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Environmental DNA for conservation

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8.1 Introduction

Biodiversity must be documented before it can be conserved. However, it may be difficult to document species with few individuals (Thompson, 2013; Goldberg et al., 2016), thus it requires a multitude of tools to detect species that occur in low numbers or are elusive (see the various chapters in this volume). One tool that has become useful for conservation efforts utilizes environmental DNA, which is DNA shed into the environment by organisms (eDNA; Taberlet et al., 2018). Typically this involves taking environmental samples such as soil, water, air, or using biological surrogates for sampling biodiversity (e.g. leeches, sponges, carrion flies, etc.; Schnell et al., 2012; Calvignac-Spencer et al., 2013; Lynggaard et al., 2019; Mariani et al., 2019) and using laboratory approaches to concentrate, isolate, and test for target DNA through polymerase chain reaction (PCR) amplification (Taberlet et al., 2018). The utilization of eDNA for species detection is part of a larger field of non-invasive DNA sampling, which more broadly includes collecting DNA passively from wildlife, through collection of faeces, saliva, feathers, hair, or other methods of sampling shed DNA. Environmental DNA has been used to document presence/absence of a target species (Ficetola et al., 2008a, 2008b; Hunter et al., 2017) or to quantify relative abundance for biodiversity from varied environments such as the arctic (e.g. Leduc et al., 2019; Von Duyke et al., 2019), marine (e.g. Port et al., 2016; Jo et al., 2017; Stoeckle et al., 2018), freshwater (e.g. Lacoursière-Roussel et al., 2016; Doi et al., 2017), and tropical (e.g. Schnell et al., 2012;

Gogarten et al., 2020) ecosystems. The application of this technology includes the detection of invasive species, pathogens (including DNA and RNA), species of conservation concern, and biodiversity (Acevedo-Whitehouse et al., 2010; Rees et al., 2014; Sakai et al., 2019).

When detection of a rare or cryptic species is the goal, often a suite of tools, such as acoustic sensors (Chapter 4), camera traps (e.g. Chapter 5; Mallet & Pelletier, 2014; Burton et al., 2015; Caravaggi et al., 2017; Wearn & Glover-Kapfer, 2019), traps such as light (Conrad et al., 2007; e.g. McLeod & Costello, 2017) or Peterson/Tomahawk or corral (e.g. Garden et al., 2007; Costello et al., 2017), trawling (Jones, 1992; Heino et al., 2011), continuous plankton recorders (e.g. Richardson et al., 2006), bait stations (e.g. Shardlow & Hyatt, 2013; Costello et al., 2017), faecal DNA mark-recapture (e.g. Waits & Paetkau, 2005; Schwartz et al., 2007), and other methods are reliably used for detection. Each method has trade-offs, including species wariness to new instruments in their environment and difficulty detecting evidence due to weather or vegetation, among other issues. Further, for species that exist at low population sizes, failure to confirm presence does not necessarily suggest its absence for many reasons, including a low DNA signal in the environment (Ellison et al., 2006; Thompson, 2013). The ability to isolate DNA from a target species without the need to capture animals provides another tool that can help biodiversity surveys (Darling, 2019). In particular, eDNA can be inexpensive to implement in the field and can be accomplished through the collection of samples easily obtained from the environment or the use of a single species to detect

others (e.g. blood or carrion feeders). However, like other tools, there are trade-offs with this method, which are described in detail later in this chapter (Cristescu & Hebert, 2018).

Largely, eDNA has been used to detect the presence/absence of a target species in the environment. Presence of DNA and, in some cases, relative frequency across sites is assessed through PCR platforms that quantify the amplification of DNA but do not provide an estimate of the number of individuals present, such as real-time PCR, specifically quantitative PCR (qPCR), or droplet digital PCR (ddPCR) (Goldberg et al., 2016; Deiner et al., 2017b; Baker et al., 2018; Klymus et al., 2020). However, genomic approaches, such as next-generation sequencing (NGS) that allow for simultaneously sequencing multiple species from a single environmental sample has been used to estimate relative abundance (Valentini et al., 2009; Bohmann et al., 2014; Valentini et al., 2016; Porter & Hajibabaei, 2018). Multiple studies comparing eDNA to other tools for sampling biodiversity demonstrate that eDNA can do as well as others (Valentini et al., 2016; Gogarten et al., 2020), be an important complement to other tools, or even perform better at detection, assessing diversity, etc. than other tools (Valentini et al., 2016; Table 1, Deiner et al., 2017a; Boussarie et al., 2018). For conservationists, it is important to understand the practical applications of eDNA, what questions it can answer, its uses and limitations, and how one might implement this tool.

8.2 New technology

8.2.1 Data collection—study design

Sampling environmental DNA has rapidly become an important tool in the arsenal of non-invasive tools for detecting biodiversity and for use in conservation efforts (e.g. Thomsen & Willerslev, 2015; Cristescu & Hebert, 2018; Taberlet et al., 2018; Mize et al., 2019; Zinger et al., 2019). Endemic and elusive species such as the Rocky Mountain tailed frog (*Ascaphus montanus*) and Idaho giant salamander (*Dicamptodon atterimus*, Pilliod et al., 2013), invasive species such as bullfrogs (*Lithobates catesbeianus*, Ficetola et al., 2008a, 2008b), and overall

biodiversity of species of concern within freshwater ecosystems (Thomsen et al., 2012a) were early targets for detection using eDNA in freshwater ecosystems. In marine ecosystems, the first application of environmental DNA involved parallel sequencing of multiple species, which detected 15 fish species and even some bird species (Thomsen et al., 2012b). Compilation of data from across the now extensive use of eDNA in conservation efforts has led to the development of standard protocols for detecting eDNA in various aquatic systems (see Table S1 in Goldberg et al., 2016; Stoeckle et al., 2018; Harper et al., 2019; Jeunen et al., 2019).

