimization to ensure that inhibitors are being eliminated as much as possible. The reduction of inhibitors present in samples has the potential to eliminate the need for nested PCR, thus reducing the time required for the assay as well as reducing the opportunities for cross-contamination and aiding in the potential for quantification of the results. In particular, the use of CTAB and a phenol-chloroform-isoamyl procedure as demonstrated by Hunter et al. (2019) is promising and should be assessed with eDNA filters from siren habitats. Other methods such as kits and purification columns may also be tested to determine the optimal protocol for siren eDNA detection in south Texas (McKee et al. 2015b, Buxton et al. 2018, Harper et al. 2019). Once an optimal method is identified, it will be able to be applied to other species occupying similar habitats. In the meantime, nested PCR is a reliable alternative for confirming detection when removal of inhibitors during the extraction protocol is inadequate for detection through a single round of PCR.

Filter material and pore size should also be assessed for use with siren DNA. While there have been studies that have tested the effectiveness of these variables for detection of eDNA,

they have not been assessed in south Texas or the uniquely turbid water occupied by sirens. As such, a direct comparison of pore sizes and materials is necessary to determine the most effective materials for detection of sirens. This study used large pore cellulose filters due to turbidity, electing to prioritize the amount of water filtered over the fineness of the pores. While cellulose filters have been found to be more effective than other materials for eDNA (Liang and Keeley 2013, Hinlo et al. 2017, Majaneva et al. 2018), this should also be tested in siren habitat for confirmation. In these comparisons, it will be important to consider the amount of water filtered as well as the amount of DNA obtained in comparison to pore size and filter material. Finally, the use of DNAzol as a preservative for eDNA filters is novel; while it was effective in preserving and isolating eDNA in this study, its effectiveness should be compared to other preservation methods such as Longmire's buffer and ethanol. The qPCR assay described in this study can be applied to these goals by quantifying the amounts of siren DNA obtained from this variety of methodology.

In conclusion, while further studies are needed to fully validate and optimize this assay, the methods described in this study are adequate for detection of sirens via eDNA, and have the potential to improve siren detection through eDNA without the need to visually observe this elusive species.

CHAPTER III

ENVIRONMENTAL DNA ASSAY ASSESSMENT: DETECTION EFFECTIVENESS COMPARED TO TRAPPING AND INFLUENCE OF ENVIRONMENTAL VARIABLES

Introduction

The development of an eDNA assay is not useful if it is not as effective as traditional detection methods. As such, comparisons between methods are necessary to determine the ideal method balancing detection with cost, time, and labor. This can be accomplished through simultaneous use of traditional and molecular detection methods and comparison of the success of each (Dejean et al. 2012, Takahara et al. 2013, Smart et al. 2015, Smith 2017, Wineland et al. 2019, Rose et al. 2019). In the case of sirens, the primary traditional sampling method is through trapping.

Trap types for sirens vary between studies, but include commercial minnow/crayfish traps (Gehlbach and Kennedy 1978, Hanlin and Mount 1978, Raymond 1991, Fauth and Resetarits 1999, Sullivan et al. 2000, Ford and Hampton 2005, Hampton 2009, Schalk and Luhring 2010, Johnson and Blackwell 2011, LaFortune 2015), large, bell-shaped crayfish traps (Johnson and Barichivich 2003, Sorensen 2003), and handmade traps (McDaniel 1969, Luhring and Jennison 2008, Schalk and Luhring 2010, Schalk et al. 2010). Traps also vary in material, between coated metal, uncoated metal, plastic, and mesh fabric (Sorensen 2003, Willson et al. 2005). Traps are often baited, but not always; previous studies have baited traps with chicken

gizzards (LaFortune 2015), bacon (Johnson and Blackwell 2011), sardines (Hanlin and Mount 1978, Sorensen 2003), and beef/pork liver (Hanlin and Mount 1978) but potential differences in siren diets across habitats may be a factor for consideration in bait choice (Hill et al. 2015).

Environmental variables may play a role in siren detection for both eDNA surveys and traditional sampling methods. Seasonality, in particular, is a major factor in siren ecology, but differs drastically across siren range and between species. For greater sirens (*Siren lacertina*), seasonal activity levels have been found to be highest in the summer (Alabama: Hanlin and Mount 1978) and the winter (Florida: Sorensen 2003). Lesser sirens (*Siren intermedia*) have been found to be most active in the summer (Petranka 2010), late fall/early winter (Louisiana: Raymond 1991), late winter/early spring (Texas: Hampton 2009), fall and spring (Arkansas: Sawyer and Trauth 2011), or to exhibit no seasonality (Texas: Gehlbach and Kennedy 1978). Sirens are thought to breed in late winter/early spring, and therefore activity levels could be expected to increase during this time (Godley 1983). At the same time, warmer water temperatures and greater food availability during the summer months could increase activity levels (Petranka 2010). Historical records of sirens in south Texas suggest increased activity during the expected late winter/early spring time frame. More recently, sirens were found to decrease activity in the summer and be most active during the fall, but sampling in this study did not encompass late winter and early spring, the probable breeding period for lesser sirens (LaFortune 2015). This could be due to the extreme seasonal environment of south Texas; daytime temperatures regularly increase above 100°F in the summer and into the fall, and many water bodies dry completely each summer. This recurrent summer drought can trigger aestivation in sirens, wherein they become dormant in mucus cocoons underground until the water body is refilled (Gehlbach et al. 1973).

Environmental variables also have the potential to influence siren activity and presence, but few studies have investigated the effect of environmental variables on siren habitat preferences. Water quality in particular may play a role in siren presence as sirens are obligate aquatic; however they have been reported in a wide range of aquatic habitats, including both permanent and ephemeral water bodies (Gehlbach and Kennedy 1978, Schalk and Luhring 2010, Petranka 2010, Tipton et al. 2012). Sirens have also been reported in hypoxic (dissolved oxygen, DO: <2 mg/L) water bodies (Duke and Ultsch 1990, Snodgrass et al. 1999, Tipton et al. 2012), but LaFortune (2015) was unable to statistically test the influence of DO on siren capture due to extreme variability within water bodies and time of day. Sorensen (2003) found a strong negative correlation between water temperature and capture of *Siren lacertina*, but no such correlation was found for sirens in south Texas (LaFortune 2015). LaFortune (2015) also failed to find any correlation between pH and conductivity with siren presence.

Seasonality and environmental variables have been found to influence eDNA retrieval. Seasonal activity has been shown to influence detection of salamanders (Spear et al. 2015, de Souza et al. 2016, Takahashi et al. 2018, Buxton et al. 2018), frogs (Smith 2017), turtles (de Souza et al. 2016), carp (Erickson et al. 2016), and salmon (Laramie et al. 2015a). Seasonal variations in eDNA concentrations have even been used to estimate the seasonal activity of organisms (Minamoto et al. 2017, Sigsgaard et al. 2017, Tillotson et al. 2018, Takahara et al. 2019). Environmental variables can play a role in both the production of eDNA from organisms and the degradation of eDNA in the water body (Stewart 2019). Higher temperature has been found to significantly increase the amount of DNA shed in the water column by organisms (Robson et al. 2016, Lacoursière-Roussel et al. 2016), as well as to reduce eDNA persistence in a water body (Pilliod et al. 2014, Strickler et al. 2015, Eichmiller et al. 2016a). pH has also been

found to impact eDNA persistence, with more acidic environments resulting in faster eDNA breakdown (Strickler et al. 2015, Seymour et al. 2018). DO has not been tested for a direct effect on eDNA persistence, but it likely influences species presence and activity; at the same time, an entirely anoxic water body would reduce microbial action and thus slow the degradation of DNA (Seymour et al. 2018, Stewart 2019).

Studies comparing eDNA with traditional methods are variable in methodology, but the majority find eDNA to have a higher detection rate than traditional sampling. Detection rates can be compared directly via percent of positive detection for each method, or by comparing catch per unit effort, from which statistical significance can be determined via statistical tests such as the Fisher's exact test (Jerde et al. 2011, Dejean et al. 2012, Takahara et al. 2013, Smart et al. 2015, McKee et al. 2015a, Smith 2017). When environmental variables are collected simultaneously, generalized linear models (GLMs) can be used to assess the impacts of seasonality and other environmental variables on detection (Fukumoto et al. 2015, Smart et al. 2015, Smith 2017). Occupancy models are an alternative method of comparison increasingly being used in eDNA and traditional detection comparisons while integrating environmental variables to determine the greatest influences on detection (Schmidt et al. 2013, Hunter et al. 2015, Dougherty et al. 2016, Schmelzle and Kinziger 2016, Vörös et al. 2017, Eiler et al. 2018, Wineland et al. 2019, Akre et al. 2019, Rose et al. 2019). Schmidt et al. (2013) assert that a direct comparison of detection rates is overly optimistic as it does not take into account the failure to detect through either method. Occupancy models, on the other hand, incorporate imperfect detection into the analysis, and are well-suited to the replication already present in eDNA studies (Schmidt et al. 2013). The integration of environmental variables as covariates into the model allows for the assessment of correlation with detection within the model (Dougherty et al. 2016,

Eiler et al. 2018). As such, occupancy models are the current standard in comparing detection methods and in integrating methods to determine overall occupancy as well as factors influencing detection (Nichols et al. 2008).

The objectives of this study were to compare the detection of sirens using eDNA and trapping to determine the most effective method. Additionally, the study set out to assess the impacts of environmental variables (season, weather, rain history, water temperature, trap hours, and amount of water filtered) on siren detection for both methods. It was hypothesized that siren detection would be higher with eDNA, and that environmental variables would impact detection for both methods. In particular, siren detection was expected to be highest in the spring (when sirens may be breeding), during cloudy weather (which may signal incoming rain), after rain, at moderate water temperatures, and when more water was filtered or the trap was set for a longer amount of time.

Materials and Methods

Study Area

Five locations were chosen across near Brownsville, Cameron County, Texas, USA based on accessibility and known siren occurrence (Table 3, Fig. 6). All locations were visited monthly beginning in March 2019 and ending in February 2020. Specific sampling within the month was attempted in the middle of each month, but was dependent on equipment availability, weather, and water presence for each water body. Resacas that had no water during the summer were not sampled during their dry months. Sampling at each site included setting siren traps, collecting eDNA samples, and measuring environmental variables including weather, air temperature, and water temperature.

Table 3. Sampling locations for siren eDNA and trapping.

Site Name	Coordinates
UTRGV Brownsville Campus, Lozano Banco Resaca	25.8950, -97.4872
TNC Southmost Preserve	25.8518, -97.3912
Sabal Palm Sanctuary	25.8499, -97.4193
TPWD Olmito Fish Hatchery	25.9856, -97.5313
Resaca de la Palma State Park	25.9875, -97.5643

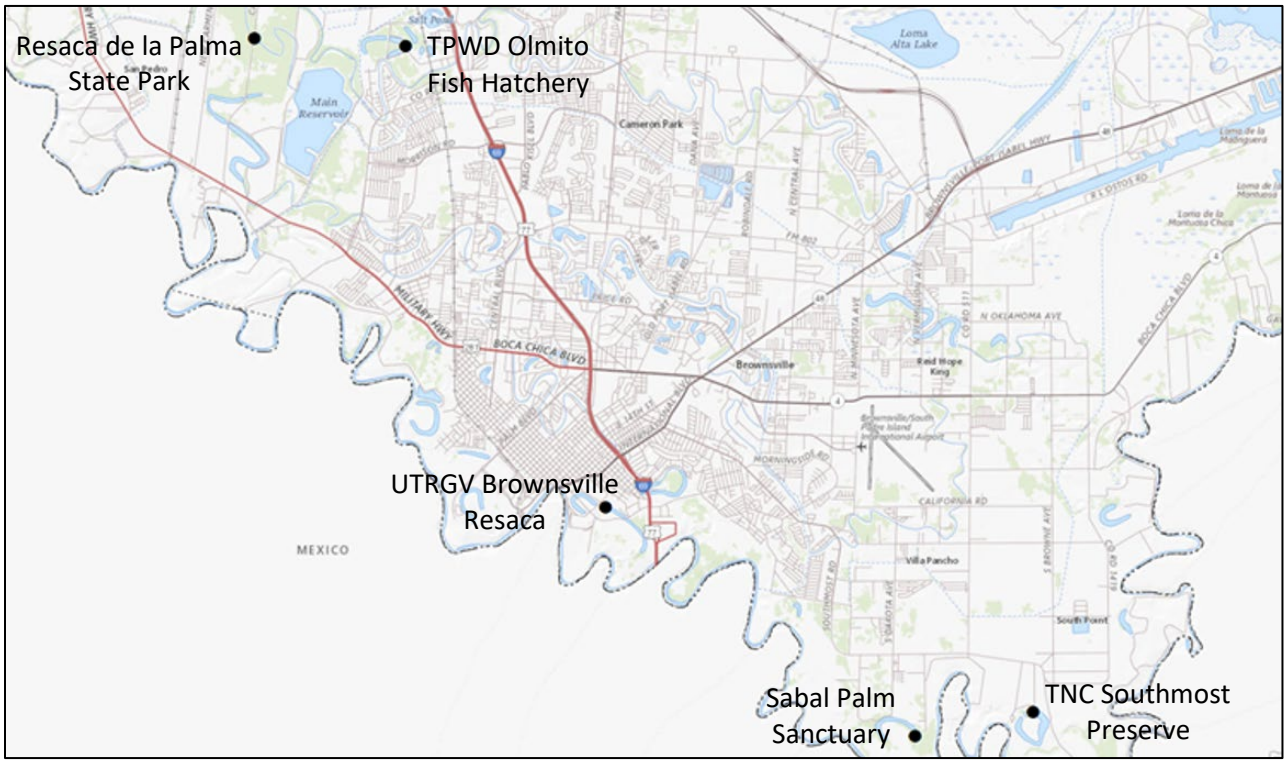

Figure 6. Sampling locations across Brownsville for siren eDNA and trapping.

Siren Trapping

Three siren traps were set at six locations at each site, for a total of 18 traps per site, per month. Occasionally, the amount of water present at a site was not adequate to place traps at all six locations, and the numbers of traps were reduced accordingly. The traps used were vinyl-dipped, two-piece metal minnow traps, cylindrical in shape with funnel ends (Academy Sports + Outdoors FSH2FE6065, Houston TX USA, Fig. 7). Traps were baited with chicken liver in small, perforated plastic containers within the traps, and were set with small foam flotation devices to avoid accidental drownings. Traps were set in shallow water along the edge of the water body within vegetation where possible to maximize the chances of siren capture. Traps were set with a focus on overnight deployment, with the average time set of 21.96 h (standard deviation: ± 1.79 h). The times traps were set and retrieved were recorded to determine the total trap hours for each sampling event. Trapped sirens were photographed, swabbed, and tissued (tail clips) before being released according to the TPWD-approved wild-caught species capture regulations and the animal care protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Rio Grande Valley (AUP18-28).

Figure 7. A Rio Grande siren captured in a metal minnow trap. Photo by Erich Schlegel.

Siren eDNA

Water samples were collected and processed according to the eDNA methodology described in Chapter 2. At each site, one blank and three eDNA samples were taken. eDNA samples were taken before traps were set to avoid stirring up the sediment. The amount of water filtered was recorded for each sample. Extracted DNA was amplified via the nested PCR protocol described in Chapter 2. Final purified PCR product was confirmed via Sanger sequencing. Samples with sequences matching published siren sequences by at least 95% in GenBank were considered positives. At least two out of three replicates were required to consider the sample as positive. If any amplification in field blanks indicated potential contamination, all replicates from those samples would be excluded. If any amplification was detected in the lab blanks, all samples were re-run to ensure exclusion of false positives.

Environmental Variables

Water temperature was measured at each water body using a Hach HDQ Portable Water Quality Meter (Hach HQ40D, Loveland, CO, USA). DO, pH, conductivity, and oxidative-reductive potential (ORP) were also measured using the Hach meter with associated probes. Probes were floated in the water column without disturbing sediment whenever possible. Weather and rain history were recorded based on observations prior to and during setting the traps, while daily high air temperature was obtained from the NOAA Climate Data Online database for the corresponding zip codes. Season was defined by the Northern Hemisphere meteorological seasons (NOAA 2016), weather was binned into the categories "sunny", "partly cloudy", and "cloudy", and rain history was categorized as rain within the past 24 hours (+) or no rain within the past 24 hours (-). Month was also noted.

Statistical Analyses

Single-season occupancy models were run in program PRESENCE 2.12.37 (Hines 2006) to assess siren detectability via trapping. As all sites were assumed to be occupied based on previous collections, ψ was set to a constant of 1 for all models. For sample-specific models, simple single-season models were used. First, a null model was run with no covariates. This was followed by the inclusion of covariates individually to determine their independent effect on detection. Due to model constrictions, any sampling covariates with even a single instance of missing data could not be included in the occupancy models. As water quality parameters (DO, pH, conductivity, and ORP) were sporadically missing data due to occasional probe malfunction, these measurements were not included in the models. All included covariates were sampling covariates; no site covariates were included. Covariates were then combined into additive models

to account for interactions between environmental variables. Models were ranked according to AIC. A global model was run including all covariates with 10,000 simulations to assess model fit. 95% confidence intervals for the coefficients for covariates were used to determine whether parameters could be considered informative.

To compare trapping to eDNA, a similar method was used via the program PRESENCE. Single-season multi-method occupancy models were run as described above, with the addition that all θ values were also set to a constant of 1. The model (with no covariates) output the base detection rate for each method; further models including covariates to assess impacts to detection were not possible due to the nature of the eDNA dataset. A Fisher's exact test was used in R v3.4.4 to determine whether the difference between the two methods was statistically significant.

Results

Trapping

Altogether, 66 sirens were captured across study sites over 12 months of sampling. Sabal Palm Sanctuary proved to be the most productive site for siren capture, with a total of 34 sirens captured, while no sirens were ever captured at the Fish Hatchery. The most productive month for siren capture was October 2019, with 15 sirens captured, while no sirens were captured in June or August at any site. Peak months for siren capture were March–May and September–December, indicating greater capture in the fall and spring with reduced capture in the summer and winter (Fig. 8). Sirens were trapped at a wide range of water quality parameters (Table 4).

