The Promise and Pitfalls of Environmental DNA and RNA Approaches for the Monitoring of Human and Animal Pathogens from Aquatic Sources

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Novel forensics-inspired molecular approaches have revolutionized species detection in the wild and are particularly useful for tracing endangered or invasive species. These new environmental DNA or RNA (eDNA or eRNA)–based techniques are now being applied to human and animal pathogen surveillance, particularly in aquatic environments. They allow better disease monitoring (presence or absence and geographical spread) and understanding of pathogen occurrence and transmission, benefitting species conservation and, more recently, our understanding of the COVID-19 global human pandemic. In the present article, we summarize the benefits of eDNA-based monitoring, highlighted by two case studies: The first is a fibropapillomatosis tumor-associated herpesvirus (chelonid herpesvirus 5) driving a sea turtle panzootic, and the second relates to eRNA-based detection of the SARS-CoV-2 coronavirus driving the COVID-19 human pandemic. The limitations of eDNA- or eRNA-based approaches are also summarized, and future directions and recommendations of the field are discussed. Continuous eDNA- or eRNA-based monitoring programs can potentially improve human and animal health by predicting disease outbreaks in advance, facilitating proactive rather than reactive responses.

Keywords: environmental DNA, environmental RNA, endangered species, pathogens, SARS-CoV-2, ChHV5, fibropapillomatosis

Novel molecular approaches have recently revolutionized how field biologists detect and trace endangered species and problem invasive species in the wild (Thomsen and Willersley 2015, Ruppert et al. 2019, von Ammon et al. 2019). Exciting new techniques applied to environmental DNA and RNA (eDNA or eRNA) have begun to be used for the dynamic surveillance of human and animal pathogens (Peters et al. 2018, Miaud et al. 2019, Kumar et al. 2020, Randazzo et al. 2020, Yetsko et al. 2021). eDNA or eRNA is genetic material that has been shed from an organism into its surrounding environment (Seymour 2019). Aquatic environmental samples are particularly well suited to eDNA- or eRNA-based approaches, although DNA or RNA has been recovered from a wide variety of environmental sample types (Thomsen and Willerslev 2015, Seymour 2019). Genetic material, which is shed into the environment by multicellular organisms and their pathogens alike, can be extracted and analyzed in great detail with advanced methodologies that are applied to eDNA or eRNA to amplify or translate the molecular material into data suitable for downstream analyses and interpretation (Ficetola et al. 2008, Diaz-Ferguson and Moyer 2014, Rees et al. 2014b, Seymour 2019). These genetic tools are being employed to better understand the occurrence and transmission of pathogens hampering wildlife species conservation and, more recently, to monitor the virus driving the current global COVID-19 (coronavirus) human pandemic, as well as other human pathogens (Barnes and Turner 2016, Adams et al. 2019, Wu et al. 2020, Urban et al. 2021). Wildlife and human diseases are not mutually exclusive; 60% of emerging human pathogens are zoonotic (transmitted from animals to humans), of which 70% originated in wildlife populations (including the Ebola outbreak of 2014, the Zika outbreak of 2015, West Nile and Marburg viruses); therefore, it is imperative that both animal and human disease and the many factors that intertwine them are studied in conjunction

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(Cunningham et al. 2017). Such combined approaches to human and veterinary medicine are championed by the One Health and EcoHealth fields (Cutler et al. 2010, Cunningham et al. 2017, Whilde et al. 2017, Dyson 2018, Duffy and Martindale 2019, UF IFAS 2020). These emerging fields examine the interconnections between human, animal, plant, and environmental health and are driving forward the interdisciplinary study of emerging global disease (Whilde et al. 2017, UF IFAS 2020). Several publications have arisen from such studies, including an analysis of all known human emerging infectious diseases that confirmed that the majority (particularly viruses) are of animal origin (Kilpatrick and Altizer 2010, Cunningham et al. 2017). Further analysis has shown that, since these studies, emerging infectious diseases have increased in frequency, particularly those from wildlife hosts (Cunningham et al. 2017). The field of disease ecology—the study of the interconnected role of host, pathogen, environment and evolution—aims to understand the transmission and impact of infectious diseases on susceptible populations and is therefore an ever-evolving field that will benefit substantially from the repurposing of eDNA- or eRNA-based techniques for pathogen detection to assist global pathogen and disease monitoring efforts (Kilpatrick and Altizer 2010).

Before discussing how these approaches were repurposed for pathogen surveillance, it is worth outlining how they originally arose from an urgent need to improve biodiversity monitoring capabilities. Earth's rapidly declining biodiversity is a major challenge of the twenty-first century, requiring swift action to mitigate the sixth mass extinction event (Ceballos et al. 2020). To estimate local and global biodiversity loss and coordinate efficient conservation efforts, accurate and reliable biological monitoring and biodiversity evaluation is crucial to calculate species abundance and changes to population sizes over time (Ficetola et al. 2008, Rees et al. 2014b, Yates et al. 2019). It is also crucial to understand ecosystem dynamics and develop and implement succinct conservation management policies to conserve elusive, vulnerable and endangered species from the threats they face, both natural and anthropogenic (Seymour et al. 2018, Adams et al. 2019). These challenges, along with the limitations of traditional field monitoring approaches, have encouraged the rapid development and adoption of molecular biology-based approaches to detect DNA shed or excreted from target species, including assessing the biodiversity of microbial assemblages. eDNA- or eRNA-based approaches can greatly complement more traditional methodologies such as visual sightings, camera traps, estimation surveys, and lab culturing of microorganisms from environmental samples (Jerde et al. 2011, Deiner et al. 2017, Qu and Stewart 2019, Strand et al. 2019, Tang et al. 2019).

However, accurate and efficient detection and monitoring of endangered species may not be enough to adequately protect and conserve vulnerable populations. Accurate detection and monitoring of the pathogens affecting a species' survival is also essential in order to devise and implement appropriate mitigation policies (Miaud et al. 2019). Pathogenic viruses have the ability to infect susceptible species without presenting obvious symptoms (Miaud et al. 2019). Parasitic diseases and pathogenic microalgae (including those that produce toxic harmful algal blooms) can result in the mass mortalities of freshwater and marine organisms, from a wide range of taxa (including humans) globally (Huver et al. 2015, Peters et al. 2018, Sato et al. 2019). Consequently, accurate, sensitive, noninvasive techniques are required to detect and monitor pathogens outside of host systems and to efficiently monitor disease ecology, wildlife conservation management, and the risk of infection to humans from aquatic transmitted pathogens (Huver et al. 2015, Miaud et al. 2019, Sato et al. 2019). Current practices rely on single species from a designated habitat to assign scores to infer a community-based assessment—as opposed to using biodiversity to track changes in populations (Seymour et al. 2020). It will be important for future effective biomonitoring to incorporate traditional community biomonitoring scores with molecular-informed population-based assessments, such as eDNA metabarcoding and eRNA shotgun sequencing, in order to incorporate a wide range of taxa that is traditionally overlooked (Broman et al. 2020, Seymour et al. 2020). Therefore, methodologies originating from the study of microbial diversity—and, subsequently, megafauna presence and diversity—are now being refocused on disease-causing microbes of animals and humans.