Protocols for collecting eDNA samples are influenced by the research question (presence/absence, relative abundance, species composition, etc.) and the target species, among other issues (Goldberg et al., 2016; Wilcox et al., 2018; Zinger et al., 2019). Because field sites introduce unique biotic challenges (e.g. pH of the water, the water body type and size, season) to detection probabilities, pilot studies are critical (Barnes & Turner, 2016; Goldberg et al., 2016; Davis et al., 2018). Before one can determine how many samples are needed within a single water body or across a target region with variable biotic conditions, we need to know the probability of detecting a species under those conditions. Further, the processing of eDNA has several steps (DNA capture, isolation, and amplification) where detection may vary. Using multistage occupancy analyses with data from a pilot study it is possible to identify the detection probability at different water body types, under different biotic conditions, and across laboratory processes (Figure 8.1: Goldberg et al., 2016; Hunter et al., 2017; Davis et al., 2018; Doi et al., 2019; Erickson et al., 2019; Sepulveda et al., 2019).

Another complex issue for eDNA studies is calculating costs, then comparing these costs to other pertinent tools, and finding the right balance between costs and information gained. One study has explored these issues and provided a phone application that can be easily used and tailored to individual needs (Davis et al., 2020). In some cases, eDNA can be prohibitively costly and, in others, there is a balance between effort, cost, and information gained with this monitoring method. As the field progresses, so may the tools that make it easier for all levels of practitioners to assess

Column 1: Select sampling method. In this example, we selected to sample water

Swab plants

Collect water samples directly

Pump water and collect sample on filter

Column 2: Select sites, number of sites to sample, and number of field replicates per site. Occupancy modeling results from pilot study – need 20 field replicates per site and 10 sites for 90% probability of detection

Pond

Stream

Lake

Column 3: Laboratory work load example - Samples from 1 site sent to lab = 20 samples

Extract DNA: 2 extractions per sample = 40 samples

PCR: 2-8 technical replicates. Here we chose 3 = 120 amplifications resulting from sampling 1 site

How many positives are enough to decide to commit resources to investigate further? It can be as few as 2 positives (red stars) for a single site, as long as negative controls are negative, but this risks committing resources to a false positive.

Figure 8.1 Study design and sampling strategy are critical to successfully detecting low numbers of target species using environmental DNA (eDNA). It is critical to understand the influences of each step of the process, from eDNA capture, isolation, and amplification to biotic influences (pH, water diffusion and dispersion, size and type of water body, time of year, etc.). Pilot studies (see example in this figure) conducted in the target geographic region, along with statistical assessments such as occupancy modelling (Davis et al., 2018) are necessary to determine the appropriate sample size per water body and the number of replicates required in the lab to maximize detection probability. It is also prudent to decide the parameters of what defines a positive that is relevant to management actions and tolerance for false positives or false negatives.

sample size needs, sampling design, costs, and required effort for individual needs, as has been done for faecal mark-recapture studies (Lonsinger et al., 2015). Overall, consultation with a lab experienced in eDNA studies is ideal, and there are useful resources for those wanting to begin the process of using eDNA¹.

8.2.2 Data collection—targeting single species

Many eDNA studies use DNA primers targeted to a particular species of interest. The development of an assay for a novel target species requires laboratory validation. Further, understanding detection limits and DNA persistence through the use of captive target taxa is critical before applying the assay to

¹ 1 <https://ednaresources.science/>
2 <https://www.fs.fed.us/research/genomics-center/edna/>

3 <https://github.com/boopsboops/seadna-protocols/tree/master/docs>
4 http://ednasociety.org/eDNA_manual_Eng_v2_1_3b.pdf

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complex and unpredictable field conditions. Captive studies involving target taxa allow testing of eDNA assays on known quantities of shed DNA or for known periods with other biotic parameters held constant (e.g., Williams et al. 2018). Assay validation should also include the testing of primers for species-specificity, meaning they do not cross-amplify in non-target species that may be present in the target sampling area. Further, replicated dilution series of positive controls to assess limit of detection, limit of blank, and limit of quantitation is necessary for assay validation and requisite understanding of potential for false-positives and false-negatives (Goldberg et al., 2016; Klymus et al., 2020). There are standardized methods for eDNA assay validation methods and reporting of critical parameters (Klymus et al., 2020). The current body of eDNA validated assays has revealed that eDNA is ephemeral and heterogeneously distributed in the environment (Furlan et al., 2016), that sensitivity of laboratory molecular methods is affected by environmental inhibitors (e.g. turbidity, pH, water chemistry, etc.), and that there is detection variability of low quantity/quality DNA from the environment (Barnes & Turner, 2016; Goldberg et al., 2016). Thus, each assay validation provides further refined data on the utility and applicability of eDNA as a tool in varied environments and across species.

8.2.3 Data collection—targeting multiple species

Biodiversity documentation and monitoring through eDNA is best informed by the ability to identify all DNA in a single sample. The method of species-specific targeting in environmental samples does not allow for biodiversity assessment but rather provides presence/absence data or, in some cases, relative quantification of population size of a single species (Goldberg et al., 2016). NGS allows DNA fragments of a mixed-species sample to be sequenced simultaneously and thus identification of a suite of species (Figure 8.2; Thomsen et al., 2012b; Taberlet et al., 2018). The potential of NGS application to eDNA is that the number of copies of sequences represents the presence of DNA cells from individuals and can be translated to the

abundance of each species and then extrapolated to the sampling area.