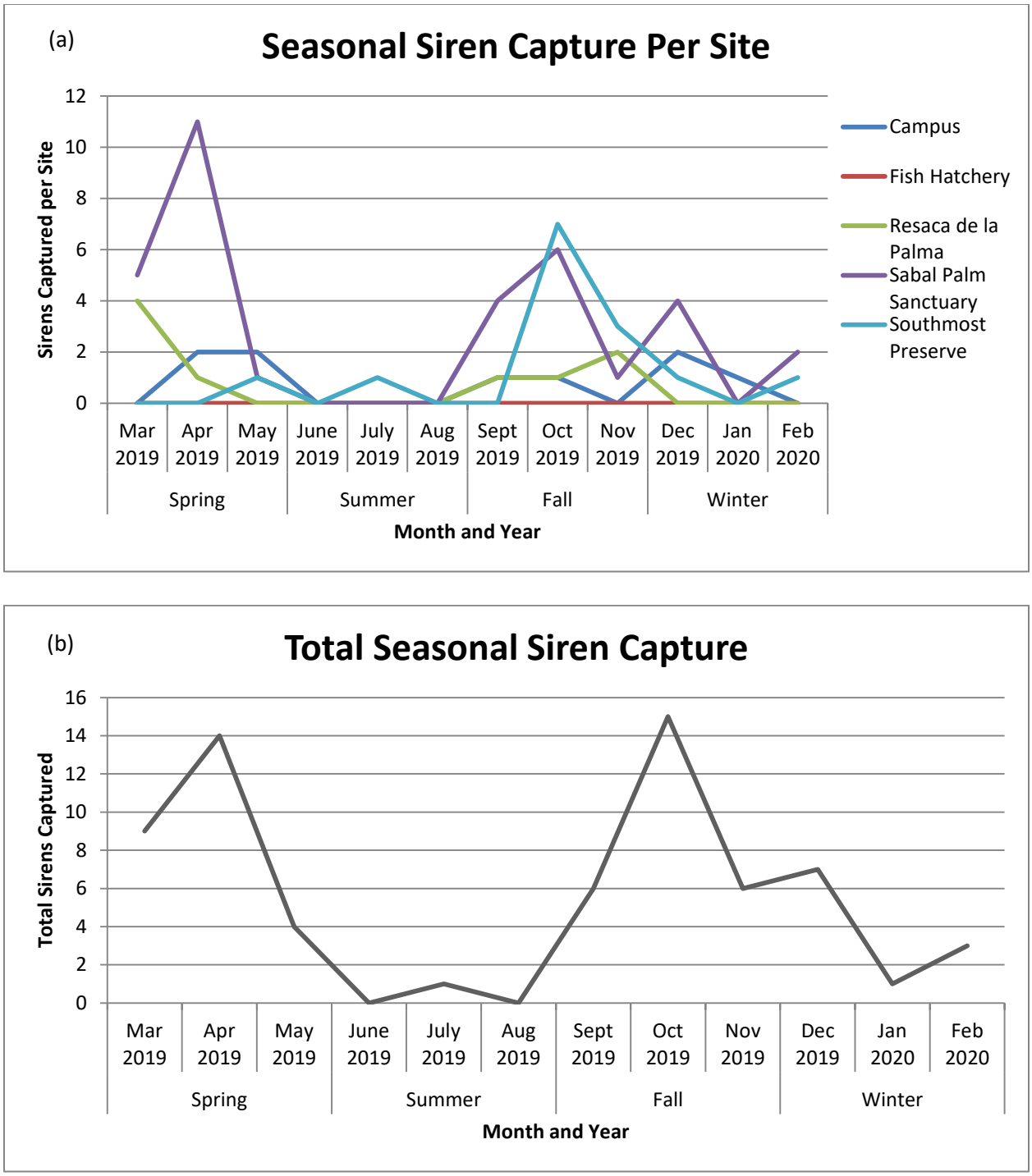

Figure 8. Siren detection via trapping over 1 year for each site (a) and overall (b).

eDNA

A majority of samples were positive for siren eDNA, though eDNA was not able to be collected due to lack of water on 4 occasions. Of the 46 sample sets tested, 29 were positive for all three replicates, 16 were positive for two of the three replicates, one was positive for one of the three replicates, and no samples were negative for all three replicates. 45 out of 46 had positive detections with at least two samples matching the sequences for siren, meeting the threshold for a positive eDNA location. This resulted in a 97.82% success rate, with a 2.17% chance of nondetection despite siren presence via concurrent trap data. Sirens were detected via eDNA at a slightly wider range of water quality parameters than trapping samples (Table 4).

Table 4. Water quality measurement ranges corresponding to siren detection for both trapping and eDNA.

	Trapping	**eDNA**
DO (mg/L)	0.187 – 7.747	0.17 – 9.213
pH	7.697 – 9.323	7.413 – 9.527
Conductivity (µS/cm)	575.333 – 2155	575.333 – 2155
ORP (mV)	20.767 – 201.467	20.433 – 301.067
Temperature (°C)	13.567 – 32.733	13.567 – 35.983

Occupancy Models for Detection

Trapping

All water quality measures except water temperature were excluded from analysis due to data gaps that could not be accommodated in the program. Based on seasonal activity patterns observed in siren capture, the month variable was also excluded due to correlation between month and season. A correlation analysis was performed on the remaining covariates, finding a strong relationship between the high air temperature and the average water temperature (0.76), and a moderate relationship between water temperature and season (-0.50). Weak relationships were found between season and air temperature (-0.28), season and recent rain (0.24), weather and recent rain (0.33), and water temperature and recent rain (-0.27) (Table 5). Due to the strong correlation, high air temperature was excluded from the model. The covariates tested in the models include season, hours set, weather, recent rain, and water temperature. All covariates were considered sample-specific, and no site covariates were used. Continuous covariates (hours set and water temperature) were standardized via a Z-transformation. Categorical covariates were either binary (recent rain) or were converted to indicator variables.

Table 5. Correlation coefficients for covariates.

	Season	***Hours Set***	***Weather***	***AirTemp High***	***RecentRain***	***AvgWater Temp***
Season	1					
HoursSet	-0.02719	1				
Weather	-0.04818	0.11746	1			
AirTempHigh	-0.28202	-0.00962	-0.08045	1		
RecentRain	0.23908	-0.02881	0.331562	-0.3843	1	
AvgWaterTemp	-0.49784	-0.11405	0.043671	0.763721	-0.26716	1

There was no evidence of strong under- or overdispersion based on the ĉ value of 1.0582. The model containing water temperature, weather, and season was best supported by ΔAIC values, followed by the global model (Table 6). 95% confidence intervals of each parameter in the top model indicate that water temperature, the season fall, and sunny weather were uninformative parameters. Likewise, in the global model hours set, water temperature, recent rain, the seasons fall and winter, and sunny weather were determined to be uninformative parameters (Table 7).

Table 6. Candidate models predicting siren detection probability via trapping, where K is the number of parameters, AIC is the Akaike's information criterion, ΔAIC is the change in AIC, ω is the model weight, and -2L is the -2 Log Likelihood.

Model	K	AIC	ΔAIC	ω	-2L
ψ(.),p(season+water+weather)	7	255.64	0.00	0.5579	241.64
ψ(.),p(global)	9	257.60	1.96	0.2094	239.60
ψ(.),p(season)	4	258.38	2.74	0.1418	250.38
ψ(.),p(water+season)	5	259.28	3.64	0.0904	249.28
ψ(.),p(water)	2	271.04	115.40	0.0003	267.04
ψ(.),p(weather)	3	271.79	16.15	0.0002	265.79
ψ(.),p(.)	1	273.50	17.86	0.0001	271.50
ψ(.),p(rain)	2	273.84	18.20	0.0001	269.84
ψ(.),p(hours)	2	275.13	19.49	0.0000	271.13

Table 7. Best supported models for siren detection via trapping, including parameters, coefficient estimates (β), standard error (SE), and 95% confidence intervals (LCI-UCI). Informative parameters are indicated in italics.

Model	Parameter	Indicator	β	SE	LCI	UCI
ψ(.),p(water+ season+weather)	Water Temp		-0.409	0.240	-.880	0.062
	Season	*Spring*	*0*	*0*	*0*	*0*
		Summer	*-2.8412*	*1.060*	*-4.918*	*-0.765*
		Fall	-0.288	0.429	-1.129	0.554
		Winter	*-1.074*	*0.517*	*-2.089*	*-0.060*
	Weather	Sunny	-0.157	0.439	-1.018	0.704
		PartlyCloudy	*0*	*0*	*0*	*0*
		Cloudy	*-1.183*	*0.498*	*-2.159*	*-0.208*
ψ(.),p(global)	Water Temp		0.088	0.173	-0.251	0.428
	Hours Set		-0.269	0.262	-0.782	0.243
	Recent Rain		0.649	0.488	-0.307	1.606
	Season	*Spring*	*0*	*0*	*0*	*0*
		Summer	*-3.039*	*1.076*	*-5.149*	*-0.930*
		Fall	-0.497	0.475	-1.427	0.433
		Winter	-0.898	0.537	-1.951	0.155
	Weather	Sunny	-0.048	0.440	-0.910	0.814
		PartlyCloudy	*0*	*0*	*0*	*0*
		Cloudy	*-1.374*	*0.522*	*-2.397*	*-0.351*

Occupancy model analyses with covariates were not possible for the eDNA dataset due to the high proportion of positive samples.

Multi-Method

Comparing only the two methods of trapping and eDNA for siren detection via a multi-method occupancy model revealed stark differences in detection probability. In the null model with no covariates, probability of detection for trapping was 0.4565 (95% CI 0.3198–0.6001) and for eDNA was 0.9783 (95% CI 0.8612-0.9969). This indicates that in general, sirens were more likely to be detected via eDNA than by trapping. Detection methods compared via a Fisher's

exact test indicate a significant difference in detection rates between eDNA and trapping (p = 1.019e-9).

Discussion

Seasonality

By detecting sirens through trapping, it was determined that Rio Grande sirens exhibit seasonal activity peaks in the spring and fall; in particular, March, April, and October were highly productive months for siren capture. For lesser sirens, activity peaks have been documented in late fall/early winter (Raymond 1991) and late winter/early spring (Hampton 2009). Rio Grande siren activity seems to be most similar to these studies, but multi-year monitoring could allow for more specific activity patterns to be determined. It is possible that these spikes in activity correspond with breeding events, but breeding times for Rio Grande sirens have not been investigated. Lesser sirens are thought to breed in later winter/early spring, so it is possible that the peaks in activity in March and April are associated with breeding (Godley 1983).

A more likely variable that may influence siren seasonal activity and detection via trapping is the availability of water. During the summer months, several water bodies in this study dried down completely, making sampling impossible. Water was also reduced during some of the winter months. In Brownsville, the highest average monthly rainfall occurs in spring and fall, when sirens were most often captured (Enciso and Wiedenfeld 2005). Additionally, water is often purchased to refill resacas in protected areas in order to mimic the historic Rio Grande flood seasons for the benefit of native and migratory species; this water is most often purchased in the spring and fall, when flow rates of the river peak (Robinson 2010, USFWS 2012). Of the sites sampled in this study, Resaca de la Palma State Park, TNC Southmost Preserve, and Sabal

Palm Sanctuary are known to purchase water for their resacas. As agencies have budgets in place for water purchase, it is impractical to keep water bodies filled in the summer and winter, despite the presence of species that may use the water, such as sirens. In particular, the extremely high temperatures in the summer result in faster evaporation of the water, making it even more difficult to keep the area filled (Enciso and Wiedenfeld 2005). Additionally, resacas historically have dried down in drier times of the year; as such, management often focuses on replicating historic floods, and allowing resacas to dry and fill based on rainfall throughout the rest of the year (Robinson 2010, USFWS 2012). Since sirens require water to be active, it follows that activity would be reduced in response to reduced water in the summer and, to a lesser degree, in the winter.

It is also noteworthy that while sirens were not captured at the Fish Hatchery during this study, eDNA data indicates their continued presence. In 1958, 81 sirens were captured and preserved from this location by A. G. Flury, and can now be found at the Texas Natural History Collection at the University of Texas at Austin. Lafortune (2015) captured one siren at this location, and sirens have been anecdotally observed in the resaca between 2015 and 2018. It remains a known siren site, despite lack of siren capture in this study. It is possible that sirens were not captured due to the nature of the habitat at the Fish Hatchery; very little edge vegetation was present, and no emergent or submerged vegetation was observed. Sirens may be more spread throughout the water body due to a lack of vegetation to cluster within.

Individual Detection Methods

Occupancy models were run to determine the impacts of variables on detection for trapping. The individual variable with the greatest impact on detection was season, which is

unsurprising based on observations of siren seasonality. When additive models were included, the model including season, water temperature, and weather was the best fit. When each parameter was assessed to determine whether it was informative, only parameters within season and weather were found to be informative. Summer and winter both had a strong negative influence on detection, as would be expected. Unexpected, however, was that cloudy weather had a negative influence on detection as sirens aestivate during dry periods as well as periods of extreme high temperature, it was thought that perhaps sirens could sense incoming rain and would become more active in preparation. Upon looking closer at the data, it was determined that the two largest trap successes were in sunny and partly cloudy weather respectively. As such, it can be assumed that the model's determination of cloudy weather as a negative informative parameter may be due to chance rather than biological significance, as any possible biological reason for this has yet to be identified.

For eDNA, detection was overwhelmingly positive to the point where the impact of environmental variables could not be determined. While all sites were known to contain sirens, the consistent detectability of sirens via eDNA is noteworthy. In terms of the single negative sample, it is possible that the addition of another sample set (three eDNA samples from a different location in the water body) could be enough to detect sirens; however, as only one sample set was negative at one location at one sampling instance, eDNA was highly accurate in detecting sirens, with approximately 98% of sample sets obtaining siren DNA. All negative controls yielded negative results, indicating that the sampling method was valid and was not subject to false positives. As all water bodies were known to contain sirens, this result is very nearly perfect detection. However, the eDNA sample size for this study is small; it is possible

that the addition of more samples from more water bodies over a longer period could change the detection rate.

Comparison of Trapping and eDNA

When the two methods are compared, it is clear that eDNA is far more effective at detecting sirens in all cases when compared to trapping. Essentially, when setting 18 traps in a water body known to be occupied by sirens, there was approximately 45.65% probability of detecting a siren, while when taking one set of eDNA samples (three replicates), there was approximately 98% probability of detecting a siren. This follows similar findings in other studies regarding the utility of eDNA compared to conventional surveys for amphibian detection (Dejean et al. 2012, Fukumoto et al. 2015, Smart et al. 2015, McKee et al. 2015a, Smith 2017, Vörös et al. 2017, Eiler et al. 2018, Wineland et al. 2019), but is the first of its kind to document the efficiency of eDNA for sirens. This opens many possibilities for surveying siren distributions more effectively. As conventional methods (trapping) were found in this study to be less consistent and more dependent on seasonality, eDNA could be used to map siren distributions without needing to capture an animal in hand.

Additionally, the effort exerted for detecting sirens using eDNA was lower than that exerted for trapping. For trapping, setting traps required visiting the site twice. While physically setting and pulling the traps does not take long, preparing the traps can take up to an hour. Trapping also often requires wading into the water body to set and pull the traps. Additionally, physically capturing and handling animals requires special collections permits, particularly for protected species; these permits are not required for collection and analysis of eDNA. Collecting a single sample set for eDNA would take less than half an hour in the field without requiring

wading into the water body, followed by lab work including approximately 3 h for filter extraction, and 6 h for nested amplification and sequencing preparation. As many samples can be processed simultaneously in the lab, the overall time invested in processing eDNA samples is less than with trapping, as well as requiring less travel and less intense field work. For detection, eDNA is the clear winner in terms of saving time and effort, and successfully detecting sirens.

Despite the promise of eDNA, it is important to remember that a species' complete biology and ecology cannot be determined from eDNA samples alone, and since so little is known of the Rio Grande siren, further in depth studies are necessary to better understand the needs of the species. As such, eDNA could be applied to preliminary surveys for sirens, thus potentially reducing the overall trap effort required to capture sirens by identifying water bodies containing siren DNA which could then be targeting for trapping. eDNA is also not appropriate for determining abundance. Altogether, eDNA is an additive tool for siren research, not one that should replace all conventional studies.

Altogether, future researchers can use the information acquired in this study to inform further research regarding sirens. For siren detection alone, eDNA should be used preferentially over trapping. eDNA can be applied for preliminary studies, to map distributions, and to inform trapping locations. Trapping efforts should be focused on the spring and fall, with summer being avoided whenever possible. Additionally, communications with landowners and agencies regarding water presence should be maintained to focus trapping efforts on times when the water body is adequately full.

CHAPTER IV

LITERATURE REVIEW OF ENVIRONMENTAL DNA METABARCODING METHODS AND APPLICATIONS

Introduction

Environmental DNA or eDNA describes the genetic material present in environmental samples such as sediment, water, and air, including whole cells, extracellular DNA and potentially whole organisms (Ficetola et al. 2008, Barnes and Turner 2016). eDNA can be captured from environmental samples and preserved, extracted, amplified, sequenced, and categorized based on its sequence (Deiner et al. 2015) From this information, detection and classification of species is possible. eDNA may come from skin, mucous, saliva, sperm, secretions, eggs, feces, urine, blood, roots, leaves, fruit, pollen, and rotting bodies of larger organisms, while microorganisms may be obtained in their entirety (Taberlet et al. 2012a, Bohmann et al. 2014, Barnes and Turner 2016). eDNA production is dependent on biomass, age and feeding activity of the organism as well as physiology, life history, and space use (Barnes and Turner 2016, Goldberg et al. 2016, Hering et al. 2018). Despite being a relatively new method of surveying, eDNA has already proven to have enormous potential in biological monitoring. Conventional methods for surveying richness and abundance are limited by taxonomic identification, may cause disturbance or destruction of habitat, and may rely on methods in which it is difficult to detect small or elusive species, thus making estimates for

entire communities impossible. eDNA can complement these methods by targeting different species, sampling greater diversity, and increasing taxonomic resolution (Deiner et al. 2017). Additionally, eDNA is capable of detecting rare species, but not of determining population quality information such as sex ratios and body conditions, so it is ideal for supplementing traditional studies (Goldberg et al. 2016, Deiner et al. 2017). Regardless, it has useful applications in detecting the first occurrences of invasive species, the continued presence of native species thought to be extinct or otherwise threatened, and other elusive species occurring in low densities that would be difficult to detect by traditional means.

Degradation of eDNA in the environment limits the scope of eDNA studies, as often only small segments of genetic material remain, particularly in warm, tropical regions. Additionally, the varying lengths of time to degradation based on environmental conditions and the potential of DNA to travel throughout media such as water can affect inference of fine-scale spatiotemporal trends of species and communities (Coissac et al. 2012, Taberlet et al. 2012a, Goldberg et al. 2016, Deiner et al. 2017, Hering et al. 2018). Despite these drawbacks, eDNA still has the potential to determine relative or rank abundance as some studies have found it to correspond with biomass, though the variation inherent in environmental samples makes it difficult to quantify (Bohmann et al. 2014, Hering et al. 2018). While eDNA has numerous applications in conservation, monitoring, and ecosystem assessment, as well as others yet to be described, the highly variable concentrations of eDNA and potential heterogeneity through the water body makes it essential that the procedure is optimized, ideally with a pilot study for each new application to ensure that the sampling design is appropriate to detect the target (Carew et al. 2013, Goldberg et al. 2016, Deiner et al. 2017).

While the definition of eDNA seems straightforward, the lines between different forms of DNA become blurred, particularly in comparison to community DNA, which is described as bulk organismal samples (Deiner et al. 2017). A question arises regarding whole microorganisms captured in eDNA samples: do these organisms alter the classification of the sample to a community DNA sample? Additionally, the classification of genetic material from feces is problematic and often referred to as eDNA (Deiner et al. 2017). Differentiation between the two is important as community DNA indicates organismal presence at a particular time and place, while eDNA may have come from a different location, from predator feces, or from past presence, however this differentiation is often impossible (Creer et al. 2016, Deiner et al. 2017). For the sake of simplicity, this paper will consider eDNA loosely classified as including many sectors of DNA biodiversity research, including fecal analysis and bulk samples when they are applicable to biodiversity research and ecosystem analysis.

Methods in eDNA research have recently expanded to be able to assess whole communities from a single sample. This process involves metabarcoding, which can be precisely defined as the use of general or universal polymerase chain reaction (PCR) primers on mixed DNA samples from any origin followed by high-throughput next-generation sequencing (NGS) to determine the species composition of the sample. This method has been common in microbiology for years, but is only just finding its footing in assessment of macroorganisms (Coissac et al. 2012, Creer et al. 2016, Deiner et al. 2017). Ecosystem-wide applications of eDNA metabarcoding have the potential to not only describe communities and biodiversity, but also to detect interactions and functional ecology over large spatial scales, though it may be limited by false readings due to contamination or other errors (Bohmann et al. 2014, Ficetola et al. 2016, Creer et al. 2016, Hering et al. 2018). Altogether, eDNA metabarcoding increases

speed, accuracy, and identification over traditional barcoding and decreases cost, but needs to be standardized and unified, integrating taxonomy and molecular methods for full ecological study (Coissac et al. 2012, Yu et al. 2012, Cristescu 2014, Gibson et al. 2015, Hering et al. 2018).

This review is intended to provide an overview of methodological and bioinformatics considerations for eDNA metabarcoding studies, as well as to summarize published eDNA metabarcoding studies to date, describing some of the numerous applications therein. Additionally, critical considerations, areas needing improvement, and future directions will be discussed.

Methods and Technical Considerations

Within published eDNA metabarcoding studies, the methodologies often differ substantially, making cross-study comparisons impossible, but the uniqueness of each study site and target taxa likely make standardization a challenging goal (Hering et al. 2018). Regardless, a coordinated global initiative for biodiversity research and evaluation via metabarcoding has the potential to standardize methods to some degree (Cristescu 2014, Hering et al. 2018). In this section, we will briefly cover the process of eDNA metabarcoding from sample collection to data analysis.