Detecting and monitoring elusive, low-density, vulnerable species and their infectious pathogens is particularly challenging in aquatic environments, and as a result, past research has relied on traditional capture and observation-based surveys that are relatively expensive (particularly with advancements in drone and artificial intelligence usage), inefficient, and invasive (Ficetola et al. 2008, Rees et al. 2014b, Roussel et al. 2015, Goldberg et al. 2016, Boussarie et al. 2018, Evans and Lamberti 2018, Erickson et al. 2019, Tang et al. 2019). For example, Jerde and colleagues (2011) used traditional field methods to attempt to evaluate rare aquatic species detection and took over 90 hours to locate just one individual of the study species (silver carp, *Hypophthalmichthys molitrix*). This may in part have been because of the limitation of the specific traditional approach employed for deep water capture. By contrast, the increased efficacy, accuracy, sensitivity and efficiency of eDNA methods has been demonstrated (Ficetola et al. 2008, Wolffs et al. 2011, Rees et al. 2014b, Valentini et al. 2016, Deiner et al. 2017, Miaud et al. 2019). However, eDNA-based methods do not always produce significantly better results, particularly in natural large-scale environments or with species that do not shed eDNA regularly, such as crustaceans (in comparison with fish and amphibians, which are known to excrete significantly more extracellular DNA via mucus secretions; Treguier et al. 2014). Comprehensive comparisons of eDNA-based approaches with more conventional approaches have been reviewed elsewhere (Roussel et al. 2015, Thomsen and Willersley 2015, Goldberg et al. 2016,

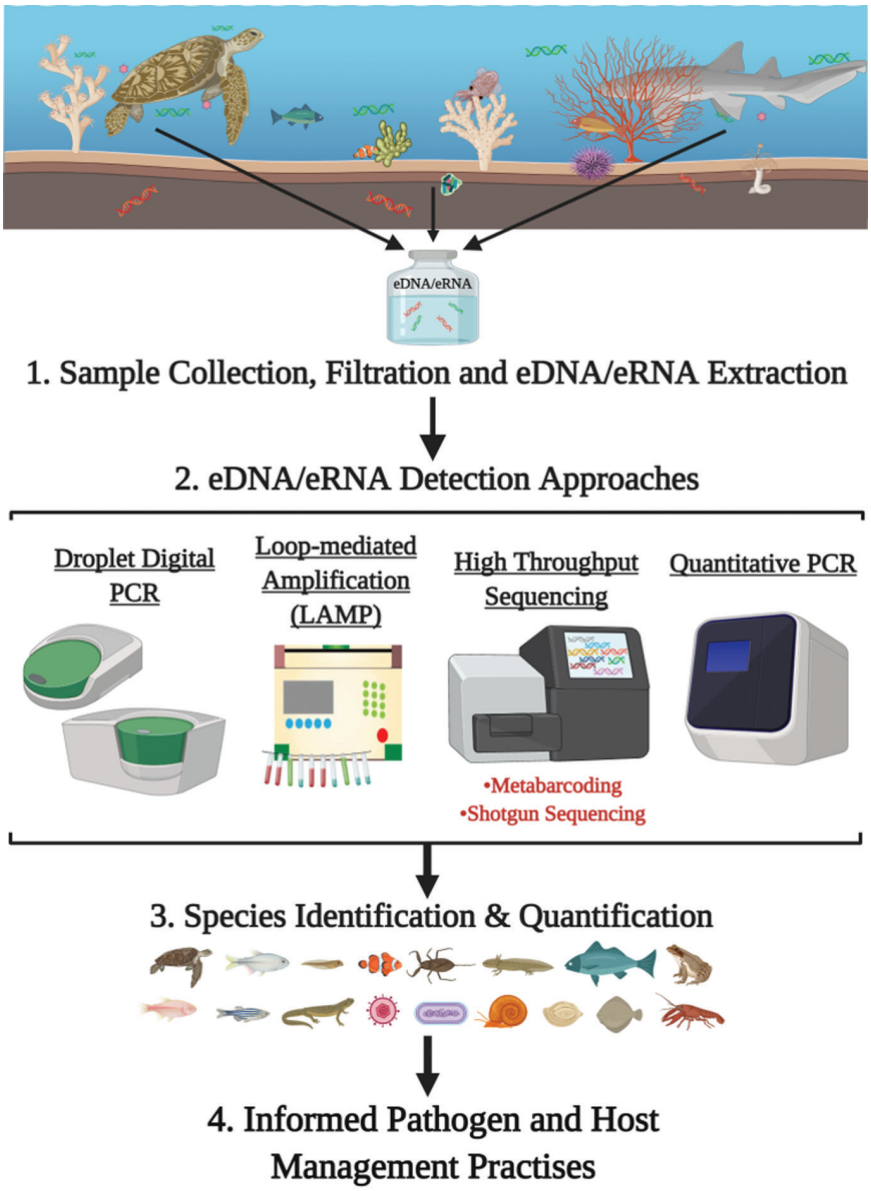

Figure 1. Schematic of eDNA methodologies for the environmental detection of pathogens and endemic species (generated using Biorender, https://biorender.com).

Smart et al. 2016, Ficetola et al. 2019, Ruppert et al. 2019, Yates et al. 2019). Similarly, Wolffs and colleagues (2011) demonstrated this increased efficacy, accuracy, sensitivity, and efficiency with respect to the detection of viral loads in a human clinical setting from fecal samples when compared with conventional viral diagnostics. Their polymerase chain reaction (PCR)-based testing was able to detect an astonishing 81 previously undetected viruses (Wolffs et al. 2011). With the development and improvement of novel applications of these molecular and genetic technologies, environmental sampling of marine and freshwater ecosystems alike has the potential to rapidly increase in accuracy and efficiency (Raemy and Ursenbacher 2018, Qu and Stewart 2019). These genetic technological advancements are crucial for detecting and monitoring low density threatened aquatic species, as well as for early detection and implementation of management responses regarding harmful pathogens (Jerde et al. 2011, Rees et al. 2014b, Barnes and Turner 2016).

The most widely employed environmental genetic detection tool is eDNA-based analysis (figure 1)—the molecular extraction and identification of released genetic material (DNA shed from sources such as urine, feces, saliva, and skin) from the environment (samples such as water, sand, soil, sediment, mud, ice and air; Diaz-Ferguson and Moyer 2014, Rees et al. 2014b, Roussel et al. 2015, Barnes and Turner 2016, Boussarie et al. 2018, Evans and Lamberti 2018, Qu and Stewart 2019, Strand et al. 2019). This rapidly advancing, noninvasive approach is capable of improving aquatic endangered species detection and early pathogen detection with a variety of applications including targeted species-specific detection and multispecies metabarcoding, by analyzing extracted DNA using a variety of methodologies such as quantitative PCR (qPCR), droplet digital PCR (ddPCR), loop-mediated isothermal amplification (LAMP) or high-throughput sequencing (table 1; Diaz-Ferguson and Moyer 2014, Roussel et al. 2015, Soliman et al. 2015, Thomsen and Willersley 2015, Goldberg et al. 2016, Boussarie et al. 2018, Evans and Lamberti 2018, Erickson et al. 2019, Strand et al. 2019, Winkworth et al. 2020). Species-specific qPCR assays have been designed for several marine pests including dinoflagellates (*Alexandrium* spp.), sea squirts (*Styela clava* and *Didemnum* spp.), the Amur River clam (*Potamocorbula amurensis*), and the Mediterranean fan worm (*Sabella spallanzanii*; Zaiko et al. 2018, von Ammon et al. 2019). Examples of the use of species-specific PCR in marine biosecurity include application to the Atlantic wedge clam (*Rangia cuneata*), the soft-shell clam (*Mya arenaria*), and the Australian tubeworm (*Ficopomatus enigmaticus*; Zaiko et al. 2018). Therefore, it is evident that eDNA has already been successfully implemented in numerous scientific studies, including one of our two case studies, the fibropapillomatosis panzootic in green sea turtles (*Chelonia mydas*; Work et al. 2015, Chaves et al. 2017, Page-Karjian et al. 2017, Farrell et al. 2018, Yetsko et al. 2021).