The use of NGS in environmental sampling is typically to amplify a single gene across all DNA in a mixed-species sample (Figure 8.2), termed metabarcoding (Taberlet et al., 2018). The adoption of metabarcoding for biodiversity assessment of eDNA has been detailed elsewhere (Deiner et al., 2017a; Cristescu & Hebert, 2018), but an overview to allow for an applied understanding will be provided here. Multiple-species DNA detection approaches promise to be just as powerful or more powerful as traditional field tools (reviewed 2011–2019 in Shu et al., 2020) in assessing biodiversity (Valentini et al., 2016; Deiner et al., 2017a; Jo et al., 2017). It is not yet possible to quantify the number of individuals identified in a sample or ensure complete detection of every species present in a sampled habitat (Bush et al., 2019), especially rare species (Deiner et al., 2017a), but the field is rapidly moving towards addressing these and other current limitations (Valentini et al., 2016; Deiner et al., 2017a, 2017b; Taberlet et al., 2018; Knudsen et al., 2019; Shelton et al., 2019). Developing an assay to target multiple species can be quite similar to an assay for a single species (Shu et al., 2020). Methods of DNA capture and isolation may vary depending on environmental sample type and targeted community being assessed (Jeunen et al., 2019). Some primers have been validated and tested for use in eDNA metabarcoding studies (Taberlet et al., 2018), yet each can introduce biases; for example, primer biases may allow one species to be over-represented in the results, among other issues (Deiner et al., 2017a, 2017b; Taberlet et al., 2018; Zinger et al., 2019). Thus, methods developed to detect biodiversity from eDNA samples must be tested using mock communities with known amounts of DNA from various species, including rare species, to make sure they can be detected in low numbers using the chosen primers (Taberlet et al., 2018; Doi et al., 2019). In some cases, species of concern may be better monitored through species-specific approaches or a combination of approaches (Wood et al., 2019).

There are currently two primary challenges in applying metabarcoding for eDNA: (1) biases caused by laboratory processes make it challenging to ascertain quantifiable biodiversity indices; (2)

Figure 8.2 Samples are taken from various environmental substrates are collected and next-generation sequencing (NGS) is used to amplify a single gene fragment or whole genomic data from all DNA in a sample. Bioinformatic pipelines such as Anacapa Toolkit (Curd et al., 2019) are used to identify Operational Taxonomic Units (OTU: Blaxter et al., 2005), groups of sequences with the chosen level of similarity and identified through comparison to publicly available DNA databases. The number of reads assigned to each OTU is the number of sequences assigned to that taxonomic designation. Additional credits: Soil in hands image courtesy of Jing/Pixabay; DNA image courtesy of geralt/Pixabay; Laptop/coffee image courtesy of Engin_Akyurt/Pixabay.

data produced are challenging to manage and analyse (see 8.2.2 Data analyses). In response to the first issue, some labs are working to find novel ways to deal with biases and use these data to estimate diversity and abundance (e.g. Shelton et al., 2019). Researchers are also developing tools to manage and analyse the data properly (e.g. Kandlikar et al., 2018). Thus, the application of NGS and approaches such as metabarcoding or even metagenomics (sequencing whole genomes rather than a single gene from a mixed-species sample) will continue to revolutionize the information available from eDNA and the utility to biodiversity assessments and thus conservation (Ruppert et al., 2019).

8.2.4 Data collection—DNA capture

Sample collection decisions are the same whether one is using an eDNA assay for single-species detection or multiple-species surveys through metabarcoding. Sample collection methodology for

collecting environmental samples advances regularly and depends on the template being targeted (e.g. water and the water type, soil, air, biological means). However, understanding the range of collection options is useful as different field conditions, target species, questions, or project resources influence the usefulness of any particular method.

Filtering water for eDNA capture is the most common approach. In freshwater systems, backpack pumps (Figure 8.1) with filtering systems are commonly used for eDNA collection (Laramie et al., 2015; Goldberg et al., 2016). Backpack pumps draw 2–10 litres of water through a filter to remove particulates and capture and concentrate DNA. Once DNA is captured on a filter, it is necessary to preserve it by applying a buffer (e.g. Longmire's buffer; Renshaw et al., 2015) or immediate freezing and maintenance of a cold chain from field to lab. In an effort to standardize collection methods, reduce field effort and field contamination in eDNA sampling, the ANDe™ (now called eDNA

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Sampler Backpack) was developed (Thomas et al., 2018) for freshwater systems. The instrument has online tutorials,² is relatively affordable, and regularly used by field practitioners (Thomas et al., 2020). Another simple and straightforward method for smaller water bodies in freshwater systems is for field personnel to scoop water into a plastic bottle, add a buffer, and maintain at ambient temperature (this can maintain integrity for up to 56 days; Williams et al., 2016). This approach does not require maintaining a cold chain in the field or using a backpack pump. Once the water is received at the laboratory, eDNA can be concentrated and captured through filtration, or other methods such as centrifugation, chemical precipitation, or electrical charge attraction with magnetic beads, which are positive and thus attract negatively charged DNA molecules (Renshaw et al., 2015; Williams et al., 2016). The decisions between filtering in the field or lab or using other lab-based methods for eDNA capture are largely based on the time field crews have to devote to eDNA collection and procedures necessary for an optimized eDNA assay where various methods have been tested for the specific target species and environmental source (e.g., Williams et al., 2016; Williams et al., 2017).