As eDNA can be applied to numerous ecosystems, collection methods must correspond with these different sample types. Microbes and pollen can be easily collected from the air, biofilms can be swabbed or scraped, water can be precipitated or filtered, and sediments can be processed (Creer et al. 2016). For water samples, large volumes of water and multiple field replicates should be used; however, the choice between precipitation and filtration (and subsequently filter size) is reliant on the target taxa, as different taxa can be isolated more

efficiently in different ways (Deiner et al. 2015, Creer et al. 2016, Goldberg et al. 2016, Hering et al. 2018). For soil and sediment samples, larger volumes of sample over larger spatial scales are required from larger size classes of organisms, and samples should be extracted from multiple locations to avoid heterogeneity of samples and to better describe the biodiversity of the area (Taberlet et al. 2012b, Creer et al. 2016). In all studies, field equipment, supplies, and personnel must be separated from areas where PCR, tissue, and organisms are processed or handled due to the high potential for contamination, and decontamination must occur between samples to maintain independence (Goldberg et al. 2016). Negative field controls are also essential to ensuring valid sampling and to identify contamination; this can include periodically filtering clean water as field blanks and processing the filters with the same protocol as the samples (Goldberg et al. 2016, Hering et al. 2018). Preservation methods are straightforward among sample types: drying, freezing at -20^{o}C, or preserving in 100% ethanol or a cell lysis buffer are all adequate methods for sample preservation (Creer et al. 2016, Goldberg et al. 2016). Immediate preservation of samples is essential to avoid degradation, which can be affected by environmental variables such as temperature, pH, and exposure to light, as well as target species (Barnes et al. 2014, Strickler et al. 2015, Tsuji et al. 2017).

Laboratory procedures tend to be more homogenous among sample types but differ between studies. eDNA should be processed in a clean lab with equipment and supplies that cannot be removed; additionally, decontamination of personnel and supplies must occur before entering the clean lab (Goldberg et al. 2016). Processing areas in the lab should be cleaned with bleach and UV periodically, and during all procedures, filter pipet tips and clean gloves should be used (Champlot et al. 2010, Goldberg et al. 2016). In addition to the field blanks discussed previously, it is important to have a lab procedure that includes positive controls, negative

controls, and replicates at all steps (Ficetola et al. 2016, Deiner et al. 2017). Kit-based extraction procedures are probably the most common, and are generally effective; however different kits may be more effective depending on the target taxa, particularly between eukaryotes and prokaryotes (Deiner et al. 2015, Creer et al. 2016). Phenol chloroform extractions are also effective, and may isolate more genetic material than other methods in certain scenarios (Creer et al. 2016, Deiner et al. 2017).

Perhaps one of the most important considerations in eDNA metabarcoding studies is PCR primer design. Different primers and regions differ in coverage, resolution, and bias between taxa. Cytochrome oxidase-I (COI) for metazoans and Ribulose bisphosphate carboxylase large chain (*rcb*L) for plants are the standards established by the barcode of life, but other regions such as 12s or 16s ribosomal RNA may be more appropriate for different taxa (Epp et al. 2012, Taberlet et al. 2012b, Gibson et al. 2015, Creer et al. 2016, Deiner et al. 2017, Hering et al. 2018). Primers for eDNA metabarcoding need to be short enough to amplify degraded samples, identical within but variable between species, and flanked by highly conserved regions to amplify a variety of species without sacrificing specificity of the target group (Epp et al. 2012). In addition to these specifications, primer choice has the potential to bias results by preferentially amplifying some target sequences more than others, as well as amplifying non-target groups (Cristescu 2014). One potential solution to this issue is the use of multiple primer sets, particularly evolutionarily independent primer sets coinciding with standardized barcodes for the target taxonomic groups (Drummond et al. 2015). Although it can reduce primer bias and increase taxonomic coverage, this method suffers from being more costly and time-consuming (Cristescu 2014, Creer et al. 2016, Alberdi et al. 2018). Another important factor in PCR and primer design is in replicates; multiple PCR replicates increase species detection and decrease

the likelihood of false negatives, but the number of replicates used often differs between studies and depends on detection probabilities, research objectives, sequencing depth, primer choice, cost constraints, and sequencing platform (Ficetola et al. 2015, Alberdi et al. 2018). It is also worth noting that the use of PCR replicates is not an adequate substitute for biological replicates; rather, both are necessary for comprehensive results (Goldberg et al. 2016). In running PCR for next generation sequencing (NGS), primers can be tagged with short nucleotide sequences to uniquely identify their source in a process commonly referred to as multiplexing. These tags, while useful, also have the potential to bias results, particularly when located on the 5' end, so they also require rigorous testing before implementation (Binladen et al. 2007, Berry et al. 2011). In the sequencing step a number of platforms are available, but Illumina sequencing currently outperforms other NGS platforms in terms of depth and accuracy (Deiner et al. 2017). It is also important to note that diversity has been found to increase with sequencing depth before reaching a plateau, so theoretically a maximum sequencing depth could be determined for individual studies (Alberdi et al. 2018).

The final technical step to be discussed is bioinformatics pathways. Unique bioinformatics pathways must be developed for eDNA metabarcoding and must be adapted for each study, previously requiring consultation with a computational specialist (Coissac et al. 2012). As technology has advanced, however, programs have been developed specifically for analysis of information from NGS data, making the method more accessible and user-friendly (Alberdi et al. 2018). NGS results in millions of reads which describe the genetic code of each strand of DNA that has been sequenced. These reads are typically grouped into operational taxonomic units (OTUs) which are not standard across sites, studies, or species, and cannot be universally applicable between them (Cristescu 2014, Hering et al. 2018). OTUs are used to

differentiate species or taxa based on similarity of genetic code as determined by sequencing, but they also need to be linked with ecological and physiological traits of species for proper identification. Various programs exist to help, but intragenetic variation hampers this process (Coissac et al. 2012, Cristescu 2014, Deiner et al. 2017). OTU clustering is based on similarity to a specific sequence and subsequent grouping under similarity cutoffs, with 97-99% similarity being a common cutoff range; however ranges for studies depend on intraspecific diversity estimated from existing reference databases, such as GenBank (Deiner et al. 2017). Species identification from OTUs is often based on similarity (BLAST), phylogeny (maxlike), character (diagnostic regions), classification algorithms, and coalescence (evaluation of quality using likelihood/Bayesian), or even species delimitation where sequences are partitioned based on differences and not similarity to a known sequence, but these should be combined with other ecological characteristics (Coissac et al. 2012, Deiner et al. 2017). Unassigned reads are another concern and their impact on analyses needs to be limited, but as so many species have yet to be described, analyses may wrongly exclude target species; poor species representation in databases may lead to incorrect identification and affect assessments of ecological quality (Deiner et al. 2017, Hering et al. 2018). Errors in false positives and negatives can be best avoided by combining metabarcoding data with direct observation and generalized occupancy models. It is recommended that occupancy models be run to evaluate detection probability, rate of false presences, and whether the level of replication is appropriate to control for false negatives (Ficetola et al. 2015, 2016, Deiner et al. 2017). Calculation of diversity indices is often used to influence diversity modelling and interpretation of results (Deiner et al. 2017). Ficetola et al. (2016) developed formulas to calculate the number of positives needed to be confident that they are not false positives:

$$\Pr(x \geq i|\text{site not occupied}) = \sum_{j=i}^{k} \binom{k}{j} \times p_{10}^{j} \times (1 - p_{10})^{k-j}$$

where p_{10} is the false positive rate, k is the number of replicated analyses on one sample, and i is the number of detections in PCR replicates from the same sample, and

$$\Pr(\text{site occupied}|x) = \frac{\psi p_{11}^{x}(1 - p_{11})^{k-x}}{\psi p_{11}^{x}(1 - p_{11})^{k-x} + (1 - \psi)p_{10}^{x}(1 - p_{10})^{k-x}}$$

where x is the number of detections, ψ is the estimated probabilities of occupancy, p_{11} is true detection, and p_{10} is false detection.

Quantitative analysis of eDNA metabarcoding data is questionable, and while there is some data that suggests a relationship between biomass and number of copies, there are many confounding variables, so multiple sampling occasions and replicates would be necessary as well as in-depth testing and optimization. Additionally, the scope of the study must be considered as general estimates may be appropriate for some studies (Goldberg et al. 2016, Deiner et al. 2017, Hering et al. 2018). There are some core programming skills required for analysis of metabarcoding data: the ability to operate from the command line, the capacity to use/manipulate/write programs in Perl or Python for analyzing large volumes of data, and familiarity with R (Creer et al. 2016). Some of the software often used for analyzing these datasets includes USEARCH, VSEARCH, CROP, swarm, UNIX, QIIME, mothur, OBITools, and phyloseq (Coissac et al. 2012, Creer et al. 2016). Most importantly, data from eDNA metabarcoding studies must be shared and accessible in its raw format with standardized metadata, ideally in cloud-based storage or common-storage databases to be used by future researchers (Creer et al. 2016, Deiner et al. 2017). Altogether, workflows are often determined by personal preference, available equipment, and cost (Deiner et al. 2015). Specificity and suggestions for bioinformatics pathways are also reviewed by Deiner et al. (2017).

It is important to note that even if all methods in an eDNA metabarcoding study are executed flawlessly with primers that bind perfectly, some error should be expected. As eDNA is composed of small fragments heterogeneously distributed in the environment, there is always a chance that these fragments will not be picked up (Pilliod et al. 2013, Goldberg et al. 2016). This supports the need for lab-based testing before entering the field and replicates across sampling areas, but also the idea that absence cannot be confirmed; even if a species is not detected, that does not mean it is not there. Additionally, a positive eDNA result can be introduced through confounding sources (Darling and Mahon 2011, Goldberg et al. 2016). Many issues with eDNA procedures may be resolved over time as the process matures, but the goal is to be focused on reducing falsehoods rather than perfect detection. Regardless, the data able to be gleaned from eDNA metabarcoding is invaluable and can be applied to a myriad of projects in a variety of ways.

Ecosystem and Biodiversity Monitoring

Advantages and disadvantages

Perhaps the most exciting and thus far the most explored avenue of eDNA metabarcoding research is the application for ecosystem and biodiversity monitoring, either for specific species groups, or potentially for entire ecosystem studies. Biodiversity is known to be declining worldwide, but it is difficult to monitor using traditional means (Thomsen and Willerslev 2015). Additionally, some traditional survey methods have relied on destructive techniques and often require extensive taxonomic expertise, making them imperfect methods of detection (Thomsen and Willerslev 2015, Holdaway et al. 2017). eDNA can remain viable for variable periods of time from weeks to hundreds of thousands of years, allowing for applications in molecular

biology, ecology, palaeontology, and environmental sciences, particularly when coupled with next-generation sequencing (Thomsen and Willerslev 2015). eDNA metabarcoding can even be automated to further streamline the procedure (Holdaway et al. 2017). Taking automation even further, supervised machine learning has been used to build predictive models for biodiversity monitoring without requiring taxonomic assignment, removing the need for human interference in development of reference databases and subsequent species assignment, an innovation particularly exciting for monitoring of extremely diverse and sensitive groups of bioindicators that would require extensive effort to fully describe (Cordier et al. 2017). eDNA has superior species detectability, requires lower effort, causes no ecosystem disturbance, allows detection without a priori knowledge of species, and can be implemented in areas where traditional surveys are impossible, making it extremely promising for the future of biodiversity monitoring (Valentini et al. 2016).

There are concerns regarding methodology with eDNA metabarcoding, as would be expected with any emerging science. Every step of the procedure has the potential to influence the results, and when these results are in turn used to influence important conservation decisions, it is essential that the procedures be optimized and standardized. Many of these concerns are discussed in the previous section, but some specifically relevant to biodiversity monitoring include: optimization of sample collection and extraction; ensuring samples are free of contamination; designing primers to have minimal bias without sacrificing resolution or spread; development of extensive reference databases; using proper bioinformatics pathways; lack of standardization in classification; and difficulty in estimating DNA degradation rates, thus allowing for the misrepresentation of species presence in areas which they may have vacated (Deiner et al. 2015, Thomsen and Willerslev 2015, Valentini et al. 2016, Holdaway et al. 2017).

There is information on populations that simply cannot be obtained via metabarcoding. Population structure and size, sex and age ratios, and individual conditions cannot be obtained by using standard eDNA metabarcoding alone and are often critical to the development of conservation plans (Valentini et al. 2016, Evans et al. 2016a, Holdaway et al. 2017). Despite this, there are ways to accurately measure biodiversity with the integration of eDNA metabarcoding. The most common solution to this lack of data is to integrate metabarcoding into the traditional monitoring framework (Holdaway et al. 2017). While eDNA metabarcoding cannot ascertain information on population structure, it can detect rare, cryptic, or elusive species from a single sample, including species that may be considered pests, diseases, or species of conservation concern (Holdaway et al. 2017). An integration of the two methods can allow for the benefits of both without being hindered by the drawbacks of using only one; as such, this integrated biodiversity monitoring approach has been recommended to be used with government monitoring projects in New Zealand, including in biosecurity and agriculture (Holdaway et al. 2017). Fully removing the need for traditional sampling in the determination of ecological networks, it may be possible to describe interactions between species and their subsequent influence on ecosystem function from metabarcoding alone. Evans et al. (2016) developed a methodology employing the use of nested tags allowing analysis of samples as individuals, enabling the construction of ecological networks from environmental samples alone. Traditional methods of describing ecological networks rely on indicator species or bipartite networks, but DNA-based networks have been found to be faster, more efficient, and more comprehensive than traditional methods, particularly as the complexity of the region increases or in poorly known ecosystems (Evans et al. 2016a). Overall, a combination of methods yields the most resolved networks, and has been suggested for use in conservation, restoration, and assessment of how

human-induced climate change affects ecosystem function (Evans et al. 2016a). Altogether, an integration of conventional and novel techniques appears to be the next step in acquiring information for land management and biodiversity monitoring. The following discusses some of the studies in which eDNA metabarcoding has already been applied for monitoring across marine, estuarine, freshwater, and terrestrial ecosystems.

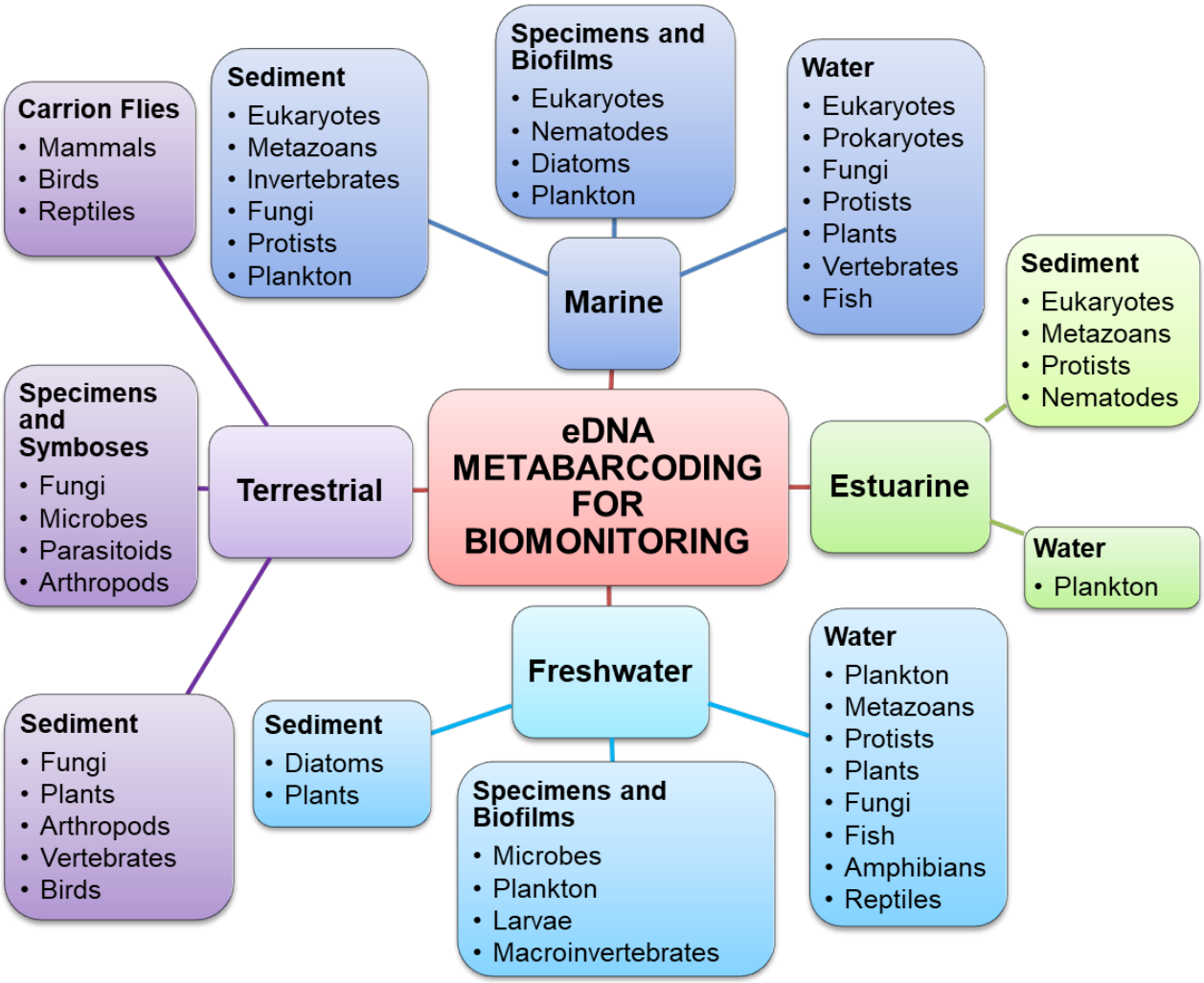

Figure 9. Schematic diagram of global ecosystem and biodiversity monitoring with environmental DNA metabarcoding.

Marine monitoring

Sediments

eDNA metabarcoding has been applied to marine environments by use of several collection procedures targeting many different groups of organisms. Marine sediments in particular are extremely species-rich, have key ecological roles and ecosystem services, and are sensitive to anthropogenic disturbance, but they have been poorly studied using traditional means and are not well understood because taxonomic work to describe the species found in marine sediments is difficult and rarely performed (Guardiola et al. 2015). eDNA metabarcoding of sediment has been used in attempts to describe marine eukaryotic communities, though it has suffered from a lack of taxonomic knowledge. Pawlowski et al. (2011) used metabarcoding on marine sediments to examine deep-sea eukaryotic richness and detected 942-1756 taxa per sample, dominated by dinoflagellates, cercozoans, ciliates, and euglenozoans; even photosynthetic taxa were present. Between 31 and 71 metazoan taxa were identified per sample despite small sample volumes, indicating free, extracellular, remnant, or resting DNA/organisms in the sample (Pawlowski et al. 2011). They determined the deep-sea floor to be a global DNA repository, describing organisms living both in the sediment as well as in the water column, making it useful for monitoring environmental changes. Interpretation of DNA data from the deep-sea floor was difficult due to lack of direct observational data, but still offers important insights into living marine organisms and the potential to study ancient marine life by analysis of deep soil cores (Pawlowski et al. 2011). A similar study by Guardiola et al. (2015) using metabarcoding on deep sea sediments identified over 1600 taxa dominated by Metazoa, Alveolata, Stramenopiles, and Rhizaria, with noticeable differences on a small scale, such as within the same core sample, between core samples, within localities, and between geographic

zones (Guardiola et al. 2015). While composition was found to change between sites, overall biodiversity was similar throughout the dataset, with some differentiation due to differences in metazoan lifestyles (Guardiola et al. 2015). Guardiola et al. (2016) also published a similar study the following year, finding over 5500 taxa dominated by Metazoa, Alveolata, Rhizaria, Nematoda, Arthropoda, and Annelida and discovering significant differences between layers of sediment, zone (slope or canyon), depth, and season. They determined that despite the use of a hypervariable region, diversity estimates were likely conservative due to the huge diversity of eukaryotes and that a lack of extensive databases limits the completeness of the study; as such, differences in taxonomic units may only be variations within taxa rather than different taxa entirely. In the same year, Sinniger et al. (2016) published a study in which they used eDNA metabarcoding of sediments to assess deep-sea benthic metazoan diversity at varying depths and locations worldwide. They found their data to be dominated by meiobenthic taxa, and while all phyla were identified, diversity within phyla remains largely unknown due to lack of studies exploring benthic metazoans and therefore a reference database that lacked specificity. Finally, Lanzen et al. (2017) tested the use of metabarcoding genomic DNA obtained from soil samples for determining diversity and community structure of meio- and microbenthic eukaryote communities, finding that sequencing depth and extraction replicates are important for optimization of diversity estimates. However, the precise number of replicates or sequencing depth will vary based on the aim of the study.