Table 1. Description of each of the eDNA- or eRNA-based methodologies discussed within this article.

Methodology	Description
qPCR	Quantitative PCR monitors the amplification of a targeted gene (DNA or cDNA) during a polymerase chain reaction and can be used to measure the presence or absence and quantity of a particular gene in a sample.
ddPCR	Digital droplet PCR is an adaptation of conventional PCR with water–oil emulsion droplet technology, that can be used to quantify and clonally amplify nucleic acid strands including both DNA and cDNA from RNA.
LAMP or RT-LAMP	Loop-mediated isothermal amplification is a technique that can be used to amplify and detect nucleic acids in a single tube with a constant temperature. Reverse transcription LAMP can be applied to the detection of RNA. LAMP methods are widely regarded as a low-cost alternative to the detection of several human diseases.
High-throughput sequencing or NGS	High-throughput (or next-generation) sequencing can be used to rapidly determine the DNA or RNA (via cDNA) sequences within a sample and can be used for a variety of approaches for eDNA including metabarcoding and shotgun sequencing. It is a highly efficient technology that is capable of sequencing hundreds of millions of DNA molecules simultaneously.
LFA	Lateral flow assays (or immunochromatographic assays) are easy to operate tests that can detect a target gene in a liquid sample (environmental or host based) rapidly and without the need for advanced personnel training or specialized facilities. LFAs run the liquid sample along the surface of a pad with reactive molecules that show a visual positive or negative signal.
CRISPR-based SHERLOCK	CRISPR-based SHERLOCK (for *specific high-sensitivity enzymatic reporter unlocking*) can detect the presence of multiple genetic targets (RNA or DNA transcribed into RNA) at one time, without the need for DNA extraction, and uses lateral flow (paper) strips. The reporter is cleaved and emits signal only in the presence of the target sequence.

An alternative environmental genetic detection tool is the analysis of eRNA, a method that uses similar detection approaches to the analysis of eDNA but that, instead, is targeted against RNA molecules (transcripts). Environmental RNA-based approaches have helped to discriminate between "contemporary intracellular (living or recently shed) and extracellularly persistent (legacy) genetic fragments" (von Ammon et al. 2019, Broman et al. 2020). Environmental RNA analysis has proven to be a reliable predictor for both living species and viral pathogens in aquatic habitats and is the accurate and efficient methodology applied in our second case study, the COVID-19 pandemic-related early viral detection from human wastewater (Kumar et al. 2020, Randazzo et al. 2020, Wu et al. 2020). With the urgent need for rapid and sensitive viral detection in response to this human pandemic, there has been a surge in more novel detection approaches including reverse transcription-LAMP (RT-LAMP), lateral flow assays (LFA) and CRISPR-based SHERLOCK testing; however, their current application to environmental detection (as opposed to blood or plasma detection), although it is promising, is still relatively limited and warrants further investigation (table 1; Dao Thi et al. 2020, Ganguli et al. 2020, Grant et al. 2020, Halliday and Pidd 2020, Lall 2020, Ragnesola et al. 2020, Sheridan 2020, Thompson and Lei 2020).

Although eDNA and eRNA-based methodologies have the potential to be efficient, cost-effective, noninvasive, and crucial to improving aquatic pathogen monitoring and communicable disease outbreak forecasting, there remains considerable uncertainty regarding methodological limitations of these novel approaches. Each of these approaches will be evaluated within the context of aquatic pathogen detection. In the present article, we will first summarize the benefits of eDNA-based applications highlighted by a sea turtle viral panzootic case study then eRNA-based applications highlighted by a human viral pandemic case study. The limitations of both eDNA- and eRNA-based approaches will be summarized, followed by the discussion of future directions of the field (table 2).

Aquatic pathogen eDNA

Endangered species conservation management policies are only effective if the data used to inform such policies are accurate and reliable (Qu and Stewart 2019), and the same is true for management of their pathogens. Early detection of threats to such species, including the presence of harmful invasive species and pathogens, is critical for successful wildlife management and conservation (Goldberg et al. 2013, Diaz-Ferguson and Moyer 2014). The extensive economic and personnel requirements necessary to conduct traditional aquatic species and pathogen detection research have limited global endangered species monitoring and impeded wildlife health assessments (Goldberg et al. 2013, Goldberg et al. 2016, Qu and Stewart 2019). Fortunately, since the increased application of novel, cost-effective, and efficient approaches, pathogen detection, and monitoring have improved (Ficetola et al. 2008, Smart et al. 2016, Miaud at el. 2019, Strand et al. 2019, Randazzo et al. 2020, Yetsko et al. 2021). For aquatic eDNA-based studies, water samples (fresh or marine) typically in the range of 0.5–5 liters are passed through filter membranes (or 0.5–2 liters for the most commonly used 0.2-micrometer filter membrane) to capture cellular and DNA material (Machler et al. 2015). However, the precise volume and filter size are still debated in the literature and needs to be selected on the basis of the water sample type (marine, freshwater, stagnant, flowing, surface, depth) and target species size (vertebrate, invertebrate, parasite, virus etc.; Moushomi et al. 2019). DNA is then extracted from the filter and species can by identified by PCR

Table 2. Summary of the benefits and limitations of environmental DNA and RNA approaches, compared with traditional approaches (such as capture- or observation-based surveys, visual sightings, camera traps, estimation surveys, lab cultures), for aquatic pathogen monitoring and host detection.

Benefits	**eDNA**	**eRNA**	**Traditional**
Host and pathogen			
Cost effective	Yes (Y)	No (N)	N
Easy and efficient sample collection	Y (more so)	Y	N
Applicable in areas in which traditional methods are not	Y	Y	N
High sensitivity	Y	Y	N
Improved ecosystem health monitoring: Early detection of aquatic pathogens or transmission vectors or harmful invasive species, advanced warning	Y	Y	N
Both endangered species and their pathogens can be simultaneously detected from the same water samples, streamlining management practices	Y	Y	N
Potential to determine the full spectrum of biodiversity and pathogen diversity from a single aquatic sample	Y	Y	N
Continuous technological advancements and improving methodologies are making them more widely available or applicable	Y	Y	Y
Degradation in environment, particularly marine settings, leading to close-to-real-time detection	Y	Y (more so)	Y
Persistence in environment after organism has vacated (legacy genetic fragments) allowing longer-term detection (particularly freshwater settings)	Y	N	N
Host only			
Noninvasive	Y	Y	N
Pathogen only			
Increased accuracy of low-density aquatic pathogen detection	Y	Y	N
Advanced warning capacity of disease outbreaks and mass mortality events	Y	Y	N
Detection of pathogens that are difficult to culture	Y	Y	N
Limitations			
Host and pathogen			
Degradation in environment, particularly marine settings, leading to false negatives	Y	Y (more so)	N
Imperfect detection: False negatives, particularly due to low or subclinical concentrations or abundances	Y	Y	Y (more so)
Imperfect detection: False positives, particularly due to increased sensitivity and contamination potential	Y	N	N
Unknown or undetected target species genetic variation	Y	Y	N
Potential marker selection variability	Y	Y	N
Detection threshold limits due to low eDNA or eRNA concentrations	Y	Y	N
For most approaches, prior sequence information for pathogens or hosts is required	Y	Y	N
Specialized storage required	Y	Y (more so)	N
Persistence in environment after organism has vacated (legacy genetic fragments) leading to false positives and temporally misleading data	Y	N	N
Host only			
Limitations on species abundance estimation (although less prevalent for pathogen infection abundances, particularly in highly prioritized human disease)	Y	Y	Y
Limited ability to discern population structure, size, or sex	N	Y	Y