Marine eDNA collection methods include sampling from shore with buckets (The eDNA Society, 2019) or on board ships with various pumping apparatus (Hansen et al., 2007; Costello et al., 2017; Westfall et al., 2017; The eDNA Society, 2019). More recently, robotic samplers, including the use of autonomous vehicles for sampling marine systems at extreme depths, have also been successfully tested (Yamahara et al., 2019). There are efforts to compare various methods for DNA capture and isolation to determine best practices for obtaining the highest marine species diversity as well as reliable presence/absence surveys (Stoeckle et al., 2018; Jeunen et al., 2019; Shu et al., 2020). The distribution of DNA in an ecosystem depends on the abundance of the species, movement of the substrate (e.g. diffusion and dispersion of molecules of traces of DNA through the environment) (Furlan et al.,

2016), and biotic factors that degrade detectable DNA (Barnes & Turner, 2016). The application of mechanistic models to examine fate and transport of eDNA in marine systems has shown promise for revealing from where and when eDNA was shed from the source species (Andruszkiewicz et al., 2019). Thus, as in freshwater systems, it is critical to conduct pilot studies to better understand the appropriate method for eDNA capture for the target marine ecosystem.

For collecting eDNA from aquatic systems, both marine and freshwater, there is not a standard for collecting at any particular depth (Shu et al., 2020). However, the collection of particles from substrate should be avoided as the DNA may bind colloidal-ly to sediment particles and be retained for long periods (Barnes et al., 2014). Thus, sampling from shorelines can help avoid stirring sediment particles into the water column.

Filtering water requires identifying the balance between the volume of water one can pump through a filter, which is influenced by the degree of water turbidity and the desire to collect as many DNA particles or cells as possible. Most researchers have found that 5µm filters can handle 3–7 L of water throughput before clogging occurs and provide the optimal balance of higher throughput and DNA capture over smaller and larger pore size filters (Goldberg et al., 2017; Thomas et al., 2018; Jeunen et al., 2019). Although there is not a standard for the amount of water that needs to be collected (Shu et al., 2020), one thing that has become clear is that pooling water samples is not useful as it causes dilution and lowered probability of detection (Davis et al., 2018; Shu et al., 2020).

Environmental DNA can also be isolated from collected soil samples where the biodiversity of microorganisms (e.g. bacteria and fungi), soil invertebrate, animals, and plants can be assessed (Andersen et al., 2012; Cristescu and Hebert, 2018). Beyond collecting samples from the environment (e.g. water and soil), various substrates may have DNA traces that reveal a particular species' presence, such as browsed twigs for ungulate species (Nichols et al., 2012; Nichols & Spong, 2014; Nichols et al., 2015). To obtain such DNA simply requires swabbing the surface and isolating the

² <https://www.smith-root.com/support/tutorials>

DNA from the swab. An emerging use of eDNA is the exploitation of biological systems to subsample biodiversity, specifically targeting species that feed on blood (e.g. leeches, flies, Schnell et al., 2012; Gogarten et al., 2020), carrion (Calvignac-Spencer et al., 2013), or filter water for food (e.g. sponges, Mariani et al., 2019). Accessing these DNA sources provides a sample of biodiversity from the ecosystem where the insects and sponges live using standard DNA extraction methods. The ability to use organisms that harvest DNA from many species will continue to provide novel approaches to surveying biodiversity.

8.2.5 Data collection-DNA isolation, purification, and amplification

There is also a need to determine the ideal approach for eDNA isolation and purification (extraction from sample matrix after concentration and capture) as one approach does not fit all study species or ecosystems (Deiner et al., 2015; Jeunen et al., 2019). There are many commercially available kits designed to extract DNA (e.g. Qiagen DNeasy Kit™ among others). Pilot studies often test multiple kits or chemical methods (e.g. phenol/chloroform extractions) to see which one works best for their study system (Deiner et al., 2015; Williams et al., 2017; Shu et al., 2020). There has been no consistent pattern of best practices for DNA isolation and purification that has emerged, thus making it clear that testing for each study remains the best option (Goldberg et al., 2016; Kumar et al., 2020).

Largely, conventional PCR or qPCR is the standard DNA amplification platform (Goldberg et al., 2016; Klymus et al., 2020) for single-species detection using eDNA (for technical details, Taberlet et al., 2018), but ddPCR is becoming an appealing alternative because it eliminates the need for a standard curve, which takes up valuable sample space on a PCR plate, and is less affected by inhibitors (Doi et al., 2015; Hunter et al., 2017; Baker et al., 2018; Hunter et al., 2018). Further, ddPCR has been shown to outperform qPCR (Doi et al., 2015) and metabarcoding in the detection of a single-target species (Wood et al., 2019). A subset of positive samples are often sequenced using

conventional approaches for DNA sequencing of gene fragments (Sanger sequencing, Deiner et al., 2017a) and comparison to publicly available DNA sequence databases³ to confirm that the positives were, in fact, the target species.

NGS allows for simultaneous amplification of all DNA fragments in a mixed-species sample. Samples from the environment are treated to the same DNA isolation and purification processes as for a single-species assay. However, the next steps involve preparing DNA into fragments with additional portions of adapters and tagged primers added to generate 'libraries' of the field obtained eDNA (this process is detailed in Chapter 7, Taberlet et al., 2018). This is a time-consuming process requiring technical expertise but is regularly performed in most genetics laboratories and can be easily applied to biodiversity conservation studies through collaboration or pay-for-service.