Looking at marine metazoan biodiversity, Fonseca et al. (2010) used metabarcoding to analyze eDNA from sediment in Scotland. From this study, they aimed to determine taxonomic richness based on identifiable taxa, to describe the breadth of unidentified microbial metazoans, and to assess the ecological importance of various groups. They argued that biodiversity

assessments are biased towards large organisms, thus leading to the lack of descriptions for millions of microscopic metazoans, a group estimated to comprise up to 60% of the animal phyla that perform important tasks in the marine ecosystem. Their study confirmed this immense lack of knowledge, for which they suggest further exploration of microbial metazoan phylogeny by use of metabarcoding in order to better understand this important and highly diverse group (Fonseca et al. 2010).

eDNA metabarcoding of marine sediment has also been used to identify invertebrate communities. Cowart et al. (2015) tested the reliability of metabarcoding by comparing it to morphological methods of identifying invertebrates associated with seagrass meadows. They determined that metabarcoding enabled them to identify greater diversity with similar spatial differences when compared to morphological surveys; however, 36% of the species identified with metabarcoding were not present in the database, indicating the need for optimization of the library and procedures before future studies are employed (Cowart et al. 2015). Aylagas et al. (2016) compared taxonomic inferences of benthic macroinvertebrates of known taxonomy between different DNA sources, different COI barcodes, and different amplification conditions. They determined that metabarcoding was comparable to morphologic surveys but was able to consistently identify more benthic macroinvertebrate taxa. eDNA metabarcoding of marine sediments has also been used to investigate the distribution of fungi diversity and potential factors influencing said distribution (Li et al. 2016). This study identified 816 taxa, 130 known genera, 36 orders, 14 classes, and 5 phyla, with Ascomycota making up 72-79% of all units, and found that species compositions varied across regions, with temperature determined to be the most important factor in fungal community distribution (Li et al. 2016). eDNA metabarcoding of sediments has the potential to quickly and accurately assess community structure, benthic health,

and response to changes in environment, making it a promising methodology for biodiversity monitoring. However, it currently continues to require integration or comparison with traditional methods until it is fully optimized (Cowart et al. 2015, Aylagas et al. 2016, Sinniger et al. 2016).

Bulk Specimens and Biofilms

In addition to sediment, marine eDNA metabarcoding can be applied directly to collected specimens, particularly of microscopic organisms that would otherwise require high levels of taxonomic expertise to identify. This technique has been applied to plankton biodiversity studies to determine the applicability of metabarcoding for evaluation of large scale trends (Chain et al. 2016). From bulk samples of zooplankton, Chain et al. (2016) discovered approximately 400 metazoan families in ports, including over 30 orders not previously reported. Zooplankton communities were also found to vary temporally and to be distinct throughout habitats (Chain et al. 2016). More recently, Djurhuus et al. (2018) compared metabarcoding of plankton samples against morphological identification, as well as the potential differences between direct seawater filtering, prefiltering, and plankton tissue metabarcoding. They found that different metabarcoding techniques revealed significant differences in taxonomic composition, with prefiltering detecting less than other methods. Deagle et al. (2018) also published a study comparing plankton metabarcoding to morphological identification; however, their study relied on the use of continuous plankton recorders which have been used to characterize plankton diversity for decades. They found that metabarcoding increased the number of species identified, increased the taxonomic resolution, and produced more detections overall compared to microscopy. Collectively, these results indicate that the potential use of metabarcoding in addition to continuous plankton recorders already in place has great potential, though it would

require a shift in perspective regarding biodiversity surveys to integrate molecular methods in what is traditionally a morphological field.

Metabarcoding of specimens has also been applied to eukaryotes as a general group. Leray et al. (2015) used eDNA metabarcoding of biofilms to describe the diversity and structuring of animals living on oyster reefs. They identified over 2,000 taxonomic units, but only 11% could be matched to reference databases and only 8% to the species level in part due to broad but nonspecific taxonomic coverage. Despite issues in coverage, they were able to determine that smaller organisms were more diverse and that spatial overlap between sites was minimal, illustrating the usefulness of the method for presence/absence surveys as well as relative abundances and comprehensive community-level diversity analyses (Leray and Knowlton 2015). Wangensteen et al. (2018) developed a DNA metabarcoding protocol for biodiversity characterization of marine hard-bottom communities using two molecular markers. They determined that the analyzed communities contained a large amount of undescribed eukaryotic biodiversity, and that while gaps in reference databases were common, they typically applied to "rare" taxa present on a low number of sequencing reads (Wangensteen et al. 2018). Specimen metabarcoding has also been applied to focal groups of nematodes and diatoms. Dell'Anno et al. (2015) compared eDNA metabarcoding to morphological data of nematodes and found that the analyses only matched at the family level due to poor resolution and incomplete reference databases for the metabarcoding methodology. As such, they called for further research on the subject before widespread application, but acknowledged its potential for studying the hyper-diverse nematodes in the deep-sea benthos (Dell'Anno et al. 2015). For diatoms, Rivera et al. (2018) explored the use of metabarcoding biofilms from the shells of sea turtles to infer turtle behavior. They compared metabarcoding and morphological approaches and determined that

each approach resulted in different diatom assemblages. Metabarcoding was able to distinguish between individual sea turtles based on diatom communities, but was limited by reference databases, while microscopy provided more ecological information on sea turtles, making a combination of both methods ideal for using biofilms to study sea turtle behavior (Rivera et al. 2018a). This study illustrates the widespread potential of eDNA metabarcoding of ecosystems, as it can be applied not only to ecosystem monitoring but also to movements of larger vertebrates. Altogether, metabarcoding is powerful and sensitive for large scale biodiversity studies, and as reference databases are expanded, the method will be further improved. It is a robust, fast, objective, and affordable method for characterizing complex communities of any size of organism with the potential to be further expanded with the use of multiple primer sets for optimal coverage (Chain et al. 2016, Wangensteen et al. 2018).

Water

Metabarcoding of water samples has been integral to the development of biomonitoring methodologies. Marine micro-organismal diversity was assessed by Brannock et al. (2016) in which metabarcoding was used to assess micro-eukaryotic diversity over time at various locations throughout the Alabama continental shelf. They found that community composition correlated with salinity, temperature, and dissolved silicate, and that it also differed seasonally. This information on diversity and community variance is important to the study of marine ecosystems, but without the use of advanced molecular techniques, it would be nearly impossible to gain a comprehensive understanding (Brannock et al. 2016). Bridging the gap between micro- and macro- organismal metabarcoding studies, Stat et al. (2017) compared shotgun sequencing to tree-of-life metabarcoding or "the use of multiple metabarcoding assays to survey a wide array of biotic diversity," in determining biodiversity in a tropical marine environment. They found

shotgun sequencing to be very inefficient due to the high number of reads required to obtain a clear picture of biodiversity, but the tree-of-life methodology was able to detect 287 taxa across the major eukaryotic divisions (animals, fungi, protozoa, plantae, chromista, bacteria, archaea) (Stat et al. 2017). However, multiple sets of "universal" primers were needed to obtain the full scope of biodiversity (Stat et al. 2017). In addition, they were able to isolate haplotypes from a specific fish genus from the same water samples, further expanding the biodiversity applications of eDNA metabarcoding (Stat et al. 2017).

Metabarcoding studies focusing solely on the identification of vertebrates from marine water samples are numerous. A study by Thomsen et al. (2012) investigated the potential of metabarcoding eDNA from seawater to determine marine fish biodiversity and compared the data against traditional fish surveys. They successfully obtained DNA from 15 species of fish, including species important for human use and species rarely recorded traditionally, as well as 4 bird species, determining that eDNA metabarcoding identified the same or more species than traditional sampling. Additionally, they found that DNA fragments degraded within days in seawater, indicating that positive eDNA signals indicate recent species presence. Kelly et al. (2014) tested the ability of eDNA metabarcoding to detect species of bony fish in a large mesocosm aquarium, discovering a correlation between the rank abundance of eDNA and species biomass. They were also able to detect non-native species used as food in the aquarium. Miya et al. (2015) focused on the development of a truly universal primer set for fish identification to the species level via metabarcoding, testing their primers against over 800 different species and calling their creation "MiFish". These primers were tested at an aquarium with known species, detecting 93.3% of all species present (123 genera and 59 families), and around natural coral reefs detecting 232 species (70 families and 152 genera), supporting their

effectiveness and universal application. These primers have since been used in numerous fish eDNA metabarcoding studies (Miya et al. 2015). One such study was published in 2017 by Yamamoto et al. (2017) in which the ability of eDNA metabarcoding to reveal fish community structure in coastal waters at 47 spatially distinct stations was evaluated and compared to 14 years of data from visual surveys. The metabarcoding study detected 128 species, 62.5% of which were seen by visual surveys, and 20 species that were expected to occur but had never before been documented, indicating that the method has potential to enhance marine ecosystem analyses and fish community surveys, particularly as it was determined to have a higher detection rate than other methods (Yamamoto et al. 2017).

eDNA metabarcoding of water samples has also been used to describe entire vertebrate communities. Port et al. (2016) tested eDNA metabarcoding for marine vertebrates along a transect gradient of habitats and compared the results to simultaneous visual surveys by divers, finding spatial correspondence between metabarcoding and visual surveys, and significant differences between habitats indicating local community compositions. Additionally, metabarcoding was found to be able to reliably detect cryptic species missed by visual surveys, though not all species were consistently detected with both methods, suggesting that the current applications of eDNA metabarcoding are best suited to augment traditional data for the monitoring of community dynamics over space and time (Port et al. 2016). Andruszkiewicz et al. (2017) published a study in which they used eDNA metabarcoding to characterize marine vertebrate communities at 10 locations by collecting water samples in replicates at multiple depths per station. They were able to identify 7 families, 3 subfamilies, 10 genera, and 72 species from 33 unique marine vertebrate taxa with all but one family already known to inhabit the area. They also found that communities differed based on sampling depth and between sampling

locations, indicating that eDNA metabarcoding is able to provide an accurate census of vertebrate communities and thus has enormous potential for biomonitoring at fine scale over space and time (Andruszkiewicz et al. 2017).

The effectiveness of marine eDNA metabarcoding has also been compared between sample types. Massana et al. (2015) analyzed the taxonomic diversity of both planktonic and benthic protist communities over space and time. They found different species compositions between water and sediment samples and that community composition varied between sites. They also determined that seasonality was important to consider, and that many samples are needed per site to adequately estimate diversity and community composition (Massana et al. 2015). Altogether, the use of sediment and water each yielded different results in community composition and spatiotemporal differences, further indicating the need to ensure that studies are optimized for the desired species groups. Overall, marine eDNA metabarcoding is non-invasive, efficient, cost-effective, and sensitive, with potential to complement other methods of biodiversity monitoring for ecological study and management on large spatial and temporal scales. Although it requires optimization in replicates, marker selection, avoidance of false negatives/positives, and reference database development, it has enormous potential across sample types and target taxa (Miya et al. 2015, Port et al. 2016, Andruszkiewicz et al. 2017).

Estuarine monitoring

Sediment

Estuarine biodiversity monitoring is often included with marine monitoring due to their similarities; however, estuaries are a unique ecosystem overlapping with both marine and freshwater ecosystems and as such some eDNA metabarcoding studies have focused specifically

on estuarine biodiversity. The majority of these studies have been concerned with the identification of benthic communities from sediment samples which often contain taxa such as nematodes that are commonly considered to be indicators of environmental health (Lallias et al. 2015, Avó et al. 2017). Morphology-based profiling of benthic communities has so far been low-throughput and ambiguous, and metabarcoding has been poorly applied to estuarine macrobenthos communities. Additionally, models to predict estuarine ecosystem diversity are limited for eukaryotes, which are a huge resource for assessing ecosystem health (Lallias et al. 2015, Lobo et al. 2017). As such, future studies are needed to explore estuarine sedimentary diversity via metabarcoding; however, some exploratory studies have been published. Chariton et al. (2010) applied metabarcoding to estuarine sediments to identify eukaryotes in human-impacted ecosystems, successfully obtaining sequencing data for thousands of eukaryotes from 54 phyla ranging from microscopic non-metazoans to large metazoans, with a large proportion of richness stemming from micro- and meiofauna that are often overlooked in traditional surveys. Chariton et al. (2015) also s published a study in which metabarcoding was used to assess benthic eukaryotic composition of five estuaries of varying conditions. They found that eukaryotic community composition reflected environmental condition and was strongly correlated with nutrients, turbidity, and pH, with the highest biotic richness occurring in the most disturbed environment. In the same year, Lallias et al. (2015) published a study in which metabarcoding was used to assess environmental impacts on microbial metazoan and protist diversity in two distinct estuaries with different histories of use by using longer primers focused for live individuals, they aimed to offer an immediate picture of the microbial community. They found that taxa were responding to hydrodynamics, salinity, and granulometry based on their life history, and that they exhibited estuary-specific similarity within salinity ranges. In development

and assessment of metabarcoding methodology, Lobo et al. (2017) created several test communities to assess the viability of metabarcoding, and then compared metabarcoding to morphological identification in field tests at four distinct estuaries. They found that field morphological methods underestimated species richness, identifying only 23 species as compared to the 61 species identified using metabarcoding. They concluded that metabarcoding was able to provide many high-quality species identifications, and sensitive enough to determine differences in species compositions between communities. Avó et al. (2017) also tested the ability of metabarcoding to accurately describe communities by comparing metabarcoding of sediments from an estuary that had been previously characterized using traditional methodology. Metabarcoding generated 25-26 of the traditionally identified genera and identified 34-43 species in total, including 7-16 new genera for the databases, while morphological analysis matched approximately 90% of the metabarcoding genera (Avó et al. 2017). Between the two methods, they found strong temporal differences, but not spatial differences in species composition, with metabarcoding appearing to be a promising alternative for the labor-intensive and time-consuming traditional monitoring methods (Avó et al. 2017).

Bulk Specimens

eDNA metabarcoding has also been applied to studies of the plankton community. Abad et al. (2016) evaluated the capacity of metabarcoding for estuarine plankton monitoring by comparing it with traditional microscopic taxonomic analysis. They found similar spatial and temporal trends of taxonomic diversity for zooplankton but not phytoplankton, possibly due to a lack of an extensive phytoplankton database. Additionally, there was high correspondence between abundances in the two methodologies, suggesting that there is potential for using

metabarcoding to estimate species abundances as well as invasive species detection, as two non-indigenous species were discerned (Abad et al. 2016).

Limitations of eDNA metabarcoding in estuaries are similar to those found in other ecosystems and applications, though the lack of studies assessing estuaries using metabarcoding results in a greater need for exploratory studies. Overall, eDNA metabarcoding of estuaries is fast, scalable, objective comprehensive, extremely sensitive, has potential for high taxonomic resolution, is able to identify species of any size, and is cost- and time-effective with the ability to enhance traditional monitoring techniques and inform environmental policies (Lallias et al. 2015, Chariton et al. 2015, Abad et al. 2016).

Freshwater monitoring

Biodiversity monitoring studies in freshwater using eDNA metabarcoding are quite similar to those in marine and estuarine environments, with the main areas of focus being in sediment, biofilms, bulk specimens, and water samples. Freshwater fauna are sensitive to environmental change, making them excellent indicators of ecosystem health, but traditional surveys are labor intensive, require physical capture which may injure individuals, and often result in low detection. eDNA metabarcoding has potential to improve aquatic biodiversity surveys; however further research of species characteristics and life history traits that may affect detection and refinement of quantification is needed (Evans et al. 2016b).

Sediments

Metabarcoding has been used on freshwater sediments to analyze plant communities. Alsos et al. (2018) analyzed the composition of lake vegetation and vascular plant eDNA at 11 lakes by using both metabarcoding and traditional vegetation sampling to determine the degree

to which modern taxa are found in sediment eDNA samples. They recorded 489 taxa by traditional surveys, of which 17-49% were detected using eDNA, while 47 plant taxa were identified using eDNA, of which 73% matched those recorded in vegetation surveys. They concluded that the taxa which were present in eDNA but not surveys were likely missed during the surveys or grew outside the survey area, and those missed in eDNA may have been found with increased sampling effort. Additionally, they determined that detection rates varied among plant families, and terrestrial plant DNA was dependent on sampling distance (Alsos et al. 2018). For aquatic vegetation, eDNA may be superior to traditional surveys; however, further optimization is needed before the procedure is ready for widespread application. Altogether, metabarcoding of sediments appears to be a promising method for biodiversity monitoring, though it requires standardization and expansion of databases before it is able to reach its full potential as a staple in biomonitoring methodology (Alsos et al. 2018).

Bulk Specimens and Biofilms

Similar to marine environments, specimen collection for metabarcoding is a popular choice for describing freshwater biodiversity, with many of these studies focusing on plankton. Liu et al. (2017) assessed the effect of prefiltering on detection of microeukaryotic plankton communities and found that approximately 25% of reads were for metazoans in both groups. Additionally, they found no significant differences in richness, diversity, and spatiotemporal patterns, suggesting that prefiltering does nothing to differentiate size classes of eukaryotic plankton in freshwater environments. In the same year, Wurzbacher et al. (2017) published a study in which they tested eDNA metabarcoding for total plankton community dynamics over time, including eukaryotes and prokaryotes in their analysis. They discovered changes in community composition over time, following the same patterns as detected by morphological

methods, but morphological methods missed the bloom of a rare dominant bacterium that was impacting the planktonic community. These results highlight the usefulness of microbial and macrobial community metabarcoding studies for understanding whole ecosystems and species interactions and community dynamics. Banerji et al. (2018) published a similar study in which they ran metabarcoding analysis on samples collected from multiple sites in a reservoir over 4 months, from which they detected over 1300 taxonomic units, including metazoans, protists, chlorophytes and fungi. They found that diversity estimates differed between sites and between taxonomic groups, suggesting that different groups of plankton respond to different environmental factors. Previous to these studies, Sohlberg et al. (2015) explored planktonic fungal communities in groundwater associated with deep bedrock fractures, finding fungi at all depths sampled at higher diversity than expected, dominated by Ascomycota. Fungi were found to be active in the fracture zones, indicating an ability to maintain cellular activity potently by adaptation to the environment. Some of the fungal sequences isolated may represent novel species that may play an important role in subsurface nutrient cycling (Sohlberg et al. 2015).. Yang et al. (2017) used metabarcoding to characterize zooplankton communities by comparing results to traditional morphological methods and found agreement between the two methods regarding species composition, spatial distribution, differences between water body type, and abundance based on biomass, indicating that metabarcoding is valid for use on a large scale for biomonitoring of ecosystems to influence conservation decisions. Additionally, they found metabarcoding to identify more species than morphology-based identification, though it was limited by biases commonly associated with metabarcoding studies such as technical biases, bioinformatics differences, lack of comprehensive databases, and an inability to detect life stage and health of individuals (Yang et al. 2017). Another study applying metabarcoding to

freshwater specimens was published the same year, in which a new method for analysis of larval fish communities using metabarcoding of bulk ichthyoplankton samples was developed and compared to individual larval sequencing (Maggia et al. 2017). They found that both methods were comparable in results, indicating the potential of metabarcoding to inform understanding of fish recruitment and river preservation, particularly in tropical regions where fish larval ecology is poorly studied due to difficulty in identification (Maggia et al. 2017). Metabarcoding of plankton communities has the potential to provide better taxonomic coverage and resolution than morphological methods, and comparison to conventional data revealed similar abundance and diversity data. However, each method included groups not seen in the other, suggesting the use of the two methods simultaneously is the best way to achieve optimum coverage and the most accurate estimates of diversity (Banerji et al. 2018)

In addition to plankton, metabarcoding of collected freshwater specimens has been applied to bulk macroinvertebrate samples to describe biodiversity. Carew et al. (2013) tested whether metabarcoding could be used to identify species of Chironomidae from field specimens, an important indicator group of aquatic ecosystem health. They found that with one gene region (COI) metabarcoding could identify 96% of species, but when using two (COI and CytB), 99% of species could be identified. They also found a strong relationship between number of sequences and number of individuals, indicating the possibility of estimating abundance using metabarcoding. Metabarcoding has been compared to morphological macroinvertebrate metrics across a gradient linked to watershed features where the two study types were found to consistently indicate the same differences within the stream gradient, again suggesting the usefulness of macroinvertebrate DNA metabarcoding for large-scale biomonitoring (Emilson et al. 2017).