or high-throughput sequencing approaches (figure 1). Protocols based on ethanol precipitation without filtration have also been applied for some studies (Rees et al. 2014a). For in-depth reviews of the various methodologies applicable to eDNA analysis please see Ficetola and colleagues (2008), Diaz-Ferguson and Moyer (2014), Rees and colleagues (2014b), Thomsen and Willerslev (2015), Goldberg and colleagues (2016), Deiner and colleagues (2017), Qu and Stewart (2019) and Ruppert and colleagues (2019).

Recent applications of eDNA-based approaches have indicated the potential for early detection and identification of invasive species and infectious pathogens such as metazoan parasites, fungi (Chytrid and Oomycota), and viruses (*Herpesviruses* and *Ranaviruses*; Diaz-Ferguson and Moyer 2014, Lodge et al. 2016, Miaud et al. 2019, Yetsko et al. 2021). Studies employing LAMP technology have successfully detected a pathogenic oomycete (*Phytophthora agathidicida*) from soil with high specificity and sensitivity

(from as little quantities as 1 femtogram of total oomycete DNA; Winkworth et al. 2020). Cost-effective LAMP assays, in comparison with the equivalent PCR-based technology, do not require a specialized laboratory, personnel, or equipment and, with optimization for use with other sample types (such as from aquatic environments), could empower both scientific and nonscientific communities to monitor the wildlife pathogens affecting their species of economic and conservation importance (Soliman et al. 2015, Winkworth et al. 2020). Current research using eDNA metabarcoding has detected various salmonid pathogens in the aquaculture industry, as well as numerous pathogenic species from seawater including two pathogenic parasites (*Lepeophtheirus salmonis* and *Paramoeba perurans*) and three microalgae pathogenic species (*Prymnesium parvum, Pseudo-nitzschia seriata,* and *Pseudo-nitzschia delicatissima*; Peters et al. 2018). These studies demonstrate the potential of applying eDNA sequencing methodologies to identify multiple species simultaneously from field samples (Barnes and Turner 2016).

One aquatic pathogen not just detected but monitored by eDNA-based approaches is the Oomycota fungus, *Aphanomyces astaci*, the causative agent of crayfish plague in European noble crayfish (*Astacus astacus*; Miaud et al. 2019, Strand et al. 2019). Researchers were able to not only detect the pathogen at a single time point but achieved consistent reliable detection over the course of the study, demonstrating the ability to track and monitor eDNA amplification over time. Traditional monitoring of this crayfish plague involved trapping and observing the survival of cage-held crayfish, a high-stress approach that has raised ethical concern (Strand et al. 2019). However, with the recent application of species-specific qPCR-based eDNA, *A. astaci* can now be detected without requiring any disturbance to crayfish. Furthermore, *A. astaci* eDNA has been detected in the environment almost 3 weeks prior to when traditional methods could detect *A. astaci*. Importantly, eDNA-based approaches detected the presence of *A. astaci* prior to crayfish mortality events, demonstrating the ability of eDNA-based techniques to provide advanced warning of impending infection and mass mortality events in addition to accurate wildlife health status monitoring and surveillance (Goldberg et al. 2013, Diaz-Ferguson and Moyer 2014, Miaud et al. 2019, Strand et al. 2019).

Taking aquatic pathogen monitoring further, Huver and colleagues (2015) used single-species qPCR to detect the pathogenic parasite *Ribeiroia ondatrae,* a nematode found in North American amphibians. This detection method—as with Winkworth and colleagues' (2020) LAMP methodology—allowed for the positive detection of *R. ondatrae* at extremely low levels (as low as 14 femtograms of this parasite's DNA from 500 milliliters of field water samples), demonstrating the ability of both LAMP-based and qPCR-based eDNA methodologies to successfully detect low density pathogenic species (Huver et al. 2015, Winkworth et al. 2020). Huver and colleagues (2015) and Winkworth and colleagues (2020) also established that *R. ondatrae* DNA was detectable from *in vitro* mesocosms after 21 days of *R. ondatrae* introduction at 25 degrees Celsius (21 days of degradation) and revealed that their qPCR amplification cycle threshold (C_t) value could be used as a significant predictor of *R. ondatrae* infection abundance. Several studies have demonstrated similar success of eDNA-based approaches in determining a link between increased pathogen abundance and host species mortalities in aquatic environments by investigating pathogen, host, and environment interactions, further positioning the eDNA-based approach as a critical tool for disease ecology and wildlife conservation (Gomes et al. 2019). In one such study (Miaud et al. 2019), *Ranaviruses* were detected by qPCR- and sequencing-based eDNA analysis in the environmental water samples of susceptible host species, demonstrating a strong relationship between environmentally shed viral load and host tissue viral load.

eDNA case study: Detection and shedding dynamics of a viral pathogen of an endangered species. With the combination of qPCR and sequencing-based eDNA approaches, studies have started to optimize aquatic species and pathogen detection at lower limits and in larger waterbodies, as well as elucidate a positive relationship between species abundance and eDNA concentration (Ficetola et al. 2008, Takahara et al. 2012, Klymus et al. 2015, Evans and Lamberti 2018, Yates et al. 2019, Yetsko et al. 2021). Some studies have even used eDNA-based approaches to detect not only harmful pathogens but their transmission vectors (mosquitoes; Schneider et al. 2016), supporting the theory that eDNA-based techniques can be extremely effective when monitoring pathogens and their vectors of disease, especially when applying a multispecies targeting approach such as high-throughput sequencing (Schneider et al. 2016, Peters et al. 2018). The case study discussed in this section, the fibropapillomatosis panzootic in green sea turtles (*Chelonia mydas*), will evaluate the application and future potential of eDNA-based optimization to investigate this aquatic pathogen and its susceptible host species.