8.2.6 Data analyses

Single-species targeted eDNA assays primarily provide presence/absence results (Goldberg et al., 2016). However, the quantitation of DNA amplification through time or DNA molecules per microliter from positive results can be used to estimate the relative abundance of a species (Doi et al., 2017). Interpretation of results need to be carefully considered as eDNA captures DNA that could have been incorporated into the water system through run-off of water crossing land and thus may include DNA from faecal samples or carcasses, which could lead to inflated abundance estimates (Merkes et al., 2014; Sepulveda et al., 2019). Further, one must decide if, given the number of replicates run for a single sample, whether a single positive among the replicates is enough to expend resources to deploy field confirmation efforts (Figure 8.1).

Bioinformatic tools are required to analyse NGS output. These analyses involve cleaning up spurious sequences generated in the process of amplification, trimming the adapters and primers off of sequences, and other data processing. The final

³ <https://www.ncbi.nlm.nih.gov/genbank/>
<https://www.ebi.ac.uk/ena>

step is matching generated sequences to either those available in publicly available databases or a laboratory-generated database for taxonomic identification (detailed in Taberlet et al., 2018; Ruppert et al., 2019). This effort can be computationally expensive and requires technical expertise, yet it can return an expansive data set of biodiversity from a simple sample of water, soil, air, etc.. In an effort to simplify data processing and analyses and make generating results more accessible to non-experts, the Anacapa Toolkit (Curd et al., 2019) was developed. This software module takes raw data and processes it, helps generate a database for taxonomic identifications, and provides statistics for biodiversity and genetic diversity assessments. As more conservation practitioners turn to eDNA for biodiversity assessments, the increasing need for user-friendly software will be further addressed.

8.3 Wider application—review of what has been done

In the realm of conservation, eDNA has become a fairly well-accepted tool in recent years (Lodge et al., 2012; Bohmann et al., 2014; Thomsen & Willerslev, 2015; Barnes & Turner, 2016; Deiner et al., 2017a; Harper et al., 2019). Environmental DNA as a tool for detection and monitoring of target species and biodiversity surveillance has largely been applied in aquatic systems (Deiner et al., 2015; Deiner et al., 2017a; Cristescu & Hebert, 2018).

Environmental DNA has been used extensively for the detection of fish species and fish biodiversity assessments across aquatic ecosystems for nearly a decade (see review, Thomsen et al., 2012b; Shu et al., 2020). This tool is capable of detecting a wide range of marine taxa from plankton and phytoplankton, fish, and mammals (Thomsen et al., 2012a; Thomsen et al., 2012b; Westfall et al., 2017; Hansen et al., 2018; Stoeckle et al., 2018; Shu et al., 2020). Environmental DNA has been used to monitor marine species of concern (Costello et al., 2017; Boussarie et al., 2018; Hansen et al., 2018) and invasive species (Westfall et al., 2017), including invasive species from ship's ballast water (Gerhard & Gunsch, 2019). Further, it has been shown to be quite sensitive;

for example, detection of killer whales was possible for up to 2 hours after animals left the area (Baker et al., 2018), and detection of species richness of shark species was increased by metabarcoding of eDNA over traditional methods (Boussarie et al., 2018). When compared to other methods of marine diversity measurements, eDNA appears to be comparable or more sensitive at times but may be limited in other cases (Boussarie et al., 2018; Jerde et al., 2019). Even so, a complete understanding of the strengths and limitations of eDNA in varying marine ecosystems and across species is still being worked out (Cristescu & Hebert, 2018; Hansen et al., 2018; Jerde et al., 2019). Environmental DNA sampling in marine ecosystems has been limited by the necessity of humans to collect water samples, particularly in deep waters. Autonomous robots have been tested and compared with the peristaltic pumps and freezing protocols used during manual capture for effective DNA capture and preservation of samples (Yamahara et al., 2019). This work demonstrates that the use of robots for collecting eDNA and assessing biodiversity has its own challenges, but also advantages, and promises to be another viable method for collecting eDNA from places where humans have difficulty accessing marine environments (Yamahara et al., 2019) and almost certainly in other systems as well.

Freshwater systems were testbeds of eDNA and many of the earliest studies focused on detecting invasive species (Ficetola et al., 2008b; Piaggio et al., 2014; Doi et al., 2015). More recently, eDNA continues to be a critical tool for detecting invasive species. For example, eDNA has been used to detect invasive dreissenid mussels in the United States (e.g. Amberg et al., 2019; Sepulveda et al., 2019; Shogren et al., 2019) and the United Kingdom (Blackman et al., 2020). Exploiting eDNA in a reservoir in Montana demonstrated that this tool was more effective at detecting invasive mussels at low densities than plankton tow samples and thus extended the seasons in which they could be detected (Sepulveda et al., 2019). Further, the ongoing novel development of eDNA assays for things such as invasive nutria (*Myocastor coypus*; Akamatsu et al., 2018), fish ectoparasites (Fossøy et al., 2020), and aquatic plants (Anglès d'Auriac et al., 2019) demonstrate the