A unique study published by Hajibabaei et al. in 2012 investigated the use of preservative ethanol as a nondestructive source for DNA from macroinvertebrates to determine biodiversity and compared this data to both the morphological identification of species in the ethanol as well as metabarcoding of all the specimens homogenized together, as is typically done with specimen metabarcoding studies. They found that ethanol-based metabarcoding identified 87% of taxa while tissue metabarcoding identified 89%, with the missing taxa for both being those of lowest abundances in the mixture. This methodology presents a solution to the need for both sample and voucher specimens by not destroying samples in order to obtain data, thus taking advantage of existing preservation standards to run molecular biodiversity surveys. It has the potential to be applied to target pests, pathogens, vectors, or other organisms associated with those that have been preserved in ethanol in addition to the larger macroinvertebrates (Hajibabaei et al. 2012). Altogether, metabarcoding of specimens has great potential in the realm of freshwater biodiversity monitoring as it has been found to reduce the cost and time of biomonitoring, though as with all metabarcoding studies thus far, it requires refinement and further development of reference databases (Maggia et al. 2017, Yang et al. 2017, Emilson et al. 2017).

The testing of biofilms for diatom diversity has been applied several times in freshwater diversity; diatoms are often a focus of freshwater monitoring due to their status as bioindicators (Kermarrec et al. 2014, Rivera et al. 2018b). Metabarcoding of biofilm diatoms has been compared to morphological surveys several times with similar results throughout. In a mock community, Kermarrec et al. (2014) found that species richness was always higher with microscopy but noted that some species detections from microscopy may have been from dead cells, which cannot always be differentiated from living organisms morphologically. Several studies have used natural freshwater communities that often function as indicators of water

quality to assess the effectiveness of metabarcoding. Zimmerman et al. (2015) found that metabarcoding consistently identified more taxa and more knowledge on taxonomic diversity than morphological methods, while Vasselon et al. (2017) determined that while the community structures of the two methods were highly correlated and the ecological quality estimates were similar, only 13% of species were shared by both approaches, likely due to incompleteness of databases, limits in taxonomic knowledge, and biased abundance estimates. Similarly, Rivera et al. (2018) found similar assemblages with both approaches structured by the same environmental factors; but the ecological assessment for each approach differed, perhaps due to the lack of a comprehensive DNA reference library, an issue that will likely resolve itself over time. Despite this limitation, the authors suggest an integrated approach of taxonomy, autecology, life forms, cell sizes, and ecological guilds from morphology, molecular data, and ecological aspects for determination of freshwater diatom biodiversity; metabarcoding offers the ability to obtain diversity data that can then be applied on different spatial, temporal, and taxonomic levels for large scale biomonitoring and management, including functional and ecological community relations and water quality assessment (Kermarrec et al. 2014, Zimmermann et al. 2015, Rivera et al. 2018b). In short, metabarcoding has great potential for the augmentation of traditional diatom surveys rather than the complete replacement.

Freshwater biofilms have also been used for the metabarcoding of microbes in general. A study by Besemer et al. (2013) used eDNA metabarcoding to test fluvial biofilms to determine the distribution of diversity of ecologically critical benthic microbes. They were able to determine from metabarcoding data alone that microbial diversity decreased downstream, particularly at confluences, suggesting that environmental and biotic factors may influence biofilm connectivity and diversity. Additionally, they found a high degree of variability in

species composition between streams not explained by geographic distance, suggesting that fluvial networks constrain microbial distribution (Besemer et al. 2013). Patterns of diversity discovered in this metabarcoding study indicate the importance of headwater communities for regional diversity and conservation as well as management of ecosystem function (Besemer et al. 2013).

Water

Water sampling is perhaps the most common method of collecting eDNA for metabarcoding in freshwater environments, largely dominated by studies focusing on fish and other aquatic vertebrates. One of the earliest studies in freshwater vertebrate metabarcoding compared eDNA metabarcoding to electrofishing to characterize stream fish communities (Olds et al. 2016). They found electrofishing to yield 12 species and an estimated richness of 16 species, while eDNA sampling detected 16 species, including 3 species that had never been detected in the water body using electrofishing (Olds et al. 2016). They determined that eDNA metabarcoding has the potential to enhance fish surveys without disturbing fish or habitat in order to better inform management decisions despite being unable to provide specific data on population quality (Olds et al. 2016). The same year, Civade et al. (2016) used eDNA metabarcoding to assess fish biodiversity in a freshwater ecosystem and investigated signal variability by testing organization of fish communities along a longitudinal gradient. They found eDNA metabarcoding to be more efficient and more likely to detect species presence than traditional sampling, and that fish species identified with metabarcoding were comparable to those identified by traditional sampling, thus giving an in-depth description of local fish biodiversity along a longitudinal system (Civade et al. 2016). Hanfling et al. (2016) compared eDNA metabarcoding of a lake fish community to long-term traditional data in an attempt to

define the qualitative and quantitative accuracy of eDNA metabarcoding. They found that eDNA detected 14 of the 16 known species in the lake from one sampling session, while the most recent traditional sampling effort had detected only 4, making eDNA much more sensitive; the two species missed by eDNA were unlikely to be in the lake at the time of sampling. They also found that the number of sequence reads corresponded with both site occupancy and abundance estimated from previous surveys, indicating that eDNA metabarcoding could potentially be used quantitatively for describing fish communities (Hänfling et al. 2016). Shaw et al. (2016) also compared eDNA metabarcoding to traditional methods, focusing on the comparison with netting surveys. They found that water samples detected 100% of netted species (including species of conservation concern, rare, and invasive species), and that five 1 L water samples per site were more effective for characterizing fish communities than smaller water samples or sediment samples. The following year, Evans et al. (2017) tested eDNA metabarcoding estimates of species richness by comparing them to traditional methods to define how spatial distribution and bioinformatics affect efficiency. They discovered that eDNA metabarcoding could detect all species found with traditional sampling plus 11 more when considering all markers separately. Despite this success with a variety of markers, they determined that when species needed to be detected by all markers, only 7 known, traditionally captured species were found. Additionally, they determined that eDNA was relatively evenly distributed throughout the reservoir but predicted that this would not be the case with moving water bodies such as streams and rivers. Nakagawa et al. (2018) assessed the performance of eDNA metabarcoding in a moving water body, focusing on stream fish species and comparing metabarcoding data against existing observational data; additionally, they tested for detection bias between upstream and downstream sampling locations. They found upstream eDNA data to be most similar to observational data,

detecting 86.4% of reported species as well as two additional species, and that eDNA results were consistent with turnover and assemblage patterns of some species (Nakagawa et al. 2018). Sato et al. (2017) explored the differences in freshwater fish biodiversity assessments resulting from pooling water for eDNA metabarcoding. They found more species in the individual samples, including moderate spatial correlation, indicating that the pooling strategy is not suitable for assessing richness, but may be useful for comparing fish communities within sites. Additionally, they found that increased replicates lead to increased sensitivity with water volumes and adequate distance between sampling points being necessary, and thus suggested small volumes from many locations for maximum detectability (Sato et al. 2017).

Expanding the scope from fish alone, Evans et al. (2015) created aquatic mesocosms to test the ability of eDNA metabarcoding to detect both fish and amphibian species in different densities and relative abundances. They found that metabarcoding was able to detect all species in all mesocosms, and were able to identify a small positive relationship between species abundance and read abundance, illustrating the potential of eDNA metabarcoding use as an index of species abundance and quantification of diversity. Valentini et al. (2016) took this a step further by designing fish and amphibian primer sets and testing them in both lab and field conditions for applicability in ecological studies and aquatic biodiversity monitoring. They tested their primer designs against 53 amphibian species, 64 freshwater fish, and 22 marine fish, initially tested with individual tissue to construct a reference database, next with pooled tissue, then with spiked water, and finally with field sampling in areas with known species compositions. Following these tests, occupancy data was compared to historical and traditional survey data, finding that the use of eDNA metabarcoding with these primers could be an applicable methodology for freshwater vertebrate biodiversity studies (Valentini et al. 2016).

Lim et al. (2016) also assessed species diversity across taxa, using universal metazoan primers on water samples from two reservoirs with known species diversity. They found signatures for over 500 species, of which approximately 100 could be identified to genus or species level, including an invasive snail and a rare fish.

Studies focusing only on amphibians have also been published. A study by Lopes et al. (2016) tested eDNA metabarcoding for anurans in tropical streams by comparing metabarcoding data to that from traditional visual and audio surveys. They successfully detected all species known to inhabit the streams with one eDNA visit, with a greater number of reads coming from more common species that are more closely tied to the streams. They also found that detection was maximized by filtering large quantities of water and sampling multiple sites along streams, but quantification was not found to be possible. eDNA detection was higher per sampling event than traditional field surveys, was able to detect various life stages that may be missed by traditional surveys, could be applied outside of the species reproductive peak, is noninvasive, and could be used on a wide taxonomic scale and large spatial scale in relatively short time periods, making it useful for anuran surveys in tropical environments (Lopes et al. 2017). Lacoursiere-Roussel et al. (2016) also focused on herpetofauna and found metabarcoding to be effective in assessing species distributions. Most recently, Harper et al. (2018) compared the sensitivity of eDNA metabarcoding to eDNA quantitative PCR (qPCR) detection for great crested newts. They found qPCR to provide greater detection for this species, but when thresholds were removed from metabarcoding, it performed as well as qPCR suggesting that a good potential methodology would be initial community surveys using metabarcoding followed by species-specific surveys using qPCR for species of concern as indicated by the metabarcoding studies (Harper et al. 2018). eDNA metabarcoding appears to be an extremely promising avenue in freshwater

biodiversity studies with the capability to collect monumental amounts of data in short periods of time without disrupting ecosystems (Shaw et al. 2016, Nakagawa et al. 2018). Although it has the expected downsides discussed in previous sections, as technology advances, metabarcoding can be expected to decrease in cost and increase in effectiveness. As in other ecosystems, it is probably best suited as a complementary method to traditional surveys (Valentini et al. 2016, Hänfling et al. 2016).

Terrestrial monitoring

Terrestrial eDNA metabarcoding for biodiversity is not an intuitive application of the methodology, but there are several important sources of terrestrial eDNA that can be traced, such as soil, saltlicks, water sources, and bulk specimens. It seems improbable that soil samples alone would allow reliable detection of non-soil dwelling species. Indeed, the majority of terrestrial eDNA metabarcoding studies focus on soil samples for invertebrates in frequent, direct contact with the soil, but other associations have been detected and studied (Calvignac-Spencer et al. 2013, Schubert et al. 2015, Lee et al. 2016). These creative workarounds are unique and certainly seem impossible without the advent of metabarcoding. Rapid survey approaches for monitoring biodiversity could help to assess and understand threats to ecosystems worldwide, and metabarcoding provides new opportunities for large-scale biodiversity studies as well as being an efficient and economical approach to studying ecosystem structure (Yoccoz et al. 2012).

Soil

Terrestrial metabarcoding began with soil sampling, with a study by Buée et al. in 2009 in which universal fungal primers were used on six distinct forest soil samples, discovering over 1000 separate taxonomic units, primarily of Ascomycota and Basidiomycota, with

Agaricomycetes as the dominant taxa. From this data, diversity analyses suggested strong spatial heterogeneity and unexpectedly high richness. Additionally, they found that analyzed sequences corresponded 73% of the time with only 26 identified taxa, thus highlighting the urgent need for more extensive sequence databases. Soil fungi also play an important ecological role but little is known about the structure, richness, and distribution of different fungal communities and ecological groups, making metabarcoding an exciting prospect for soil fungal studies in the future (Buée et al. 2009).

Other studies have focused on determining plant diversity from soil sample metabarcoding. Plant community composition can help to define the ecological quality of sites, but aboveground visual surveys are not all-encompassing; eDNA has the potential to identify active and dormant seeds, pollen, and detritus of species simultaneous with metabarcoding, thus providing a more comprehensive view of plant diversity (Fahner et al. 2016). Yoccoz et al. (2012) used metabarcoding of a short DNA fragment using plant-specific primers to estimate taxonomic diversity on 8 plant communities from two vegetation types and assessed DNA persistence by metabarcoding formerly cultivated soils. They found that plant diversity from metabarcoding was consistent with estimates from conventional surveys but revealed taxa not detectable through traditional surveys. In addition, soil samples were spatially distinct in species composition and diversity, with crop DNA sequence detection from the formerly cultivated fields dependent on the amount of time since cultivation, dropping off almost completely after approximately 50 years (Yoccoz et al. 2012). Fahner et al. (2016) also assessed soil metabarcoding for measuring plant diversity, evaluating sequence recovery, annotation, and resolution for 4 plant DNA markers first with database sequences, then with soil samples from a remote wetland. They determined that internal transcribed spacer (ITS)-2 and ribulose

bisphosphate carboxylase (rbc)-L were the most effective regions for metabarcoding vascular plants for generalized databases, and that full-length DNA barcode regions could outperform the shorter sequences in taxonomic resolution and detection. This finding is especially noteworthy as Yoccoz et al. (2012) noted the limitations of the short barcoding region in taxonomic resolution (Fahner et al. 2016).

Like in aquatic biodiversity studies, metabarcoding of soil and sediment has been applied to the characterization of eukaryotic communities. Drummond et al. (2015) compared a broad suite of markers against traditional biodiversity monitoring tools for terrestrial eukaryotes, examining the 16S, 18S, trnL, ITS, and COI regions to see if eDNA could be a proxy for traditional biodiversity sampling, which combinations of markers are best, and how sensitive the measures are to markers/parameters. They found that alpha and gamma diversities were sensitive to number of reads, while beta diversity was stable after a couple thousand reads. Additionally, they determined that the COI region had the strongest correlation with traditional sampling data, especially for vegetation and invertebrates, but that sampling efforts required at least two markers to cover all phyla (Drummond et al. 2015). Invertebrate-species metabarcoding has also been explored. Yang et al. (2014) compared the results of soil and leaf litter metabarcoding to traditional surveys for arthropods and found that the two methodologies yielded different species compositions. However, they described similar ecological information in regards to relative richness and diversity, with metabarcoding being perhaps more sensitive to habitat differences than traditional surveys (Yang et al. 2014). Soil metabarcoding can also be applied to the assessment of eukaryotic communities in response to environmental changes. Štursová et al. (2014) used metabarcoding to analyze the response of fungal soil and leaf litter communities during a bark beetle-induced tree dieback, finding that the biomass of the fungal communities

decreased, and that community composition changed with a loss of root-associated fungi and an increase in saprotrophic species (Štursová et al. 2014)

Andersen et al. (2012) used metabarcoding to attempt to characterize vertebrate taxonomic richness and structural diversity by use of soil samples from areas with known species compositions such as zoological gardens, safari parks, and farms. They found that DNA from the soil reflects overall taxonomic richness and relative biomass but may not be sensitive to recent changes in communities. Moreover, they found no impact of soil pH on DNA amplification, but that high organic content did inhibit amplification, and that DNA was capable of traveling through strata depending on soil texture and structure (Andersen et al. 2012). DNA was not uniformly distributed throughout the area, with higher concentrations found around latrine or den areas, so they concluded that spatial replicates are necessary to optimize detection. Despite this, they determined metabarcoding to be a quick and reliable alternative to traditional surveys (Andersen et al. 2012). Perhaps the most comprehensive study on terrestrial biodiversity from soil samples was published by Epp et al. (2012) in which they developed metabarcodes for fungi, bryophytes, enchytraeids, beetles and birds, and evaluated *in silico* PCR and *in vitro* PCR of surface soil and frozen sediment. They found that *in silico* primer design was effective in creating working primers for the field and that the most promising universal metabarcoding regions were located on 12s and 16s which resulted in higher coverage than other regions. They also found that there was high variation between the modern and ancient species compositions (Epp et al. 2012).

Root Associations

In a similar environment to soil samples, sampling of communities associated with roots has been used for metabarcoding biodiversity studies. Blaalid et al. (2012) investigated changes

in fungal communities associated with the root systems of *Bistorta vivipara,* a perennial herbaceous flowering plant, along a primary succession gradient using metabarcoding to detect variation in fungal richness and community composition as the ecosystems change. They identified a total of 470 taxonomic units found to correspond with between 8 and 41 fungal sequences dominated by basidiomycetes. Additionally, total taxa increased as the ecosystem developed, but average per root system did not change, though there was a high degree of patchiness in fungal distribution across root systems (Blaalid et al. 2012). Martinez-Garcia et al. (2015) also looked at root-associated fungal communities by using metabarcoding. They looked at a long-term soil sequence that included ecosystem progression and retrogression to determine the importance of host plant identity as a factor driving fungal community composition. They also combined metabarcoding methods (for determining community per site) with terminal fragment length polymorphism (T-RFLP) methods (for determining community within sites) to acquire a full view of community composition, diversity, and change over time. They found fungal communities to be highly structured by ecosystem age with strong niche differentiation. They also found that diversity decreased as the ecosystem aged, and 39% of fungal variance could be attributed to the host plant, suggesting that fungal communities are tightly linked to plant communities (Martínez-García et al. 2015).

Bulk Specimens

Bulk specimen metabarcoding has also been explored in terrestrial environments. Conventional ecosystem assessments based on morphology or barcodes are costly and time-consuming and thus less than ideal for large numbers of samples, hence the future applications of metabarcoding (Gibson et al. 2014). Yu et al. (2012) presented protocols for determining taxonomy of bulk arthropod samples via metabarcoding by constructing seven communities of

arthropods and comparing community composition and diversity between the two datasets. They found that it is possible to precisely estimate community differences and diversities by metabarcoding despite the loss of some taxonomic information, and that metabarcoding of bulk samples is effective for measuring biodiversity. In the following year, Ji et al. (2013) validated metabarcoding of bulk arthropod samples by testing it against 3 high quality standard datasets from Malaysia, China and the United Kingdom. For all locations, metabarcoding returned correlated estimates of species richness, identified treatments and responses, and returned similar statistical models in the same taxon. Gibson et al. (2014) published a similar study in which they used multiple universal primers on bulk arthropod samples to test the ability of metabarcoding to describe diversity. They found that metabarcoding was able to identify 91% of the arthropods as well as detect microbes associated with the arthropods. Additionally, they determined that metabarcoding was more effective than other methods and greatly reduced the time and cost of biodiversity studies, making it ideal for a variety of further applications in ecology, including interactions between the macro- and micro-biome (Gibson et al. 2014).

Metabarcoding of bulk specimens may also have applications in the assessment of host-parasitoid and community interactions within biodiversity studies, an area of study essential to biodiversity monitoring, but difficult to discern using traditional methods. Šigut et al. (2014) used mock samples of insect larvae and parasitoids to test the potential of metabarcoding for identification and assessed the completeness of the barcode database by comparing it to a known host-parasitoid database for the study area. In this study, metabarcoding successfully identified 92.8% of all taxa present in mock samples and was able to identify taxa consistently across all taxonomic levels. Additionally, they found that 39.4% parasitoid and 90.7% host taxa were able to be identified based on the reference database, indicating a definite need for expansion of the

parasitoid database. Metabarcoding data in this study suggests higher parasitoid diversity than traditional surveys, identifying the potential of metabarcoding to achieve more accurate identifications from reliable libraries and to better characterize diversity (Šigut et al. 2017). Miller et al. (2016) established a protocol for metabarcoding fungal communities directly from the DNA extracted from homogenized beetles and found that metabarcoding produced viable information on fungal species abundance associated with beetles particularly when large numbers of PCR replicates are used.

A unique application of metabarcoding bulk arthropod samples lies in the use of carrion fly samples to estimate vertebrate diversity. Carrion flies feed ubiquitously on dead and decaying animals and have not been found to have any distinct host preferences. This lack of preference makes them an excellent potential model for determining vertebrate diversity, assuming death rates between species are relatively constant (Calvignac-Spencer et al. 2013). Applications of this method may even be able to be applied to monitoring efforts of mortality dynamics or identification and monitoring of individual populations (Calvignac-Spencer et al. 2013). One of the earliest studies employing this creative methodology for monitoring terrestrial biodiversity was by Calvignac-Spencer et al. (2013) in which they used metabarcoding on 201 pooled carrion flies collected in Cote d'Ivoire and Madagascar, retrieving DNA from 22 and 4 species respectively, from different forest strata and different size ranges, including very rare species. Though a full spectrum of mammalian diversity was not established in this study, they estimated further sampling effort could achieve a more complete estimation of biodiversity. In addition, amphibians and birds were detected, suggesting further applications in biodiversity monitoring (Calvignac-Spencer et al. 2013). Schubert et al. (2015) screened carrion flies with nonspecific and specific primers to determine the differences in detection rates between the two primers.