One pathogen of particular concern in relation to the conservation of endangered marine turtle species and the monitoring of harmful pathogens is the turtle-specific DNA virus, chelonid herpesvirus 5 (ChHV5; Chaves et al. 2017, Yetsko et al. 2021). This alphaherpesvirus has been identified as the most likely etiological agent of a devastating neoplastic disease, fibropapillomatosis, affecting all seven species of sea turtle, particularly juvenile green sea turtles (*Chelonia mydas*) found in tropical and subtropical oceans worldwide (Chaves et al. 2017, Farrell et al. 2018). Fibropapillomatosis can result in fatalities through both direct and indirect mechanisms. Chelonid herpesvirus 5 can infect endangered sea turtle species without presenting symptoms of infection until environmental cofactors trigger tumor formation (figure 2). Therefore, diagnostic techniques capable of detecting the pathogen prior to host mortality events and outbreaks is vital in order to minimize outbreak severity,

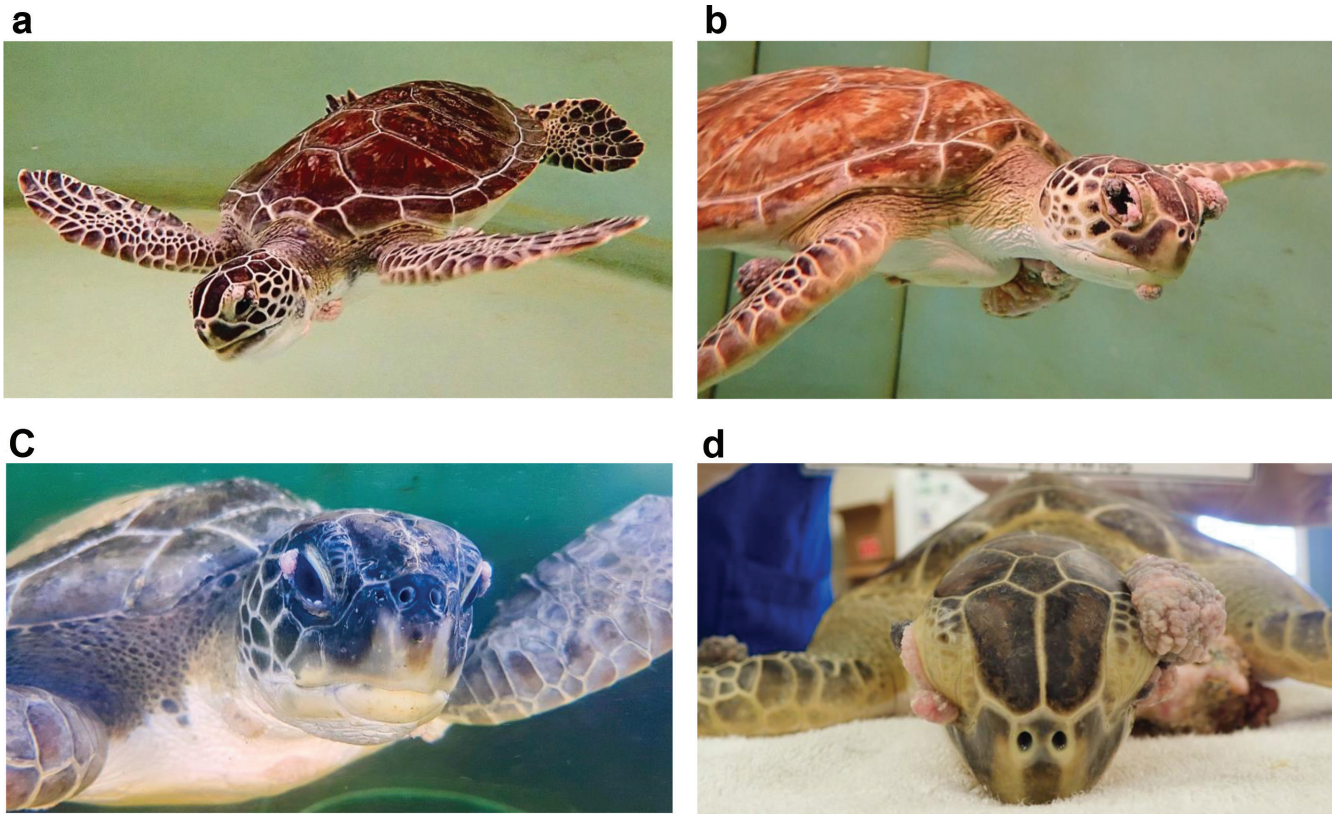

Figure 2. Juvenile Chelonia mydas patients at the University of Florida's Whitney Sea Turtle Hospital being treated for fibropapillomatosis. Photographs: Devon Rollinson-Ramia, Nancy Condron, Liam Whitmore.

where possible, or rapidly initiate recovery management plans (Page-Karjian et al. 2015, Chaves et al. 2017, Miaud et al. 2019). Furthermore, proactive early detection can help predict the spread of pathogens to nearby new and susceptible geographic locations and populations in advance, providing opportunities to implement prevention and mitigation strategies (Page-Karjian et al. 2015, Chaves et al. 2017, Miaud et al. 2019). In the case of human pathogens such as SARS-CoV-2, early detection from wastewater can enable medical facilities to prepare for an increase in patient numbers,and the reinforcement of public health measures to minimize transmission prior to mass patient presentation, diagnostic, and mortality events. Several modes of transmission have been proposed for ChHV5 and its variants, including mechanical leech or cleaner fish vectors, direct contact between turtles, viral transmission via the excretion of corporeal fluids, and epithelial viral shedding into the water column (Greenblatt et al. 2004, Work et al. 2015, Chaves et al. 2017, Page-Karjian et al. 2017, Work et al. 2020). Some evidence suggests that the escalation of fibropapillomatosis incidence from regional epizootics to a full scale panzootic may have depended on superspreaders—a few highly infectious individuals growing numerous small tumors permissive to extensive viral production, enabling exacerbated ChHV5 transmission and contributing disproportionately to secondary infections (Work et al. 2015, Chaves et al. 2017). Epithelial viral shedding in juvenile *C. mydas* appears to be inconsistent, with 65% of turtles not actively shedding, and those that are shedding only featuring epidermal intranuclear inclusions in 7% of tumors (Work et al. 2015). This provides evidence for superspreaders being primarily responsible for ChHV5 transmission, but the superspreader hypothesis is based primarily on examination of tumor tissue (Work et al. 2015) and corporeal fluids (Chaves et al. 2017, Page-Karjian et al. 2017) without direct evidence of quantifying viral levels shed into the environment.

Work and colleagues (2015) explicitly stated that "if a rapid and high-throughput sampling scheme to detect virus shedding could be developed, surveillance efforts would be optimized" (p. 1200), and consequently, the performance of wildlife health status monitoring could be improved (Deiner et al. 2017, Miaud et al. 2019). Application of eDNA-based approaches has the potential to investigate the superspreader hypothesis by quantifying the level of virus shed into the environment by different fibropapillomatosis-afflicted individuals. Such investigation would first begin in a controlled setting in which shedding rates could be confidently assigned to individual turtles. The capacity of eDNA-based methods to successfully detect and quantify environmentally shed ChHV5 from individual infected sea turtles in such a

rehabilitory setting has been recently demonstrated (Yetsko et al. 2021). In addition, fluctuations of environmental virus concentration over time have been successfully monitored and correlated to the tumor burden of the infected individuals (Yetsko et al. 2021). By comparing these environmental viral concentrations with the tumor burden (size and quantity) of individuals and the presence or absence of epidermal intranuclear inclusions, eDNA analysis has the potential to determine the relationship between sea turtle tumor status and potential viral shedding-based transmission. Theoretically, if eDNA analysis detects higher environmental ChHV5 concentration from turtles with small, numerous tumors, those individuals could be confirmed as superspreaders. Such eDNA-based findings have the potential to inform both rehabilitation and wildlife management policies, because the identification of superspreaders would highlight the need to isolate highly infectious individuals or even remove them from wild populations entirely (Work et al. 2015, Chaves et al. 2017). Conversely, if ChHV5 superspreaders do not exist or if viral shedding is linked to specific time periods and disease states, alternative mitigation strategies would be more effective. The use of sensitive, novel molecular technologies such as eDNA-based methodologies are imperative to not only monitor endangered aquatic species but to also allow for the early detection of pathogen presence in the environment of these vulnerable species and populations (Work et al. 2015, Barnes and Turner 2016, Yetsko et al. 2021). The information gleaned from such novel approaches is also crucial to enabling the rational design of management strategies on the basis of detailed quantitative understanding of pathogen shedding and transmission dynamics (Yetsko et al. 2021).