utility of this tool in the detection of early stages of invasion and thus provide a critical alarm system for implementing management actions (see review in Mahon & Jerde, 2016). The study of vector-borne pathogens is important to predicting disease outbreak and managing pathogens to protect humans, wildlife, and domestic animals. The utility of eDNA to understand the movements of hosts that cannot otherwise be easily detected is highlighted by a study of the presence of freshwater snails (*Bulinus truncatus*) that are host to a human pathogen (*Schistosoma* parasites) (Mulero et al., 2020). The development of this eDNA assay will contribute to the documentation of the distribution of the snail and thus the potential distribution of a pathogen of human health concern and is a good model for other vector-borne diseases (Mulero et al., 2020). Environmental DNA in freshwater systems has also been used to detect species of concern (Barnes & Turner, 2016; Goldberg et al., 2016; Cristescu & Hebert, 2018; Cilleros et al., 2019; Cristescu, 2019; Huerlimann et al., 2020) and is still proving its utility in finding rare target species. In Japan, a new population of an endangered salamander, Yamato salamander (*Hynobius vandenburghi*) was predicted, through habitat modelling using Geographic Information Systems (GIS), and confirmatory presence was assessed using eDNA (Sakai et al., 2019). Environmental DNA used to monitor or detect endangered species is a particularly useful tool because it does not require the capture or handling of sensitive species, which can be vulnerable to the stresses of trapping, vulnerable to pathogens transmitted by humans, or difficult to detect through trapping (Adams et al., 2019). Importantly, beyond continuing to develop the ability to quantify biodiversity using NGS approaches, the inference of genetic diversity and other population genetic parameters among populations of species of concern will greatly expand the utility of eDNA in biodiversity conservation efforts (Adams et al., 2019; Sigsgaard et al., 2020).

An emerging utility of eDNA is identifying biodiversity of communities that are too small to otherwise characterize (termed 'Biomonitoring 2.0', Baird and Hajibabaei, 2012); these include terrestrial microbial communities (e.g. water quality, Gerhard & Gunsch, 2019), soil bacterial

communities (e.g. Fierer & Jackson, 2006; Warren-Rhodes et al., 2019), pathogens (e.g. avian influenza, Koçer, 2010), and pollen grain diversity (Bell et al., 2016) in air samples (Kraaijeveld et al., 2015), and on insects (Widmer et al., 2000). Samples from soil have provided an avenue to a more robust understanding of flora and fauna over the past 400,000 years (Hofreiter et al., 2003; Willerslev et al., 2003; Thomsen & Willerslev, 2015). The study of DNA of ancient ecosystems has also been greatly advanced by the ability to isolate and amplify DNA from ice cores (Willerslev et al., 2004; Willerslev et al., 2007). Recently, eDNA was used for understanding insect diversity visiting flowers. By collecting flowers and extracting DNA from them 135 arthropod species were detected, and clusters of species had associations with particular plant species (Thomsen & Sigsgaard, 2019). The pursuit for global biomonitoring through eDNA holds much promise for documenting the biodiversity of species that are easily seen, as well as ones that are not (Porter & Hajibabaei, 2018; McGee et al., 2019).

Methods for detecting terrestrial organisms using eDNA are less common for many species and ecosystems (Harper et al., 2019). Methods developed for collection of eDNA from terrestrial wildlife have included sampling ephemeral aquatic sources such as snow for vertebrate DNA from tracks (Franklin et al., 2019; Kinoshita et al., 2019), soil samples to assess vertebrate diversity (zoo animals; Andersen et al., 2012), watering holes for coyote detection (Rodgers & Mock, 2015), and freshwater sources for invasive species such as Burmese pythons (Piaggio et al., 2014), feral swine (Williams et al., 2017; Williams et al., 2018), and nutria (Akamatsu et al., 2018). Environmental DNA has also been collected from crops (Saito et al., 2008) and depredated sage-grouse eggs (Hopken et al., 2016) to detect predator species involved in damage or predation events. Vertebrate faunal diversity has been determined when DNA was collected from blood- or carrion-feeding insects (Calvignac-Spencer et al., 2013; Lynggaard et al., 2019), thus exploiting these species as ecosystem samplers. Plants have been swabbed for eDNA of herbivores such as deer and endangered mice (Nichols et al., 2012; Nichols & Spong, 2014; Nichols et al., 2015; Lyman et al., 2019) and to assess arthropod diversity

(Bittleston et al., 2016; Thomsen & Sigsgaard, 2019). As an effort to implement a sensitive tool to elucidate the influence of ungulate grazing on plant communities, the application of eDNA was tested (Nichols et al., 2012). It was demonstrated that buccal cells from saliva left behind on browsed twigs could amplify eDNA from various ungulate species and that it could be detected for up to 12 weeks after deposition (Nichols et al., 2012). Once the method was developed, it was then applied to a broader ecosystem in Sweden where ungulate species causing damage to tree plantations and browsing preferences among ungulate species were detected all using eDNA (Nichols & Spong, 2014; Nichols et al., 2015). Such studies have demonstrated that the development and application of the eDNA assay require extensive work but provide critical conservation and management results (Williams et al., 2016; Williams et al., 2017; Williams et al., 2018). The utility of eDNA for conservation and management of terrestrial species is only now becoming realized and, with more interest, it will become as routinely applied as it is in aquatic systems.

8.4 Case study

One of the largest programmes to implement eDNA for conservation purposes is for the management of a highly invasive fish species (Asian carp: Bighead Carp, *Hypophthalmichthys nobilis* and Silver Carp, *H. molitrix*). The United States Fish and Wildlife Service (USFWS) uses water samples to monitor the progress of invasion by Asian carp⁴ (USFWS, 2018), with the goal of detecting, managing, and eventually arresting the spread of these species in the Great Lakes, where they would decimate native fisheries and recreational value of waterways as they have done in other invaded areas (Darling & Mahon, 2011; Jerde et al., 2011). The need for eDNA was identified as a critical monitoring tool and species-specific primers were developed and tested in 2009–2010 (Jerde et al., 2011). The developed eDNA assay was applied to the monitoring programme alongside electrofishing and other methods. Environmental DNA results identified

that both carp species had moved north of the electric barriers set up to block their northward dispersal. Further, eDNA was more effective at documenting the invasive front than electrofishing implemented simultaneously for comparison between the methods (Jerde et al., 2011). Later work focused on sequencing the mitochondrial genome of both carp species and developing a suite of markers that could be used together to increase the probability of detection (Farrington et al., 2015). Current eDNA surveillance uses a set of primers that capture both species at once, and then qPCR positives are further tested with species-specific primers to assess which species were detected (Farrington et al., 2015; USFWS, 2018).