They found that while specific primers increased the detection of target species, it was still possible to detect these species using general primers. They also determined that the DNA collected from carrion flies was enough to attempt genotyping individuals in addition to the intended use to estimate population abundance and assess overall mammalian diversity (Schubert et al. 2015). Lee et al. (2016) attempted to calibrate the use of metabarcoding of carrion flies by comparing the metabarcoding-derived biodiversity estimates against traditional methods including cage trapping, mist netting, hair trapping, and scat collection in two forests, one with known megafauna and one without. They found metabarcoding to consistently detect more species than other methods, and predicted that as study times progressed, it would continue to outperform traditional methods (Lee et al. 2016). Finally, Rodgers et al. (2017) conducted vertebrate-specific metabarcoding on carrion flies in an area with a well-known mammal community to test the efficiency of the methodology for vertebrate detection and monitoring by comparing metabarcoding results against diurnal transect counts and camera trapping. All surveys were conducted over the same time period (including fly collection), and metabarcoding was found to detect more species than other methods over the same time period, but less than those documented over the past 7 years of surveys (Rodgers et al. 2017). Their data suggests that metabarcoding is powerful and efficient for biodiversity studies, particularly when used in conjunction with current monitoring methods. However, more complete reference databases, PCR optimization, multiple markers, greater sampling effort, and greater sequencing depth are needed, sentiments shared by other studies (Lee et al. 2016, Rodgers et al. 2017). This unique application of metabarcoding may be a rapid, unbiased, and cost- and time-efficient wildlife monitoring methodology with capabilities to detect species and individuals, and the potential to join the standard mammal monitoring field methods. Moreover, further investigation is also

needed into carrion fly ecology before it can be fully integrated into terrestrial biodiversity monitoring efforts.

Water

Though they do not reside in the water, terrestrial animals still require water and other nutrient sources to survive. As such, eDNA can be collected from the sources and analyzed to determine species use. Assessing species presence using eDNA in forest habitats, which are often difficult to survey, particularly when containing elusive or rare species, could be a viable alternative or augmentation to traditional study methods. A study in 2017 used metabarcoding to characterize the mammalian community utilizing forest ponds in Japan, from which they were able to detect several common mammals from the area. Not all common mammals were detected, however, as pond use is dependent on individual need and likely varies greatly over space and time (Ushio et al. 2017). A similar study using universal bird primers has also been published, however variation in species and a lack of reference sequences reduced detection (Ushio et al. 2018). Another terrestrial location that may be visited more frequently is saltlicks or other nutrient centers. Environmental DNA metabarcoding has been applied to saltlicks in Borneo for detection of mammals; in this study, several endangered species were detected, but many smaller species were missed due to lack of reference sequences (Ishige et al. 2017). Regardless, this is an area of research that shows great promise with the improvement of techniques and reference databases.

Further Applications

While the prospects for ecosystem and biodiversity monitoring using eDNA metabarcoding are thrilling and in need of further research, it is not the only area in which the method can be

applied. Metabarcoding can be used to reconstruct ancient ecosystems from DNA found throughout the world, to explore the interconnectness between plants and pollinators, to assess diet without need for feeding observation or stomach flushing, to detect invasive species before they could possibly be detected via traditional means, to determine community responses to pollution, and even to assess air quality and its implications for human health. Though these are the topics covered in this review paper, more applications of eDNA metabarcoding are surely yet to come.

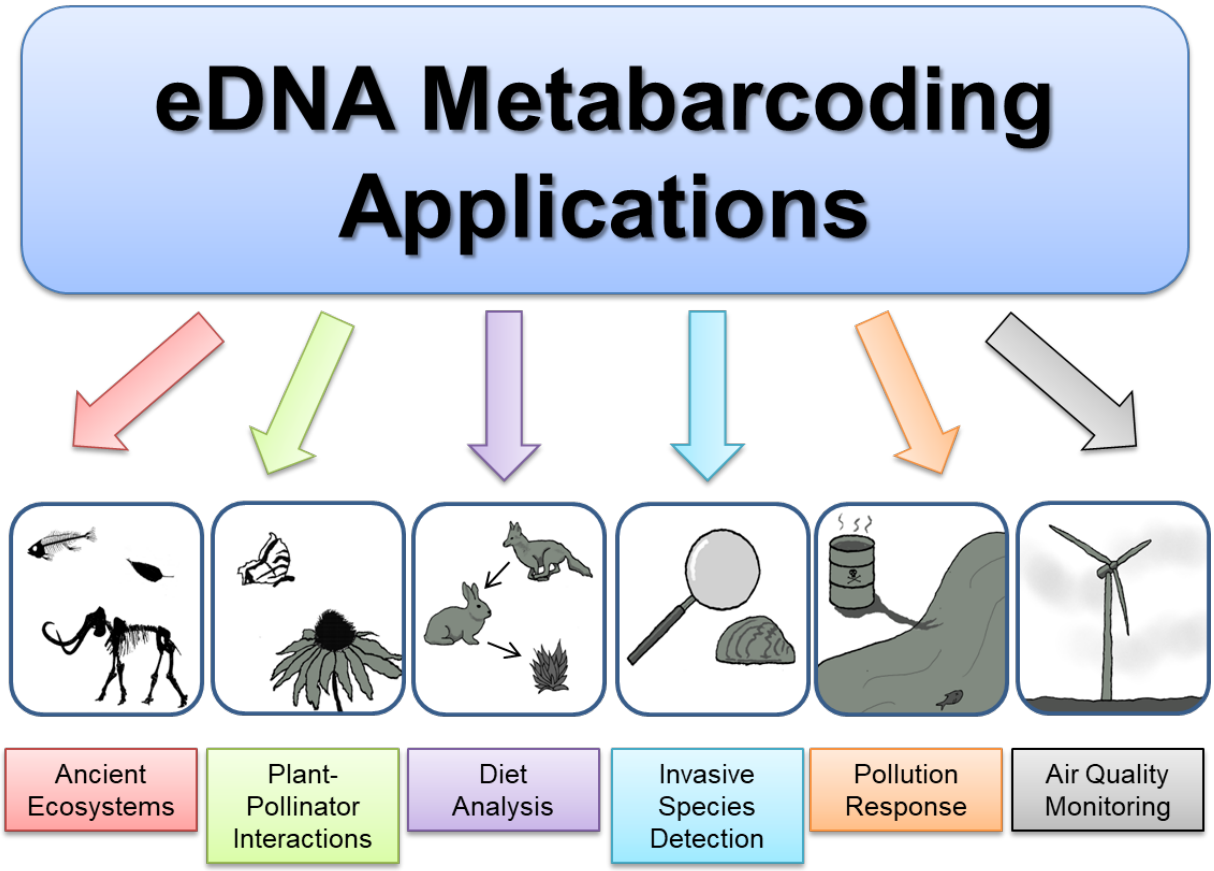

Figure 10. Applications of environmental DNA metabarcoding in aquatic and terrestrial ecosystems.

Paleology and ancient ecosystems

Environmental metabarcoding is not only applicable for monitoring the health and status of modern ecosystems but has also been used to reconstruct ancient ecosystems from carbon-dated deep sediment samples that have been carbon dated (Pedersen et al. 2013). As traditional methods of ancient ecosystem reconstruction are based on pollen and microfossil identification, they are often time-consuming and prone to human error; metabarcoding studies when used in conjunction with traditional data may be able to give a more complete view of ancient ecosystems (Sønstebø et al. 2010, Jørgensen et al. 2012). These reconstructed ecosystems can in turn be used for future conservation planning, including backwards testing of climate change models, invasive species emergence tracking, and assessment of anthropogenic influences to past biodiversity and landscape (Sønstebø et al. 2010, Jørgensen et al. 2012, Bellemain et al. 2013, Giguet-Covex et al. 2014).

One of the first studies using both metabarcoding and next-generation sequencing on ancient DNA was published in 2010, wherein Søstenbø et al. (2010) constructed a library of 842 widespread/important arctic flora species and identified chloroplast region P6 as a successful metabarcoding region for these plants. They found that using metabarcoding primers with NGS resulted in greater taxonomic resolution than traditional methods. However, using both methods together was the most successful in identifying the greatest number of taxa to the smallest resolution (Sønstebø et al. 2010). Also studying arctic plant composition, Jørgensen et al. (2012) compared sedimentary DNA retrieved from nunataks to data from detailed surveys of the past 130 years. Based on traditional surveys, biodiversity had drastically decreased since 1967 from 60 species to just eight in 2009. They determined that metabarcoding plant DNA was more successful than traditional surveys, as it was able to identify twice as many species as the 2009

survey, including 3 taxa that had never been documented on the nunatak (Jørgensen et al. 2012). As there is no animal life on these nunataks, changes in biodiversity can be assumed to directly correlate with climate, making data from this location especially valuable for climate change research (Jørgensen et al. 2012). Pederson et al. (2013) compared metabarcoding of sediment from a lake in Greenland to a previously determined biodiversity record to identify ancient plant biodiversity. Sedimentary DNA was able to identify two previously undiscovered families at the study site; however, others were missed due to the incomplete nature of genetic libraries. As such, they determined that DNA metabarcoding has the potential to reconstruct local vegetation in greater detail than traditional means, but that further work is needed on resources such as GenBank to ensure that no species are missed during the process. Despite increased rates of degradation due to climatic differences, even tropical regions can yield useful metabarcoding data from sediment. Boessenkool et al. (2014) were able to identify species from tropical sedimentary DNA to a greater taxonomic resolution than was found with pollen sampling, but more species were identified with pollen. This discrepancy could be due to DNA degradation, or it could be a product of the transient nature of pollen; where sedimentary DNA is relatively local, pollen is able to spread before landing in sediment, and thus may not give an accurate representation of species composition in a specific location (Boessenkool et al. 2014).

To achieve a more complete look at ancient ecosystems, Willerslev et al. (2014) studied not only plant diversity from sediment DNA, but also nematode diversity as a proxy for vegetation cover and the diets of ancient herbaceous megafauna as a method of confirming plant species presence and ecosystem-level interactions. By combining all results of ancient eDNA sequencing, the study was able to determine the circumpolar vegetation cover and the changes therein over time. Additionally, metabarcoding of stomach contents of ancient megafauna

reveals information regarding habitat choice, behavior, and biology of species and their influence on the plant communities (Willerslev et al. 2014). Wood et al. (2012) also used metabarcoding to analyze the diet of extinct herbivores, focusing on the moa of New Zealand. They were able to identify at least 67 plant taxa, revealing a generalist feeding ecology across available habitats. Additionally, intact seeds indicate the potential importance of moa for plant dispersal (Wood et al. 2012). Microorganisms such as fungi also play an important role in ancient ecosystems and can even be used to determine plant and animal diversity based on associated fungal presence (Bellemain et al. 2013). Metabarcoding of permafrost has been successfully used to identify fungal communities with almost double the number of taxonomic units identified than with Sanger sequencing (Bellemain et al. 2013). While this is undoubtedly useful, differences in stability during cryopreservation and bias in PCR amplification and sequencing make it impossible to fully describe ancient fungal communities, though overall changes in diversity over time may be observed (Bellemain et al. 2013). Ancient DNA metabarcoding can also be used to determine anthropogenic effects on the landscape, as explored by Giguet-Covex et al. (2014). Lake sediments were used to reconstruct plant cover and livestock farming history to determine human causes of erosive events as compared to naturally predicted erosion. High amounts of cattle and sheep DNA in the sediment indicated intense grazing and a disappearance of tree species from deforestation would reduce soil stability and contribute to erosion (Giguet-Covex et al. 2014). Pansu et al. (2015) also assessed ancient human impacts on plant communities by metabarcoding lake sediments; they were able to identify anthropogenic changes to vegetation communities indicating the establishment of an agropastoral landscape.

The majority of ancient metabarcoding studies have used sediment to determine biodiversity, but bone fragments can be sequenced to determine their species of origin (Haouchar

et al. 2014, Grealy et al. 2015). Bone fragments that are unidentifiable based on morphology can be combined and sequenced together to determine the species at a certain site where bones were found. In particular, bulk bone metabarcoding "has applications for the detection of cryptic or morphologically undiscoverable taxa, historical faunal turnover and population fragmentation" which may provide a powerful tool for species conservation as well as "identifying taxonomic assemblages, estimating genetic diversity, and assessing general ancient DNA preservation" (Grealy et al. 2015). Bulk bone metabarcoding can be combined with sediment analysis as indicated by Haouchar et al. (2014). They used a variety of general and taxa-specific primers on bone and sediment samples to assess the genetic biodiversity of a cave in Australia, from which at least 15 plant and animal taxa were identified, providing valuable insight to the ancient biodiversity of the area.

The possible applications of ancient DNA metabarcoding are staggering, but it is important to acknowledge potential flaws and limitations of current technology. Limitations of genetic libraries, differing rates of degradation between species and geographic location, and PCR bias are all major concerns (Bellemain et al. 2013, Pedersen et al. 2013). Another major concern is contamination; the presence of a modern species replicated by the primers can not only give false positives, but also false negatives by overwhelming the concentration of taxa to where low-concentration DNA is not able to replicate (Boessenkool et al. 2012). One of the contaminants most difficult to avoid is human DNA, which can cause problems when using mammal primers (Boessenkool et al. 2012). To combat this, Boessenkool et al. (2014) developed human-blocking primers to reduce the amplification of human DNA and found that species diversity increased, and new taxa were evident when the human DNA was suppressed. Other concerns include climatic/temporal limitations, biological origin/state, stratigraphic reliability,

and sterile sampling procedure (Rawlence et al. 2014). In particular, ancient DNA is limited by the climate of the region; most studies so far have employed the technique in arctic or temperate climates; tropical regions are typically both warmer and more humid, which increases the rate of DNA degradation over time. As such, microclimates such as caves may be essential to the application of ancient eDNA metabarcoding in more tropical regions (Gutiérrez-García et al. 2014)

Plant-pollinator interactions

Environmental DNA metabarcoding can be used to characterize interactions between organisms and the environment. One of the most promising areas where this is being applied is in analyzing pollinator interactions with plants. Pollinators are essential to human survival and to general ecosystem health, but many populations are declining. The worldwide decline of bee species and colony collapse has the potential to be detrimental to global food production. In determining plant species most used by pollinators, conservation plans can be constructed to ensure these species are available for pollinators to use. Previously, the methods for determining the plants visited by pollinators involved either visually observing which plants individuals visited or collecting pollen to be identified under a microscope. Both methods are time-consuming and subject to bias, with the latter requiring a taxonomic pollen expert to do the identification. Valentini et al. (2010) used universal barcodes and NGS to determine the geographical origin of honey based on the plant DNA present in the honey samples. They found the method to be simple, fast, robust, and successful in identifying plant presence to pinpoint the origin of the samples. Additionally, the nature of honey prevents DNA degradation, so multiple, longer primer sets could be used if certain regions are indistinguishable with the universal

primers (Valentini et al. 2010). Hawkins et al. (2015) tested the effectiveness of the metabarcoding method as compared to traditional, microscopic identification of pollen grains in honey. They found that the two methods had a correspondence of 92% when only high abundance taxa were considered, but that when all taxa were considered, the correspondence dropped to below 50%, with both methods finding taxa that were missed by the other. While metabarcoding is highly sensitive and able to provide greater taxonomic resolution, it is less likely to detect pollen present in low levels than traditional methods due to inherent bias in amplification and sequencing. de Vere et al. (2017) applied metabarcoding of honey samples directly to determination of bee plant preference. By testing honey in a highly diverse botanical garden, they were able to determine that bees preferentially used the same plant taxa composed of native or near-native plants found in woodlands and hedgerows, suggesting that maintenance of these natural environments free from invasive species is essential for bees to thrive (de Vere et al. 2017).

In addition to honey samples, pollen can be taken directly from pollinators for identification. Keller et al. (2015) compared metabarcoding to microscopy for mixed pollen samples collected from pollinators, testing whether primers were actually universal while being able to differentiate species. They found that at 85% match of sequencing results to genome libraries, 98.9% of samples were classified to genus and 61.6% were classified to species, with metabarcoding identifying 13 taxa missed by microscopy. Microscopy found 4 taxa missed by metabarcoding, but overall richness and abundance estimates were similar (Keller et al. 2015). Richardson et al. (2015) published two studies on metabarcoding pollen from pollinators. The first tested a multi-locus approach to metabarcoding to characterize pollen from honey bees. This study found that using multiple loci was more reliable for identifying pollen as it helps to

eliminate false positives and to avoid primer amplification bias. They also compared traditional pollen analysis to metabarcoding of pollen samples from honey bees. The results from this study indicate that metabarcoding is sensitive enough for identification of plants and is able to identify taxa missed by traditional analysis. However, it cannot quantify pollen analysis due to replication bias, and they therefore recommended that both methods be used together when quantification is necessary (Richardson et al. 2015b, 2015a). In the same year, Sickel et al. (2015) published a dual-index multiplexing method adapted from bacterial studies and applied it to the sequencing of pollen samples from multiple bee species simultaneously. They were able to adapt the method so that labelling and PCR were completed in one step and determined that 2000 to 3000 quality reads were needed in order to identify all taxa in the sample. In addition to general plant species use, metabarcoding of pollen from pollinators can be applied to look at landscape-level plant diversity of taxa most used by pollinators (Cornman et al. 2015). Networking and quantifying plant-pollinator interactions can also be accomplished by metabarcoding. Pornon et al. (2016) tested whether metabarcoding could circumvent the limits of conventional sampling in detecting and quantifying plant-pollinator interactions and found that metabarcoding of insect pollen loads revealed 2.5x more plant-pollinator interactions. Additionally, there was correlation between number of sequences with both pollen-carrying load and number of visits per plant species (Pornon et al. 2016). These interactions can be used to determine pollen transport networks and plant species choice among groups, as studied by Lucas et al. in 2018. They used metabarcoding of pollen from hoverflies to determine that there appears to be individual seasonal specialization in plant choice, while within groups there was redundancy (Lucas et al. 2018). Using eDNA metabarcoding on the pollen from migratory pollinators can reveal migratory distances and geographic ranges of migratory species (Liu et al. 2017, Suchan et al. 2018,Chang et al. 2018).

Suchan et al. (2018) used this method on a model butterfly species along the European Mediterranean coast, finding pollen from taxa native to Africa, suggesting the possibility of insect-mediated transcontinental pollination.

Pollen metabarcoding is faster and more effective than direct observation for interactions and allows the study of plant-pollinator networks across ecological gradients (Pornon et al. 2016). Additionally, it is able to detect numerous interactions that would otherwise be missed by traditional methods, thus better describing pollinator behavior and plant choice (Pornon et al. 2017). Bell et al. (2017) tested the effectiveness of metabarcoding for creating networks by sampling pollen from 38 bee species across varying environments. From this data, they were able to match bee species to pollen presence and number of interacting individuals to quantify the interactions. Though metabarcoding of pollen to determine plant-pollinator interactions has benefits including faster turnaround time, hands-off work during which researchers can focus on other activities, the ability to include all pollen grains, more standardized and known techniques, greater taxonomic sensitivity, identification of novel taxa, and more predictable costs that may sometimes be lower than traditional pollen ID, there are some downsides to the technique; namely, lack of complete libraries, potential for contamination and misidentification, and potential reduced amplification of rare species due to primer bias (Bell et al. 2017, Pornon et al. 2017).