Given the versatility of eDNA-based approaches, both aquatic endangered species and their pathogens or parasites can be simultaneously detected from the same water samples, further streamlining management practices. For example, both sea turtle eDNA and ChHV5 eDNA have been readily detected from the same samples by either single-species qPCR or deep sequencing approaches (Yetsko et al. 2021). This could be further adapted to simultaneously detect the presence of one of the proposed mechanical vectors of the disease—marine leeches (*Ozobranchus* spp.; Greenblatt et al. 2004)—to investigate the interaction between the host species and direct transmission through the water column, as opposed to vector-based transmission.

Aquatic pathogen eRNA

One alternative to an eDNA-based approach is the analysis of eRNA. eRNA analysis can also be used simultaneously to complement eDNA-based approaches (Zaiko et al. 2018, Merou et al. 2020). In recent studies of marine fanworms (*Sabella spallanzanii*) and bioindicator microeukaryote groups such as nematodes, the combination of eDNA analysis with high-throughput shotgun sequencing of eRNA has helped to discriminate between "contemporary intracellular (living) and extracellularly persistent (legacy) genetic fragments" (von Ammon et al. 2019, p. 1, Broman et al. 2020). The analysis of eRNA provides discriminating data between viable and inviable cells, and more accurate data concerning the presence and spread of pathogens in aquatic environments (Goldberg et al. 2016, Broman et al. 2020, Merou et al. 2020). Single-species qPCR-based eRNA approaches have already been successfully used in numerous wildlife health studies, including the detection and quantification of a protozoan parasite (*Bonamia ostreae*) in seawater (Merou et al. 2020). Merou and colleaues (2020) suggested higher temporal sensitivity of their eRNA-based approach when compared with their eDNA-based approach and proved the significant importance of using this genetic tool to monitor pathogen presence and transmission and gain a deeper insight into the life cycle of this uncultivable, detrimental pathogen (Merou et al. 2020). There are both advantages and limitations to the deployment of eDNA- and eRNA-based tools alike, particularly when managing invasive and pathogenic aquatic species or using aquatic samples for the detection of pathogens with terrestrial hosts. eDNA or eRNA samples enable rapid assessment of environments and added statistical power; this lends itself to species distribution mapping and pathogen presence prediction when used in conjunction with abiotic or physical model inputs, as was demonstrated by a study of salmonid parasites in river habitats (Carraro et al. 2018). Furthermore, Zaiko and colleagues (2018) completed a thorough assessment of the performance of several environmental detection technologies with respect to obtaining marine biosecurity-relevant biodiversity information and found that although species-specific eDNA or eRNA qPCR or ddPCR was better for biosecurity applications (with eDNA performing 10% higher overall with regards to feasibility, quantification, cost efficiency, early warning, and low environmental impact), eDNA or eRNA metabarcoding was most suitable for obtaining species inventory information.

eRNA case study: Detection and surveillance of a human pandemic-inducing coronavirus in human wastewater by aquatic eRNA. A pathogen currently of major significance to human populations worldwide is the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; Randazzo et al. 2020, Wu et al. 2020). This single-stranded RNA virus is the third zoonotic coronavirus in as many decades to emerge, but the only one with pandemic potential, and it has rapidly developed into a pandemic—COVID-19—with over 86 million cases and 1.8 million deaths worldwide as of 6 January 2021 (Johns Hopkins 2020, Mackenzie and Smith 2020, Randazzo et al. 2020, Rohde 2020). Symptoms of this zoonotic disease include fever, cough, diarrhea, and respiratory distress that can result in organ failure, viral sepsis, and death (Randazzo et al. 2020). Because of the rapid widespread nature of the disease—and the lack of an easy diagnosis, a treatment, and a vaccine—novel measures must be employed to assist in monitoring, tracking, and predicting the spread of localized

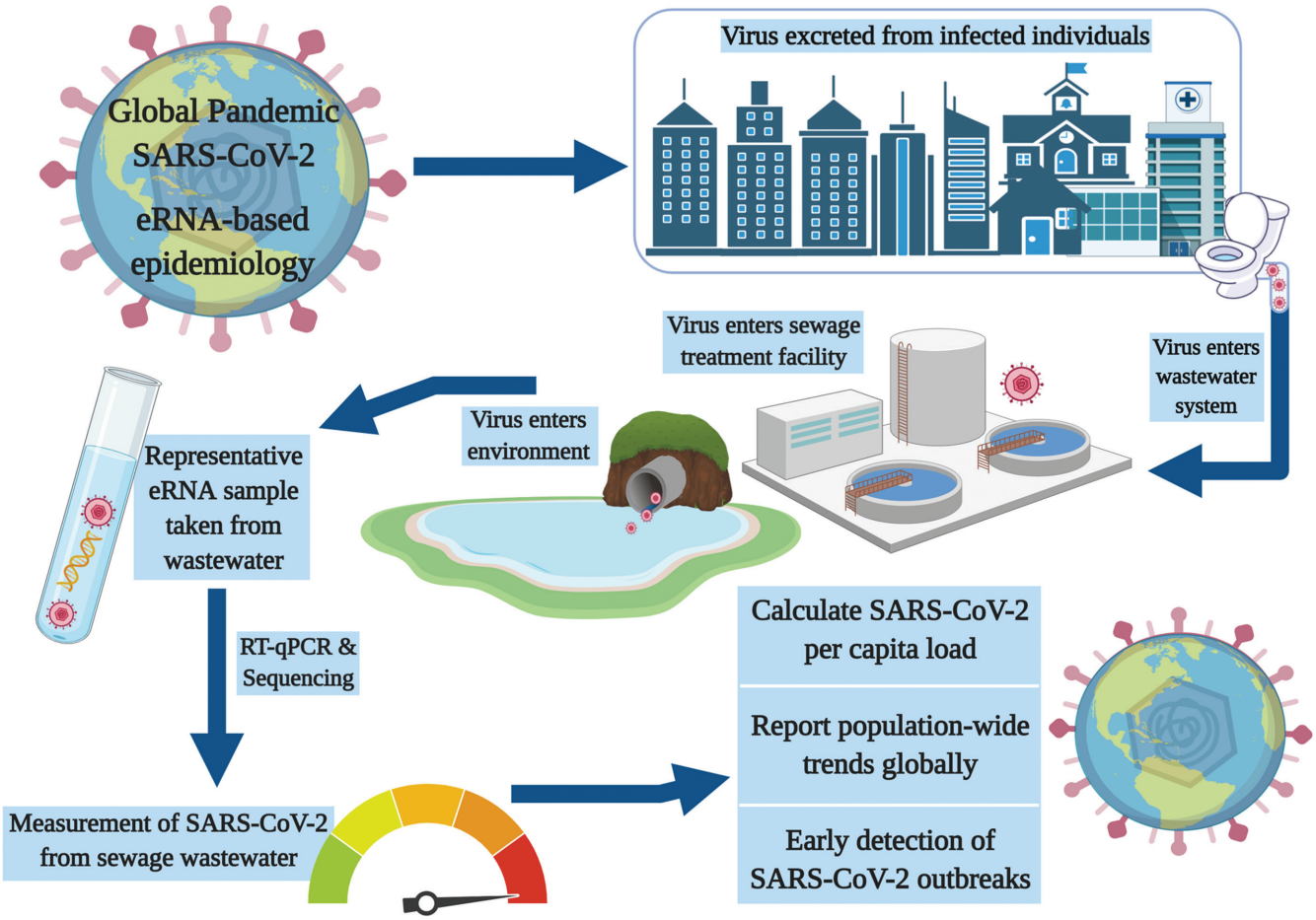

Figure 3. Schematic representation of the application of how eRNA approaches were applied to detect the SARS-CoV-2 virus from human wastewater during the COVID-19 pandemic.

outbreaks throughout this pandemic (Randazzo et al. 2020, Street et al. 2020).