Some of this programme's critical components are collaborations with state agencies within the affected and potentially affected areas, and the carefully developed Quality Assurance Project Plan (Woldt et al., 2015; USFWS, 2018). This plan outlines the field and laboratory methods, laboratory validation and calibration results, quality control and assurance procedures, and criteria for declaring positive detections. It also describes the responsibilities of each participating partner agency. This project continues to refine its sampling strategy and its understanding of detection probabilities given the freshwater ecosystems where they are monitoring. Using their vast sampling and processing capabilities, they have used occurrence modelling (similar to occupancy models) and simulations to assess their probability of detection across time, habitats, and biotic factors (Mize et al., 2019). This work identified the ideal sample size per site, the best time of year, and types of water bodies to sample to accomplish the highest probability of detection of both invasive species of carp in the target freshwater ecosystem. The work to monitor these invasive species and manage the invasion using eDNA as a primary tool has not been without controversy (Darling and Mahon, 2011), but the USFWS has aimed to address the concerns through ongoing research (Jerde et al., 2011; Klymus et al., 2015; Erickson et al., 2019; Mize et al., 2019) and the Quality Assurance Project Plan (QAPP), which is regularly adapted and updated (USFWS, 2018). In many ways, this project leads the way as an example of how eDNA can be implemented on a broad scale to

⁴ <https://www.fws.gov/midwest/fisheries/eDNA.html>

aid and inform conservation management of native biodiversity and long-term monitoring of invasive species.

8.5 Limitations/constraints

Different organisms shed DNA differently and at different rates. DNA persistence in the environment is affected by numerous environmental variables (e.g. pH, temperature, UV, microbial organisms), and these may change across time (Taberlet et al., 2018). Further, eDNA is not distributed homogeneously in the environment and is affected by dispersion and diffusion in an aquatic habitat (Furlan et al., 2016). The co-occurrence of DNA inhibitors in the environment may reduce detection probability (McKee et al., 2015). Currently, eDNA cannot be used to assess a species' population size, but NGS allows relative comparison of signal across samples and some labs are successfully using the technology to provide abundance and biodiversity data. The attempts to address the limitations and constraint of eDNA application is addressed in more detail under future directions later in the chapter.

8.6 Social impact/privacy

There have thus far not been any privacy concerns documented associated with the use of eDNA. However, one might imagine an instance where a private landowner will not allow access to their property for a resource manager or researcher to monitor for an endangered species or a potentially invasive species on their property. However, the waters downstream can be sampled and successful detection could lead to further action or enforcement against the property owner. This could potentially lead to concerns about privacy, but this has yet to become an issue.

As with other tools that allow us to better understand biodiversity and associated anthropogenic impact, it is hard to account fully for social impacts. Environmental DNA has not been widely used to make management decisions without confirmation of eDNA results with another tool (Sepulveda et al., 2020). However, using the Daubert standard, which is used to evaluate reliability in United States Federal Courts, eDNA was determined to be a

viable, stand-alone method for rigorous decision-making under law (Sepulveda et al., 2020). The use of eDNA data in a structured decision-making framework could rigorously inform management actions (Sepulveda et al., 2020). With the application of eDNA to conservation management, the contributions of data from this tool to biodiversity conservation would be an important social impact. Environmental DNA has been shown to be a viable tool in detecting rare and elusive or small organisms, and now is proven to be valid data in a legal arena (Sepulveda et al., 2020). Thus the impacts to society through policy actions based on eDNA data will increase.

8.7 Future directions

Largely, those using eDNA are processing samples in the laboratory on benchtop instruments (e.g. rtPCR instruments, MiSeq, PacBio, etc.), but some are testing the applicability of handheld devices directly in the field (MinIon, NGS device, Jain et al., 2016; Deiner et al., 2017a) (Biomeme, rtPCR, Sepulveda et al., 2018). The promise of this advancing technology is that one can collect environmental samples and process them within an hour or two from a laptop and an instrument the size of a USB flash drive. The handheld NGS device, MinIon, has been used effectively in the field in Antarctica to obtain long-read sequences from ancient and modern microbial mats found in the environment (Johnson et al., 2017) and sequence ray-finned fish mitogenomes from water (Deiner et al., 2017a). The MinIon introduces more sequencing error than desirable for most eDNA applications, and challenges with sensitivity and susceptibility to PCR inhibition of the Biomeme platform means they are not yet ready to become the standard for eDNA studies yet (Sepulveda et al., 2018; Thomas et al., 2020). However, handheld field-deployable platforms are clearly the next advancement to come to eDNA applications. These developments aim to minimize sample contamination by having all collection steps completely contained within the instrument, making field-based sampling easier and detection more rapid (e.g. Sepulveda et al., 2018; Thomas et al., 2018; Thomas et al., 2019). This will

further facilitate the implementation of eDNA monitoring programmes to detect rare and endangered species or invasive species early in the invasion process when they might be halted.