Diet analysis

Perhaps one of the earliest applications of metabarcoding was in non-invasive diet analysis from fecal matter or stomach contents. Traditional diet analysis is labor intensive and involves expert morphological identification of partially or fully digested food material which is

particularly difficult for species that are rare, small, aquatic, nocturnal, fast-moving, elusive, or that consume primarily soft-bodied organisms (Boyer et al. 2013, Berry et al. 2017). As such, a new method was needed to fully characterize the diets of species in the wild. Metabarcoding has shown to be a viable alternative and has been found to result in more in-depth characterization of diet than other methods for both herbivores and predators, particularly when standard forward and reverse primers are combined with a blocking primer to reduce amplification of the host species (Vestheim and Jarman 2008, Deagle et al. 2009, 2010, McInnes et al. 2017a, Guillerault et al. 2017). Metabarcoding of feces and stomach contents has further applications beyond diet characterization. It has been used to study resource partitioning, dietary overlap and competition, impacts to forestry and fisheries, comparative conservation strategies, range management, habitat use, species interactions, large-scale monitoring, ecological impacts of changing populations, and human dimensions (Valentini et al. 2009, Kowalczyk et al. 2011, Shehzad et al. 2012a, 2012b, Baamrane et al. 2012, Quéméré et al. 2013, Hibert et al. 2013, Soininen et al. 2013, 2014, Jakubavičiūtė et al. 2017). The metabarcoding approach to diet analysis is fast, simple, robust, widely applicable, typically cheaper than traditional sampling and identification, often has high taxonomic resolution, and is able to detect DNA from digested materials even for soft-bodied or gelatinous prey (Valentini et al. 2009, Shehzad et al. 2012a, Boyer et al. 2013, Buglione et al. 2018). It has even been described as "one of the most accurate approaches available for increasing the understanding on the feeding habits of animals" (Buglione et al. 2018). Despite these definite advantages, there are still drawbacks to using metabarcoding for diet analysis. Poor database quality and imprecise taxonomic resolution are issues yet to be overcome. Despite this, it can be expected that as technology advances, metabarcoding will make these issues merely a problem of the past (Hibert et al. 2013, Yoon et al. 2017).

One of the most important specific methodologies for characterization of herbivorous diet using metabarcoding was developed by Valentini et al. in 2009: the *trn*L approach. This approach has been found to be able to identify about 50% of plant taxa to species from degraded DNA from feces by amplifying a 100-150 base pair region of the P6 loop of the chloroplast *trn*L intron, and can be applied to herbivorous birds, mammals, insects, and molluscs (Valentini et al. 2009). This method has been applied to numerous herbivore diet analysis studies since its development, including an experiment by Kowalczyk et al. (2011) studying European bison restored after extinction in the wild. In this study, the *trn*L approach of metabarcoding was used on feces from bison of different feeding groups (intensively fed, less intensively fed, non-fed in forest, and non-fed in agriculture surrounding forest), and found that when fed, bison consumed more herbs, grasses, and sedges, but when not fed they consumed more woody materials (Kowalczyk et al. 2011). As supplementary feeding has a negative impact on bison ecology and health, a conservation plan can be developed based on their dietary choices when human-provided food is not available; notably, the species of trees consumed could be identified and were determined to be insignificant to the forestry industry, further supporting the change to a non-feeding management style (Kowalczyk et al. 2011). Also using the *trn*L approach, Baamrane et al. (2012) evaluated food habits of the endangered Moroccan dorcas gazelle by metabarcoding fecal pellets and comparing results against a previously compiled database of plant species found on the study site. Plant preferences were able to be identified and data was compared to the results of a microhistological study in the 1980s. The modern study found less taxonomic richness, but a much more diversified diet, potentially due to changes in nutritional quality of plants over time (Baamrane et al. 2012). Hibert et al. (2013) used the *trn*L approach to study the diet of tapir, an elusive large rainforest herbivore. They were able to achieve taxonomic

resolution to genus, and found great correlation with plants already known to be consumed by tapirs in the region, but new families of plants were also identified. Additionally, seasonality of diet could be observed by their use of metabarcoding tapir feces throughout the year (Hibert et al. 2013). In the same year, Quemere et al. (2013) studied the dietary plasticity of the golden-crowned sifaka lemur in Madagascar by using the *trn*L approach on fecal material compared to a large DNA reference database. They discovered remarkable dietary diversity including over 130 identified species from varying habitats, suggesting a high flexibility of foraging strategies, including consumption ofcultivated and naturalized plants, and thus confirming the species' ability to adapt to human-induced landscape changes.

Metabarcoding has also thoroughly characterized the diet of lemmings. Through metabarcoding of stomach contents, Soininen et al. (2013) found that lemmings primarily ingested grass, but that vascular plant consumption differed between habitat types, suggesting regional variation in lemming diet. Metabarcoding of stomach contents of several species of lemmings has revealed differences in composition and temporal variation (Soininen et al. 2014). Metabarcoding of lemming feces has been used to assess diet overlap and potential for competition in sympatric lemming species. The species were found to utilize the same plant as their primary food source in proportion with resource availability but differed in other dietary items, indicating a high potential for competition in populations that were not exhibiting any actual competition (Soininen et al. 2015). Kartzinel et al. (2015) used metabarcoding and next-generation sequencing of fecal material from 7 large mammalian herbivore species in Kenya to determine resource partitioning between species. They found diet to be similar within species and strongly divergent between species, even when considering similar groups (i.e. grazing mammals may eat a similar percentage of grass, but different species will consume different

grasses), suggesting that food choice is more closely linked to plant species than type. Most recently, metabarcoding was used to characterize the diet and seasonal variations therein of the vulnerable Italian hare from only 22 fecal pellets, detecting 99 taxa of plants (Buglione et al. 2018). Applications of metabarcoding of herbivorous diets can be combined with those used for carnivorous diets to characterize the wide variety of food items consumed by omnivores, such as brown bears, which consume plants, invertebrates and vertebrates (de Barba et al. 2014).

Metabarcoding of feces and stomach contents can also be used to characterize the diets of carnivorous invertebrates. A study published in 2013 used metabarcoding of feces to analyze the diet of a carnivorous and critically endangered land snail, finding that the snails mostly consume earthworms that either live in the leaf litter or come to the surface to feed on the leaf litter at night, suggesting that the snail will eat any earthworm it encounters in the leaf litter (Boyer et al. 2013). This knowledge influences conservation decisions without having to harm or disturb the snails in their natural environment. In the same year, Pinol et al. (2013) published a study which investigated the diet of a generalist arthropod predator (linyphiid spider) using metabarcoding without a predator-blocking oligonucleotide. Of this data, only 6% of usable reads were from prey, but this amounted to over 61,000 reads and was enough to identify many prey taxa and to reach the conclusion that the spider exploits a large range of prey. Additionally, this study reached the conclusion that it is possible to characterize diet without developing a blocking oligonucleotide, which would increase the time, testing volume, and cost.

Metabarcoding of vertebrate predator feces emerged around the same time as did that of herbivores. Deagle et al. (2009) used 4 metabarcoding primer sets and a blocking oligonucleotide on feces of Australian fur seals in order to compare the process to traditional diet analysis. They identified 62 species in the seals' diet (including cartilaginous prey missed by traditional

identification due to degradation), and were able to identify the most important prey species to the seals as well as location-based differences in prey composition. Also studying aquatic mammalian predators, Berry et al. (2017) applied metabarcoding to Australian sea lion scats to find 6 classes from 3 phyla for over 8 taxa altogether, some of which confirmed the consumption known dietary items while others had not previously been recorded as prey for these sea lions. These results indicate that the Australian sea lion is an opportunistic predator primarily consuming demersal and benthic fauna of different species composition between study sites (Berry et al. 2017). Metabarcoding of feces has also been used to compare diets of sympatric mammalian predators, such as the study by Bohmann et al. (2011) with African free-tailed bats. They found a large diversity of prey in the samples that were identified to species level at times and were able to determine the differences between the two species' diets. A similar study was recently published by Arrizabalaga-Escudero et al. (2018) in which they used metabarcoding on the feces of two Mediterranean bats in order to determine dietary differences; similar to Bohmann et al. (2011), they were able to characterize the diet of each species and determine the overlap therein (Arrizabalaga-Escudero et al. 2018). Shehzad et al. (2012a,b) developed a methodology for studying mammalian carnivore diet via metabarcoding of feces using the leopard cat as a model species, and then applied the same methodology to snow leopards. To do this, they used a vertebrate primer set on feces samples and tested the methodology both with and without a predator blocking oligonucleotide. In the leopard cat, they found 18 prey taxa including mammals, birds, amphibians, and fish, with most taxa and reads coming from rodents, confirming previous assumptions that the cat has an eclectic diet focused on rodents. The methodology was adapted for use with snow leopard feces where they observed 5 large ungulate prey items including domestic livestock. This knowledge of snow leopard diet can help to

influence conservation strategies regarding livestock predation and retaliatory killing (Shehzad et al. 2012a).

Deagle et al. (2010) ran another metabarcoding study in which they pyrosequenced markers from feces of captive little penguins to see if sequences reflected proportions of species consumed. They found that the same species that were fed were detected, but the proportions were much different, and discovered that DNA can persist in feces for at least 4 days based on identification of species ingested by the penguins before they were taken into captivity for testing (Deagle et al. 2010). McInnes et al. (2017) also studied seabird diet via metabarcoding by developing scat collection protocols for detection of food DNA in vertebrate scat by using shy albatross as a case study for methods that could be potentially applied to numerous species. They found that fresh scat yielded highest food sequences while collection from dirt reduced food sequences and increased contamination, that food DNA detection varied throughout breeding season, and that older individuals produced scat with more food DNA. They then proceeded to apply this procedure to assess albatross consumption of gelatinous zooplankton, finding that while fish were the primary dietary item, cnidarians were also prevalent in samples and detectable at all breeding and life stages (McInnes et al. 2017b). They compared this data with fishery statistics regarding jellyfish catch to discover a relationship that may indicate that jellyfish consumption in albatross is selective and contributes a significant factor to diet. This study would have been near-impossible before metabarcoding, as gelatinous zooplankton dissolve quickly once consumed (McInnes et al. 2017b).

Finally, metabarcoding has been used to characterize the diets of fish species including catfish, stickleback, and toothfish. Using the feces of a non-native catfish, Guiierault et al. (2017) compared metabarcoding to traditional prey identification finding 11 species with DNA and five

with stomach content analysis for a total of 14 species, with two species identified using both methods. Even more amazingly, they were able to detect prey indirectly via parasite presence: while eels were not detected, an eel-specific parasite was, indicating that the catfish do in fact consume eels. Jakubaviciute et al. (2017) were able to identify 120 prey taxa consumed by the three-spined stickleback, a fish whose population is currently steeply increasing. From this unusually high prey diversity they were able to predict the potential impacts of the sticklebacks' increasing populations (Jakubaviciute et al., 2017). Applying metabarcoding to the stomach contents of Antarctic toothfish, Yoon et al. (2017) were able to identify a total of 19 species and to determine the most important prey species for the toothfish. Additionally, they successfully obtained genotypes of prey items via next-generation sequencing and identified 17 more haplotypes, further expanding knowledge of toothfish diet. The scope of dietary metabarcoding studies is already wide and can only be expected to grow as technology improves and techniques are refined.

Detection of invasive species

Invasive and non-native species are some of the greatest threats to ecosystems as our planet becomes more connected. In particular, aquatic vessels have incredible potential to unknowingly transport dangerously prolific species into new habitats where they are capable of outcompeting native species. Current methodologies for aquatic microbiota inventory are diverse and rely on labor-intensive sampling and expert taxonomic identification (Borrell et al. 2017). Additionally, many marine pests cannot be identified morphologically at the larval/juvenile level, so they may go unnoticed at the early stages of infestation when they have the highest prospects of successful management (Pochon et al. 2013). Many factors can influence the

transmission of species via vessels, such as activities performed on the boats, types of boats, and the season: motorboats have a higher rate of species translocation, and propagation is more likely during warmer months (Fletcher et al. 2017, Pochon et al. 2017). Invertebrates are able to survive in bilge and ballast water for at least 16 days, with protozoan species in particular able to multiply during that time and emerge from the pumps unharmed, possibly long distances away from their native environment (Zaiko et al. 2015a, Ardura et al. 2015, Fletcher et al. 2017). Metabarcoding offers a potential method to identify these species early and implement management plans, even for benthic organisms at the planktonic larval stage (Zaiko et al. 2015a). It is extremely sensitive, able to detect DNA at concentrations from 0.02 to 0.64% of the total biomass analyzed, able to detect a variety of taxa simultaneously, and able to be applied to both freshwater and marine environments (Pochon et al. 2013, Hatzenbuhler et al. 2017).

The practice of using metabarcoding to detect nonnative species was first used in marine environments. Ardura et al. (2015) sampled from ballast waters in a ship across its journey from the North Sea to the tropics and found that mollusk species could still be detected, including the invasive European mud snail. In the same year, Zaiko et al. (2015a,b) published two studies comparing results of metabarcoding to traditional identification. In ballast waters, metabarcoding found several noteworthy species that had been missed by traditional methods. In a study with a focus on plankton communities not associated with a vessel, they identified five different non-indigenous species, four of which were not found with traditional monitoring. In 2016, Zaiko et al. explored the scope of marine eukaryotic metabarcoding by sampling from settlement plates in a busy port. They found more than 400 taxa (several of which were non-indigenous), but even at poor resolution, data was consistent with the 25 taxa found by morphological analysis. In a study testing whether a minimum sampling effort for metabarcoding could compare with previously

compiled nonnative invertebrate species, Borrell et al. (2017) found three nonnative species matching the results of traditional studies from only three 1 L bottles of water per port at eight ports. Testing metabarcoding on 30 bilge water samples, Fletcher et al. (2017) were able to identify five different non-indigenous species in 23 water samples, all at high taxonomic resolution. Pochon et al. (2017) took bilge water sampling beyond the status quo, using both eDNA and eRNA metabarcoding to test the potential for invasive identification. They found that eDNA and enhancer RNA (eRNA) gave different results in terms of species presence, with eDNA showing mostly fungi while eRNA showed mostly ciliates and metazoans. They hypothesized that this was due to the increased level of cell activity of ciliates and metazoans, and therefore increased eRNA output (Pochon et al. 2017).

As previously mentioned, metabarcoding is applicable to freshwater ecosystems as well as marine. In 2016, Brown et al. sampled zooplankton in Canadian ports to attempt to identify non-indigenous species via metabarcoding and successfully identified 25 non-indigenous species, 11 of which had never previously been reported in their locations. Blackman et al. (2016) used metabarcoding of freshwater samples from rivers and found it possible to reliably differentiate between native and invasive gammarid species, and that the method could be applied to the assessment of geographic distribution of invasive species. In the following year, Klymus et al. (2017) developed two metabarcoding primers to use with eDNA from the Great Lakes, focusing on the detection of invasive gastropod and bivalve species. Their dual primer design allowed for inference of relative abundance of species, as well as detection of species missed by morphological survey. Most recently, Borrell et al. (2018) used eDNA metabarcoding in two estuaries to test detection of non-native species, and despite limited sampling, one invasive was identified and later confirmed by classic DNA barcoding studies of the estuaries.

Metabarcoding is a powerful tool, and is a more comprehensive method of monitoring and checking for invasive presence than traditional identification (Zaiko et al. 2015b, Brown et al. 2016). Additionally, the cost of metabarcoding is currently comparable to other methods and is expected to decrease as it becomes more common (Borrell et al. 2017). As with other applications, it does have drawbacks. For one, it may bias biodiversity assessments by capturing signals from dead organisms, extracellular DNA, or false readings due to taxonomic selectivity and restricted sensitivity of primers (Fletcher et al. 2017). False readings, either positive or negative, can be problematic in invasive species detection; false positives could trigger an unwarranted and potentially expensive response, while false negatives can result in an invasion that could have potentially be prevented (Darling and Mahon 2011, Ficetola et al. 2015). Another drawback to this method is the current lack of comprehensive genetic databases, and the unavoidability of primer and amplification bias make quantification impossible and require careful analysis of results (Brown et al. 2016, Hatzenbuhler et al. 2017). Regardless, metabarcoding is a unique tool that can only be expected to improve over time from its already wholly applicable and useful state.

Pollution response

Uses of eDNA metabarcoding extend to the ability to track ecosystem responses to pollution. While many pollutants do not have DNA and thus cannot be detected, they often cause changes in microbiological communities affected by them. Studies can be done to determine which species are most likely to be present or absent in the case of a specific pollutant to help determine the severity of the pollution. Until that point, responses to pollution can be monitored in areas with known pollutants or in controlled situations to determine differences in community

composition. Perhaps some of the more high-profile studies of pollution response are those which aim to determine differences in communities surrounding offshore oil drilling platforms or in response to oil pollution events. Smith et al. (2015) used statistical analysis of microbial communities to identify environmental contaminants at a nuclear waste site, and then applied the approach to hydrocarbon contamination from the Deepwater Horizon oil spill. They found that even after the contaminants had been fully degraded, the bacterial communities affected were altered based on the interaction, indicating that bacterial communities have the potential to be used as environmental sensors to evaluate human impacts. Coelho et al. (2016) used microbial activity profiling to evaluate responses to ocean acidification and oil hydrocarbon contamination in lab conditions, determining that the resulting lower relative abundance of sulphate-reducing bacteria may be coupled to changes in sediment archaeal communities. Additionally, they found increased abundance of hydrocarbonoclastic fungi and changes to the sediment chemistry due to the changes in the microbial community, all of which impair the ecosystem's ability to recover from oil contamination events. In terms of community responses surrounding offshore drilling sites, Lanzen et al. (2016) found changes in sediment communities surrounding oil platforms, with metazoans appearing to be the most sensitive eukaryotic taxa to the contaminant effects of drilling, while Laroche et al. (2018) determined that bacteria were highly responsive to drilling activity as well, noting the presence of hydrocarbon-degrading taxa at the petroleum impacted stations, indicating presence of taxa highly specific to site conditions. Both studies identified potential pollution indicator species. Another important sphere of pollution to be considered is that of marine fisheries. Products from these facilities have the potential to affect the marine microbiome, but traditional monitoring of these species is time-consuming and requires extensive specialization. Environmental metabarcoding can be used to estimate community

composition, and to identify indicator species. Foraminiferal responses have been studied multiple times, and candidate bioindicator species identified therein. Pawlowski et al. (2014) found high variation between communities collected near the fish farms versus at a distance, with species richness decreasing nearer to the farms, while Pochon et al. (2015) found clear shifts in community structures between farm sites and control sites and differences in structure between high and low flow environments. Bacterial communities may also be important for assessing the impact of fisheries on ecosystems. Dowle et al. (2015) tested the impact of organic sediment enrichment from a salmon farm on bacterial communities within the sediment along a gradient of distances from the farm. They found stark differences between bacterial communities near the farm as compared to control sites, and identified a strong relationship between bacterial communities and total organic matter, suggesting that metabarcoding bacterial communities may be a useful tool in assessing impacts of aquaculture (Dowle et al. 2015). Stoek et al. published two studies in 2018 regarding microbial response to fish farming, one assessing benthic bacterial communities and the other testing protist presence. They found that deltaproteobacteria were predominant in immediate vicinity of cages, while gammaproteobacteria dominated less impacted sites, indicating changes in community structure due to salmon farming. Additionally, alpha and beta diversity correlated with macrofauna diversity metrics, indicating similar responses to farming, thus making bacterial eDNA metabarcoding a potential alternative biomonitoring method. For protist response, they found that ciliate communities were the most powerful indicators of fisheries pollution when compared to traditional macrofaunal surveys, and that the use of ciliates can allow for an even finer scale resolution (Stoeck et al. 2018a,b).

Aquatic communities may be subject to pollution and contamination beyond that of oil drilling and commercial fisheries, further extending the potential applications of environmental

metabarcoding. Xie et al. (2017) studied eukaryotic community response to different land use types and chemical contaminants, finding that structures of eukaryotic communities were significantly different between land-use types and identifying key contaminants such as pyrethroids, Mn, Zn, Pb, Cr and polycyclic aromatic hydrocarbons (PAHs). Negative associations of eukaryotic classes with these contaminants suggest that there is some degree of toxicity. Other studies focused on specific contaminant effects. Chariton et al. (2014) examined the responses of benthic communities exposed to elevated concentrations of triclosan, a common antimicrobial agent, and found a decline of metazoan diversity, especially in the taxa Chromadorea, Bivalvia, and Kinorhyncha. Frontalini et al. (2018) tested the response of benthic foraminifera communities exposed to different concentrations of Hg, finding that Hg pollution is detrimental to foraminifera with diversity decreasing as Hg levels increase. Potential bioindicators of Hg pollution were also identified (Frontalini et al. 2018).