SARS-CoV-2 has been detected from environmental air sampling using eRNA-based approaches, and recently infective SARS-CoV-2 virus has been recovered from air sampling (Lednicky et al. 2020). Although SARS-CoV-2 is a primarily airborne virus, studies have suggested that it can also be transmitted via fecal–oral transmission from symptomatic, asymptomatic, and newly recovered individuals alike (Randazzo et al. 2020, Wu et al. 2020). Fecal viral RNA has been detected from wastewater by targeted single-species real-time qPCR up to 10 days after clearance from the respiratory tract, suggesting that wastewater (figure 3) could provide invaluable information regarding epidemiological surveillance. This, coupled with the method's high sensitivity of 0.02%–0.1% (2 positive people in 10,000 to 1 positive person in 1000) further supports the applicability of this method for sensitive surveillance and early detection of infected persons with SARS-CoV-2 (Ahmed et al. 2020, Orive et al. 2020, Randazzo et al. 2020, Wu et al. 2020). The eRNA analysis of wastewater to track large-scale outbreaks also has the potential to be significantly less invasive, faster, simpler, and cheaper than individual human testing (Randazzo et al. 2020). Recently, similar methodologies have been applied to norovirus, hepatitis A, influenza, and poliovirus surveillance, suggesting widespread applicability of eDNA- or eRNA-based monitoring of human pathogens (Wolffs et al. 2011, Naujokaityte 2020, Randazzo et al. 2020, Street et al. 2020, Wu et al. 2020).

Studies have shown that not only can SARS-CoV-2-specific eRNA be detected via real-time qPCR from wastewater sites around the world (Spain, Denmark, the United States, the Netherlands, the United Kingdom, Greece, Canada, Italy, India, and Australia), but this technique has been used to identify the persistence of the virus in the environment prior to the diagnosis of initial human cases and during periods when no human cases are even documented (Ahmed et al. 2020, Gill 2020, Kumar et al. 2020, Naujokaityte 2020, Randazzo et al. 2020, Wu et al. 2020). This highlights the potential of eRNA to provide advanced warning of human disease outbreaks. The wastewater detection of SARS-CoV-2 eRNA increased rapidly prior to medical detection of human outbreaks in those regions, with environmental virus concentration peaking at the

same time or before the number of human-detected cases, providing advanced warning of a surge in infected individuals (Kumar et al. 2020, Randazzo et al. 2020). For example, Randazzo and colleagues (2020) recorded viral concentration peaking on 9 March 2020 in Valencia at 6 $\log_{10}$ genomic copies per liter, whereas the number of clinical cases didn't peak until the start of April (approximately 8000 cases), providing early detection of a SARS-CoV-2 surge. This has profound implications for healthcare system early warning, resource provisioning and policy management regarding prophylactic quarantining. These initial studies provide substantial evidence for the sensitivity, reliability and cost effectiveness of wastewater eRNA analysis for human epidemiological surveillance, however methodologies will need to be adapted to account for temperature, in-sewer travel time and molecular assay validation for enveloped viruses such as coronavirus (Gill 2020, Naujokaityte 2020, Randazzo et al. 2020, Street et al. 2020, Wu et al. 2020). Simultaneously, nonenvironmental detection of SARS-CoV-2 has developed rapidly in response to the significant threat of this pandemic (Dao Thi et al. 2020, Ganguli et al. 2020, Grant et al. 2020, Lall 2020, Ragnesola et al. 2020, Sheridan 2020, Thompson and Lei 2020). As a result, several methodologies have been optimized to the point of such high sensitivity and specificity that their potential for detection outside the human body is remarkable. In response to the need for faster test results, in-field testing and nonspecialized personnel, RT-LAMP has emerged as a successful alternative to RT-qPCR technology. Reverse transcription-LAMP has identical benefits for eRNA detection as LAMP has for eDNA detection. Namely it is highly time and labor efficient, does not require specialized equipment and is extremely portable (Ganguli et al. 2020, Thompson and Lei 2020). The optimization of RT-LAMP for the detection of SARS-CoV-2 eRNA in human wastewater not only has great potential but is highly feasible considering the success of adapted LAMP-based detection for environmental soil pathogens (Dao Thi et al. 2020, Thompson and Lei 2020). The management of pathogens with such eDNA or eRNA field-based testing, with no requirement for specialized equipment, would be invaluable in many areas of the world—particularly, rural, remote, and lower-income locations in which lab-based assessments may not be as readily available or may be cost prohibitive. A second rapid diagnostic testing technology, LFA, has been rapidly optimized to counteract the implementation costs and logistical problems with reagents necessary for RT-PCR (currently the primary pathogen eDNA or eRNA detection tool) during the COVID-19 pandemic, although RT-PCR remains the gold standard (Grant et al. 2020, Ragnesola et al. 2020). Lateral flow assays have been successfully implemented to detect viral pathogens such as influenza and have the potential to aid patient diagnostic needs in low- and middle-income countries with specific and sensitive detection of SARS-CoV-2 antibodies (Grant et al. 2020, Han et al. 2020, Ragnesola et al. 2020). However, LFA's potential and use for eRNA detection is unexplored in the literature as of yet. The status and development of CRISPR-based diagnostic tests are in a similar situation (Lall 2020, Sheridan 2020). Although it is extremely sensitive (eliminating much of the uncertainties associated with traditional rapid diagnostics), easy to use, and resource efficient (i.e., time, money, and expertise), the optimization of such technologies for the environmental detection of human pathogens has not yet been reported (Lall 2020, Sheridan 2020). However, with recent optimizations to use such technology in the fields of ecology and conservation biology, CRISPR-based SHERLOCK methodologies are already capable of efficient species identification in the field (via corporeal fluids such as mucus), expanding the future possibilities of this technology to be adapted for eDNA detection or species identification in similar aquatic environments in which these bodily fluids are secreted or shed (Baerwald et al. 2020, UCDAVIS 2020). With the persistence of the COVID-19 pandemic and technologies that have been rapidly investigated during a time of immense scientific advancement, there has never been a more promising time to apply what has been learned during this pandemic to monitor and mitigate future epidemics and pandemics in both human and wildlife populations. Therefore, it is evident that optimization and global implementation of eRNA- or eDNA-based analysis has the potential to help prevent and prepare populations all over the world for anything from localized disease outbreaks to worldwide mass mortality events (Ahmed et al. 2020, Gill 2020, Hart and Halden 2020, Randazzo et al. 2020).