The current state of using genomic approaches does not yet deliver on the promise of sequencing every single fragment of DNA from a mixed-species, environmental sample. Biases introduced from primers and other reagents, steps in the laboratory, or analytical process of NGS, which includes metabarcoding and metagenomics (whole-genome sequencing), do not allow quantification of the number of individuals or reliable detection of rare species quite yet. However, recent studies are advancing methods that allow for quantification from NGS data and it is highly likely in the near future that we will be able to use genomics to regularly assess abundance and reliably detect rare individuals (Bush et al., 2019; Doi et al., 2019; Shelton et al., 2019; Singer et al., 2019; Zinger et al., 2019). The recent application of a part of the bacterial immune system, clustered regularly interspaced short palindromic repeats (CRISPR) paired with Cas enzymes, has altered (Knott & Doudna, 2018), and will continue to provide innovation for, genomic sequencing applications that are directly relevant to eDNA as a biodiversity conservation tool (Phelps et al., 2020). As progress towards a single-species biosensor for environmental deployment, a proof-of-concept study demonstrated the use of CRISPR/Cas to induce fluorescence in the presence of a fish species (*Salmo salar*, Williams et al., 2019). This study is the first to apply CRISPR/Cas technology to eDNA and hints at the future promise of emerging technologies coupled with environmental sampling.

The collection of RNA from the environment may be another avenue for exploring biodiversity as well as pathogens (Cristescu, 2019). The use of eRNA would allow for a better understanding of gene expression in different habitats, but RNA is a very unstable molecule and thus hard to capture (Cristescu, 2019). Despite this, RNA has been captured and isolated from the environment, including from various fossil types (Cristescu, 2019). There is evidence from one study using metabarcoding that RNA for biodiversity studies may provide more

agreement to morphological surveys than DNA (Pochon et al., 2017). The isolation of RNA from the environment allows the detection of RNA viruses, such as the detection of SARS-CoV-2 in human wastewater in the Netherlands (Medema et al., 2020). The detection of this virus, which is shed in human faeces, occurred before human cases of the COVID-19 disease caused by the virus were reported (Medema et al., 2020). Thus, eDNA may serve as an early warning signal of an upcoming outbreak for this and other zoonotic pathogens. Studies have demonstrated the persistence of Avian Influenza Viruses (AIV), an RNA virus, in wild waters (see review in Stallknecht et al., 2010). These AIV viruses in water can infect wild birds, and thus the environment can serve as a transmission pathway (VanDalen et al., 2010). Highly pathogenic AIV subtypes were detected from eDNA near a poultry farm outbreak site (Borchardt et al., 2017). As AIV affect wildlife, livestock, and human health, it is an important model for the utility of eDNA in detecting zoonotic pathogens in the environment. It will be important for conservation scientists to continue to pursue the application of eDNA for pathogen detection for early warning systems and elucidating transmission pathways.

Loop-mediated isothermal amplification (LAMP) method is not a traditional PCR (Lee, 2017) and thus does not require expensive instrumentation. The isothermal amplification produces a highly specific and efficient amplification product if the target is available in a sample. This method is simple to apply in the field and has been used to identify bacterial, viral, parasitic, and fungal pathogens (Fu et al., 2011), yet it has not been widely applied to environmental detection or monitoring for conservation purposes. One advantage of a LAMP assay is it can easily be deployed as part of a biosensor through technology that already exists (Jones, 2016). Biosensors utilizing a LAMP assay and technology such as handheld dipsticks for single-species detection have already been developed for human pathogen detection (Prompamorn et al., 2011) but can produce false positives and require further development to achieve the promise they hold for the future of eDNA (Zasada et al., 2018). An ongoing effort to apply LAMP to eDNA and develop

a handheld dipstick to detect invasive carp⁵ has been in development (Merkes, 2020), but this is currently the only LAMP assay being tested for eDNA.

Recently, the University of California Conservation Genomics Consortium started an eDNA programme involving citizen scientists called CALeDNA (Meyer et al., 2019). This focuses on sampling soils and sediments within the University of California Reserve System and posting results online. This effort is a tremendous undertaking and offers a huge promise of providing a better understanding of biodiversity across California, documenting what is currently there, and monitoring biodiversity changes through time (Meyer et al., 2019). If successful and popular, it is likely that other groups will aim to undertake similar large-scale eDNA landscape biodiversity research approaches. On a national level, Canada has made efforts to develop and evaluate the utility of eDNA for fisheries and develop national standards for its use in the management of biodiversity in aquatic ecosystems (Baillie et al., 2019). This effort has led to the creation of a national technical working group charged with integrating eDNA into federal scientific research programmes that guide resource management decisions (Baillie et al., 2019). These types of regional or federal efforts to standardize the use of eDNA are important to the integration of this tool in biodiversity conservation and management of invasive species.

In this world of fast-paced technological advances, not all new methods prove useful in an applied context. Although eDNA has not been used regularly in biodiversity conservation for more than a decade, it has proven to be an extremely practical and informative tool. The utility of eDNA is supported by ongoing advancements and development of novel applications. There is no easy way to standardize the application or methods of eDNA as the conservation question, and the target system must drive the selection of a range of options at every step. However, guidelines

now exist for the best practices of optimizing a sampling scheme and sample processing for eDNA applications (Goldberg et al., 2016; Jeunen et al., 2019; Klymus et al., 2020; The eDNA Society, 2019; Shu et al., 2020). Further, the ranks of experienced eDNA practitioners have expanded globally; thus, it is fairly easy to find expert consultation. Therefore, it is now practical and prudent to adopt eDNA in the service of biodiversity conservation efforts.

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⁵ <https://www.usgs.gov/centers/umesc/science/developing-a-portable-lamp-assay-detecting-grass-and-black-carp>

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