This technique has been used in terrestrial as well as aquatic environments. Bell et al. (2014) compared fungal and bacterial communities of 66 soil samples from the rhizospheres of planted willows and six unplanted control samples at the site of a former petrochemical plant by metabarcoding, finding that fungal communities changed drastically between sites, and bacterial communities changed to a lesser degree between sites with a decrease in diversity in hydrocarbon contaminated sites. Additionally, they found that willow phylogeny influenced fungal diversity in these sites. Heavy metals are also a concern in polluted soils. Azarbad et al. (2015) tested microbial communities associated with forests in metal (Zn and Pb) polluted soils in Poland and found that the metal pollution level impacted bacterial community structure, but not taxon richness or community composition; rather, the relative abundance of specific bacterial taxa changed. In the same year, Zappelini et al. (2015) reported bacterial and fungal communities

from both chlor-alkali residue (high in Hg and As) and nearby undisturbed soil to determine community composition. They found a dominance of gammaproteobacteria and Pseudomonas sp. at the study site, and minor differences between genera of fungal communities. Foulon et al. (2016) compared microbial community composition at two sites with varying environmental conditions, but both contaminated with Zn, Cd, and Pb: Pierrelaye, which was a sandier soil type enriched with P, C, and total N, and Leforest, which was a more silt/clay soil type with a lower pH and higher amounts of Cd and Zn (Foulon et al. 2016). They found that Ascomycota dominated overall poplar soil, root samples at Pierrelaye, and unplanted soil, while Basidiomycota was more dominant in root samples from Leforest (Foulon et al. 2016). The dominance of an ectomycorrhizal community at Leforest was potentially favored due to strong resistance to environmental stress, while at Pierrelaye where soils were more fertile and resources more available, other species are able to thrive (Foulon et al. 2016). Most recently, fungal communities at an Hg-contaminated phytomanagement site of poplar trees were characterized using environmental metabarcoding (Durand et al. 2017). Communities were found to be negatively affected by Hg concentration, and aboveground and belowground fungi communities were found to be almost completely isolated from one another (Durand et al. 2017).

Even areas specifically denoted to contain pollution are capable of having an effect on wildlife. In 2017, Klymus et al. tested eDNA metabarcoding as a survey tool to examine potential use of uranium mine containment ponds as water sources by wildlife by testing ponds near mines and one mine containment pond using two markers for vertebrates. They detected two species missed by traditional surveys, including the presence of tiger salamanders in the containment pond. Finally, while perhaps not a response to a pollutant in the traditional sense, environmental metabarcoding can be used to test the effects of invasive species on native

communities. Wangensteen et al. (2017) used multigene metabarcoding to observe littoral community responses to three invasive, canopy-forming seaweeds. They found that the presence of invasive seaweed had a significant effect on the understory community for two of the three species. Overall, pollution response studies have found metabarcoding to be more sensitive in revealing community changes than traditional surveys; however, it is still subject to amplification bias, interpretation uncertainties, and other roadblocks previously discussed (Klymus et al. 2017b, Frontalini et al. 2018).

Air quality monitoring

DNA metabarcoding can also have applications in human health; determining the microbiome in the air can influence health decisions regarding allergens, pathogenic organisms, and air pollution. Current methods in studying airborne allergens are microscope-based, labor intensive, and cannot identify to the genus level in all relevant groups (Kraaijeveld et al. 2015). By collecting samples from the air and applying metabarcoding and next-generation sequencing technology, it is possible to determine the composition of the airborne microbiome and its potential implications for human and ecosystem health.

Metabarcoding of pollen was found in 2014 to be more efficient and more sensitive than current pollen monitoring techniques; however, a simple pollen count is needed in addition to metabarcoding data in order to properly quantify airborne pollen (Kraaijeveld et al. 2015). Leontidou et al. (2018) created a standardized procedure for sample preparation and DNA extraction for airborne pollen metabarcoding by comparing metabarcoding results to morphological identification. They found that even in complex samples with more than 46

families, plants can be identified efficiently by metabarcoding with greater taxonomic resolution than by morphological identification.

Airborne fungal diversity is another important facet of the aerial microbiome. Presence and abundance of taxa in fungal airborne samples can be variable over time and impacted by climatic conditions, and because many mycelium fragments and spores have the potential to be toxic or cause allergies, it is important to describe airborne fungal diversity (Banchi et al. 2018). Previous studies have relied on a few indicator species and taxa of key importance, but with metabarcoding all taxa can be analyzed. Oh et al. (2014) used metagenomics to study airborne fungal diversity and found over 1,000 unique OTUs, with a set of 40 potentially allergy-inducing genera identified accounting for approximately 20% of the airborne fungal diversity. Banchi et al. (2018) specifically compared metabarcoding to traditional airborne fungal analysis and found that metabarcoding provided a 10x more comprehensive determination of taxa compared to microscopy; even so, some taxa were only identified in one technique or the other, making both methods necessary for comprehensive characterization of the fungal community.

While the most frequent use of environmental metabarcoding is in natural environments, the methodology can be applied to indoor locations as well. In particular, airborne microorganisms are a critical concern in hospitals, where some have the potential to cause hospital-associated infections. Tong et al. (2017) compared results of metabarcoding air samples and traditional culture-based methods to describe the microbial diversity. In this study, few bacteria were grown after cultivation, but sequencing revealed the relative abundance of fungi, archaea, bacteria, and viruses. Knowing the species present can inform infection control plans in the case of an outbreak, as sampling different areas of the hospital can offer a better idea of what control methods are necessary (Tong et al. 2017). Cao et al. (2014) applied a similar method of

characterizing the full microbiome; however, their study was outdoors in Beijing during a severe smog event. While particulate matter air pollution is well-understood, inhalable microorganisms have not been well studied, and most studies have been purposed for taxonomic identification rather than pathogenic potential (Cao et al. 2014). This study used metabarcoding to characterize the airborne microbial community and was able to identify most taxa to the species level with the results. In addition, they discovered the presence of some respiratory microbial allergens and pathogens (Cao et al. 2014).

Future directions

For eDNA metabarcoding to truly take off, current assessments of ecological quality would need to be adapted to the eDNA metabarcoding framework. These changes must be feasible on a large scale, particularly when considering thresholds between countries and the differences between traditional and molecular methods. To calibrate, molecular methods would need to be applied simultaneously with existing systems in key environmental gradients which would likely be accomplished during development and testing of molecular methods (Hering et al. 2018). As such, a comprehensive eDNA monitoring program could be possible in just a few years across a variety of environments and taxa, thus providing a global framework for ecosystem modelling and function to inform management decisions. To handle this shift, reference databases would need to be expanded to include identifiable sequences for all target biodiversity, and advanced facilities will need to be created to house the growing data libraries. Wide-spread implementation would benefit from optimized bioinformatics pathways with user-friendly interfaces, as well as general improvements to the eDNA collection and extraction

methodologies, particularly in terms of accounting for degradation. Metabarcoding has the potential to complement higher cost biodiversity measures and even replace them in some instances, especially as it becomes more widespread and the cost goes down (Bohmann et al. 2014, Creer et al. 2016, Deiner et al. 2017). Ecological indicators are essential for conservation science, but full biodiversity assessments that are rapid, cost-effective, and noninvasive are also important to conservation biology (Ji et al. 2013, Deiner et al. 2017). While eDNA metabarcoding cannot give information as to the health or demographics of a population, it has the potential to assess long-term changes in ecosystems and local extinctions over time, including for modeling the impacts of climate change on populations (Deiner et al. 2017).

eDNA also has applications in citizen science by engagement of citizen collection of eDNA using commercially available sampling kits, involving the public in biodiversity sampling in a way that could be complementary to already established methods. However it is limited by the difficulty in visualizing and explaining data to laypeople. More user-friendly methods of data exploration are needed (Bohmann et al. 2014, Deiner et al. 2017, Harper et al. 2018). It may even be possible to automate eDNA sampling in the future, but further research is needed into the temporal longevity and spatial dispersal of eDNA in all ecosystems for studies of abundance and richness to be considered fully valid (Barnes et al. 2014, Bohmann et al. 2014, Deiner et al. 2017). Metagenomics, or shotgun sequencing of environmental samples without relying on a PCR amplification step, avoids primer bias and could theoretically be used for biodiversity studies. However, because it requires a huge increase in sequencing power to acquire adequate depth, the cost is often prohibitive. As technology advances, this may become a commonplace method of biodiversity monitoring (Taberlet et al. 2012a, Yu et al. 2012, Cristescu 2014, Creer et al. 2016, Deiner et al. 2017, Hering et al. 2018). There is a definite need to let methods mature as

eDNA metabarcoding is a developing field, and thus apply the evolution of the method to reliable indices and ensure both forward and backward compatibility (Hering et al. 2018).

In conclusion, the potential of eDNA metabarcoding in biological research seems almost limitless, but the technique requires scientific collaboration and coordination. As technology advances and procedures are optimized, metabarcoding will likely emerge as an essential tool for a number of scientific tasks not only in biodiversity monitoring but also in the numerous areas discussed above. eDNA metabarcoding brings together several fields for the assessment of communities for purposes ranging from ecosystem restoration to human health, making it extremely versatile and vital to the future of molecular research.

South Texas Amphibian Monitoring Applications and Recommendations

There have been very few studies on amphibians in south Texas, and analysis of community structure and dynamics is lacking. This is due at least in part to the difficulty in studying these species; the arid climate requires unique adaptations to avoid desiccation which in turn make finding them difficult and knowledge of breeding times and seasonality is necessary to ensure proper detection (Buxton et al. 2018). There are several species that are listed as threatened by the state with minimal published information influencing this listing and detection is necessary for assessment of conservation status and the development of conservation strategies. Amphibian species of conservation concern in south Texas include the Rio Grande siren (*Siren intermedia texana*), black-spotted newt (*Notopthalmus meridionalis*), sheep frog (*Hypopachus variolosus*), Mexican tree frog (*Smilisca baudinii*), white-lipped frog (*Leptodactylus fragilis*) and Mexican burrowing toad (*Rhinophrynus dorsalis*) (TPWD 2018). In addition to detection for species monitoring, collection of environmental variables can help to predict future presence and choose likely locations for sampling. Basic knowledge of species'

life histories and biology can help inform the development of a sampling methodology of these species, while knowledge of location and habitat characteristics has the potential to make possible more in-depth studies of amphibian community dynamics and species interactions, further increasing knowledge to inform conservation decisions for imperiled amphibian species in south Texas.

Environmental DNA metabarcoding has the potential to be applied to the monitoring of south Texas amphibians. As the climate is semi-arid and many waterbodies are ephemeral, species such as the Mexican burrowing toad may remain underground or otherwise protected from desiccation until adequate rain occurs for breeding. As such, the time after rain offers a unique opportunity to collect water samples and characterize amphibian communities via metabarcoding. Valentini et al. (2016) created a batrachia primer set designed for metabarcoding of all amphibian species. In order to assess the applicability of this primer set for south Texas amphibians, several tests would need to be completed.

First, the primer set should be compared to genomes of south Texas amphibian species via GenBank or another genomic library. It is possible that the target regions of all species will not be available; however, this comparison should still be attempted. This step allows for preliminary confirmation that the primer set will amplify the region for individual species. The region of amplification should be noted and compared between species in order to assess identifying characteristics of the sequence.

Next, the primer set should be used with tissue samples from each of the target species. Using the protocol described by Valentini et al. (2016), repeatable amplification of tissue DNA should be ensured. Additionally, PCR products for each species should be separately sequenced for complete confirmation; sequences should be compared to those identified in the previous step

to confirm effectiveness. For species lacking genomic data, this will serve the dual purpose of confirming amplification and providing the amplified sequence for the species. As with the previous step, amplified sequences should be compared to each other to ensure determine the unique identifying factors between species.

The assay should then be tested with simulated water samples and filtration, as would be done in the field. For this, resaca water should be spiked with equal amounts of tissue DNA or synthetic oligomers from all species and processed using a standard protocol. These mock samples should be treated as true field samples. The field and extraction protocol previously described in Chapter 2 may be used as it has been found to be effective for the turbid water bodies of south Texas. Extracted DNA should be amplified using the previously tested primer set with the defined protocol before being pooled and prepared for NGS as designated by the sequencing service used. Results from the sequencing run should be grouped into OTUs, and identification of each species should be confirmed. Additionally, any extreme levels of primer bias should be noted and assessed. During these tests, multiplexing may also be tested. Amplification and potential bias of identification tags for various species should be assessed; tags resulting in extreme bias should be excluded from future use with the primer set.

For final confirmation of the applicability of the assay, it should be tested against natural water bodies. Call surveys, dip netting, trapping attempts, and historical data may be used to estimate the species most likely to be detected. Call surveys and sampling for adults should occur immediately after heavy rains. For several species of conservation concern tied to ephemeral water bodies, larvae require approximately 30 days to metamorphosis, allowing a greater sampling window for larval amphibians (Fouquette 2005, Judd and Irwin 2005, Livezey and Wright 1947, Mulaik and Sollbereger 1938). As the larvae should continually shed DNA into the

water body, eDNA sampling should occur within 30 days of heavy rains. NGS results should be analyzed to determine whether expected or known species were detected; a lack of detection may require tweaking of field methods and sampling timing. Multiplexing, if previously determined to be effective, may be used to reduce sequencing cost. Future tests may assess the ideal timing post-rain for maximum detection.

If found to be effective, eDNA metabarcoding could allow for the characterization of entire communities from water samples. As with the siren-specific assay, this information may be used to look at distributions or as a preliminary tool to inform in-depth sampling efforts. Additionally, amphibian metabarcoding can be used to assess occupancy-level interactions between species. Multiple sampling visits can determine how amphibian communities change in water bodies over time since rainfall. Altogether, eDNA metabarcoding for south Texas amphibians could open countless avenues of research regarding the largely understudied and unique species found here.

CHAPTER V

SUMMARY AND CONCLUSIONS

The aim of this study was to develop an eDNA assay for the Rio Grande siren, to assess the effectiveness of the assay, to determine the impacts of environmental variables on both the assay and traditional siren detection via trapping, and to review the literature of eDNA metabarcoding in order to design a research plan for implementing the best molecular methods for detection of south Texas amphibians. An eDNA assay was developed and optimized for use with Rio Grande sirens. Due to high turbidity in the waters that sirens inhabit in south Texas, large pore cellulose filters were necessary to filter large amounts of water and were found to be effective in detecting sirens. Additionally, the use of a manual automotive fluid evacuator was effective in allowing water to be filtered in the field, eliminating the need to immediately place water on ice or carry heavy batteries into the field. DNAzol was found to be an effective storage buffer for eDNA filters, particularly due to its long-term stability at room temperature. The primers designed in this study were found to be effective in amplifying siren DNA from specimens collected across Texas while excluding amplification of sympatric amphibians in the Rio Grande Valley. The use of the nested primers developed in this study further increased the utility of the assay by reducing the effects of inhibitory substances, increasing specificity, and enabling amplification and sequencing of the low copy number samples common to eDNA studies. Furthermore, the development of a qPCR assay allows for future studies in

quantification of siren eDNA. As sirens can be highly secretive, the ability to detect siren presence via a water sample is exciting and novel, opening new avenues for siren research, particularly for initial detection and determination of species presence.

While the eDNA assay for detection of sirens is effective, it can still benefit from optimization. Improved extraction protocols that remove inhibitors and eliminate the need for nested PCR would greatly increase the efficiency of the assay. Additionally, a comparison of filter materials and pore sizes would be beneficial to determining the ideal filter for turbid south Texas waters. The use of the storage and extraction buffer DNAzol should also be evaluated in comparison to other established eDNA preservation methods.

Following development, the assay was compared to the traditional siren survey method of setting baited minnow traps overnight. Siren detection was significantly greater via eDNA than via trapping; in fact, all but one eDNA sample was positive for siren DNA. When environmental variables were assessed, strong patterns emerged between siren trapping and season, indicating that sirens were more likely to be captured in the spring (March–May) and fall (September–November) than in the summer or winter. The exact reason for this is not certain; it is possible that this encompasses the times that sirens are breeding. More likely, the increase in siren capture may be due to increased availability of water; further studies assessing siren detection in variable water availability would be useful. Regardless, studies requiring the capture of sirens in south Texas should focus trapping efforts in the spring and fall. While cloudy weather was also found to impact siren detection via trapping, a biological reason for this has not yet been identified. Due to the high detection success of sirens via eDNA, the impacts of environmental parameters on detection via eDNA were not able to be assessed. Based on the findings of this study, eDNA can be reliably applied to survey efforts for detection of sirens. It is recommended that eDNA is

seen as an additional tool and not a sole method used to assess a species' biological, ecology, or conservation status.

A logical next step to applying molecular methods to the detection of sirens in south Texas is to expand detection capabilities to entire amphibian communities. Amphibian species are extremely understudied in south Texas, and knowledge of their community structures is entirely lacking. eDNA metabarcoding offers an option to enhance the ability to study south Texas amphibian communities. In this thesis, metabarcoding methods were reviewed, a plan for development of a metabarcoding assay for south Texas amphibians was outlined, and potential research topics and applications were identified. The ecological uniqueness of the region and the species within offer countless possibilities of studies, and metabarcoding could greatly improve the capacity for implementing monitoring programs. In this time of mass amphibian extinction, the ability to sample effectively in multiple capacities is essential.

Altogether, the findings of this study lay groundwork for future studies in eDNA, metabarcoding, and the Rio Grande siren. The methods described can be replicated for future siren eDNA studies or adapted for other eDNA studies of species in similarly turbid habitat. A time window for optimal siren capture has been identified. Additionally, a plan for the development of a metabarcoding assay for south Texas amphibians can be followed to increase sampling capabilities in this unique region.

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APPENDIX

APPENDIX

Museum ID	Field ID	Species	State	County	Locality
	DRD 5444	*Siren intermedia*	TX	Cameron	Lozano Banco Resaca
	DRD 5289	*Siren intermedia*	TX	Harris	Katy Prairie Conservancy
TNHC 112457	DRD 5156	*Siren intermedia*	TX	Kinney	Imperialist Creek
	DRD 5457	*Siren intermedia*	AR	Craighead	near Jonesboro Municipal Airport
MMNS 5978		*Siren intermedia*	MS		
ODWC 3849		*Siren intermedia*	OK		
	DRD 5421	*Rana catesbeiana*	TX	Refugio	Co Rd 1360, ca. 2.3 rd km W jct Co Rd 2511
TNHC 112113	DRD 5122	*Rana berlandieri*	TX	Cameron	TX Hwy 100, ca. 2.6 rd km E jct TX Farm Rd 539
TNHC 112004	DRD 5041	*Hypopachus variolosus*	TX	Hidalgo	Brushline Rd, ca. 2.6 rd km S jct TX Hwy 186
TNHC 112082	DRD 5171	*Gastrophryne olivacea*	TX	Hidalgo	Brushline Rd, ca. 1.1 rd km S jct TX Hwy 186
TNHC 112166	DRD 5120	*Anaxyrus speciosus*	TX	Kenedy	US Hwy 77 exit ramp to Sarita,
TNHC 112149	DRD 5137	*Incilius nebulifer*	TX	Cameron	6005 Danubio Ct, Brownsville, TX 78526
	DRD 5405	*Incilius horribilis*	TX	Willacy	Co Rd 1420, ca. 1.3 rd km N jct Co Rd 498
TNHC 112175	DRD 5021	*Scaphiopus couchii*	TX	Cameron	TX Farm Rd 1420, ca. 2km N jct TX Farm Rd 508
	DRD 5801	*Smilisca baudinii*	TX	Cameron	Lozano Banco Resaca
	DRD 5990	*Rhinophrynus dorsalis*	TX	Starr	South Texas College
	DRD 6000	*Leptodactylus fragilis*	TX	Zapata	Co Rd 2687, ca. 7.5 rd km NE jct US Hwy 83
	DRD 5176	*Ambystoma tigrinum*	TX		
	DRD 5165	*Notopthalmus meridionalis*	TX	Cameron	Palo Alto National Battlefield

BIOGRAPHICAL SKETCH

Krista M. Ruppert graduated from Texas A&M University in 2016 with a Bachelor of Science degree in Wildlife and Fisheries Sciences with a concentration in Vertebrate Zoology. During her undergraduate, Krista worked with the Texas A&M AgriLife Extension Wildlife Unit on the Reversing the Quail Decline in Texas Initiative. After graduation, she began an independent research project at The Wilds in Cumberland Ohio, where she studied mesocarnivore interactions via camera trapping. It was here that she cultivated a love for amphibians. Following this project, Krista was accepted to the University of Texas Rio Grande Valley in 2017 to earn her Masters of Science in Agricultural, Environmental, and Sustainability Sciences, earning her degree in August 2020. Krista will be pursuing a doctoral degree in Forest Resources with an emphasis in wildlife at Mississippi State University beginning in August 2020, where she will be studying gopher frog ecology and conservation under Dr. Scott Rush. Krista can be contacted at krista.ruppert@aggienetwork.com or at www.kristamruppert.com.