Aquatic pathogen eDNA and eRNA limitations

There is extensive evidence in the literature promising eDNA and eRNA-based techniques to be groundbreaking new approaches to pathogen detection and monitoring (including those examples outlined above (Roussel et al. 2015, Deiner et al. 2017, Adams et al. 2019, Erickson et al. 2019). The rapid application of eRNA-based methodologies to understanding and tackling the COVID-19 pandemic is a particularly striking case, given the disease's very recent emergence. Sample collection of bottles of water, soil, or sediment is relatively simple. Assays developed for the detection of pathogens from host samples can often readily be repurposed for detection of that pathogen from eDNA samples (Page-Karjian et al. 2015, Yetsko et al. 2021). qPCR- and sequencing-based eDNA and eRNA studies have successfully detected similar numbers of (or indeed more) cryptic, rare, and endangered species; diseases; and pathogens than traditional methods, and they require less sampling effort and can be used in areas in which traditional methods cannot (table 2; Thomsen et al. 2012, Goldberg et al. 2016, Valentini et al. 2016). LAMP-based eDNA studies have even developed field-based methodologies to enable rapidly deployable environmental detection of invasive and pathogenic species without the need for specialized equipment; this could have a dramatic impact in many parts of the world including those with fewer resources to spare and more rural or remote locations (Winkworth et al. 2020). However, there

are currently only very limited studies exploring the ability of LAMP-based assays to directly detect pathogens from the environment (primarily fungi from soil); therefore, this methodology needs significant optimization to be deployable in all environmental types (marine, freshwater, air, sand, and ice) for a variety of pathogens including bacteria and viruses (Wong et al. 2018). It is consequently extremely crucial that researchers also understand and consider the substantial limitations of each of these rapidly developing molecular approaches (table 2).

Financial costs. For each particular study, extensive evaluation of study design should be considered to ensure the cost effectiveness of an eDNA- or eRNA-based approach as opposed to traditional approaches, because, as the current costs stand, on the basis of study design, eDNA-based methods can be more expensive than traditional methods and eRNA-based approaches are significantly more expensive than eDNA-based approaches (Smart et al. 2016, Broman et al. 2020, Winkworth et al. 2020). However, the continued rapid fall in high-throughput sequencing costs, increased multiplexing potential of qPCR or ddPCR (which partitions the sample before amplification, allowing for rare or low-abundance detection), and the development of more cost-effective and resource-conscious methodologies such as LAMP-based assays is bridging these economic gaps (Smart et al. 2016, Broman et al. 2020, Winkworth et al. 2020). As an example, it has been estimated that when detecting New Zealand mudsnails, an invasive species in the United States, it is four to eight times less expensive to use qPCR-based eDNA approaches—at US$35–$80 per sample—than traditional approaches (collecting, sorting, and identifying samples) that cost US$300 per sample (Goldberg et al. 2013). Goldberg and colleagues (2016) have compiled guidelines and critical considerations to mitigate these limitations and improve the scientific study and reporting of eDNA, including study design, method selection, assay validation, and minimum reporting suggestions. In addition, the cost of high-throughput sequencing and the ability of qPCR or ddPCR multiplexing continue to fall rapidly, making eDNA- or eRNA-based studies ever more financially attractive. Many companies are even offering packaged extraction, bioinformatics, and assignment deals that are getting more cost effective and resource efficient (vis-à-vis the time and personnel required).

False negatives. A significant limitation of eDNA- and eRNA-based techniques is imperfect detection—the occurrence of false positives and false negatives—and can occur whether using qPCR or sequencing-based approaches (Ficetola et al. 2015, Roussel et al. 2015, Goldberg et al. 2016, Guillera-Arroita et al. 2017, Boussarie et al. 2018, Erickson et al. 2019, Sutter and Kinziger 2019, Trujillo-Gonzalez et al. 2020). False negatives occur when pathogens or species are not detected despite their environmental presence. The rapid development of eDNA-based approaches is directly in response to this methodology's tendency to produce far fewer false negatives for rare or elusive taxa than most conventional sampling approaches. However, they are still possible, particularly with eRNA-based studies. False negatives can occur when unknown genetic variation in a target species or pathogen occurs (Goldberg et al. 2013) or when species-specific DNA or RNA concentrations are beneath the detection threshold because of small DNA or RNA quantities shed by the target species or pathogen, recent absence of the target species or pathogen in the sample area, or environmental conditions (salinity, acidity, ocean currents, UV radiation, and season) affecting the dispersion, dilution, preservation, and extraction of eDNA or eRNA (Roussel et al. 2015, Goldberg et al. 2016, Evans and Lamberti 2018, Raemy and Ursenbacher 2018, Seymour et al. 2018, Trujillo-Gonzalez et al. 2020). For example, qPCR-based eDNA techniques employed to detect subclinical ectoparasite (*Neobenedenia girellae*) infections in barramundi fish (*Lates calcarifer*) at border controls resulted in false negatives as they were unable to reliably detect the ectoparasite eDNA when it was at low subclinical concentrations (fewer than two copies per microliter; Trujillo-Gonzalez et al. 2020). Although several technologies are advancing our ability to screen for and detect low abundance pathogens from human or animal patient samples (including several low-abundance pathogens at once), such as FLASH, a next-generation CRISPR diagnostic that uses CRISPR-Cas9 enrichment to detect several known pathogens from a single sample, the applicability of these technologies to aquatic pathogen environmental detection has yet to be assessed (Quan et al. 2019). The degradation of DNA and RNA (which can significantly affect nontargeted high-throughput sequencing as a result of taxonomic misidentification) in aquatic environments varies considerably (Goldberg et al. 2016). Studies have shown that eDNA persists longer in freshwater (7–25 days) compared with seawater (less than 7 days) in both field and mesocosm environments, but this persistence varies depending on the numerous combined factors stated above (Dell'Anno and Corinaldesi 2004, Barnes et al. 2014, Keskin 2014). One study in particular showed that inshore marine eDNA degraded more rapidly compared with pelagic eDNA, and eDNA decay rates ranged between 10 and 50 hours (Collins et al. 2018). The reduced preservation of eDNA (and even more so eRNA) in marine environments could however be advantageous for some applications, allowing close-to-real-time detection of the presence of an organism and its potential pathogen, because the eDNA or eRNA is not able to disperse far before degrading substantially. The enhanced persistence of eDNA in freshwater would suggest that if the study's application is temporally sensitive—as in the case of monitoring potential COVID outbreaks—eRNA may represent a better choice of genetic material, whereas for marine-based studies, the use of eRNA-based methodologies might not be so critical for avoiding legacy detection because of the shortened persistence of eDNA in marine environments. Furthermore, eRNA may be more prone to

false negatives in marine environments that are harsher than freshwater or wastewater environments. Inhibition is an additional constraint for both eDNA and eRNA and can vary between locations or even between seasons in the same location (Shamblin 2007, Jane et al. 2015, Sanches and Schreier 2020). Estuarine waters contain increased levels of PCR inhibitors that, even if target DNA or RNA is present, can mitigate amplification to the point of false negative detection during qPCR (Jane et al. 2015, Sanches and Schreier 2020). Several filters, reagents, and methodologies have been developed to counteract PCR inhibitors found in the environment but without foresight to remove these from environmental samples, data can be significantly negatively affected (Shamblin 2007, Sanches and Schreier 2020).

False positives. Conversely, partly as a result of eDNA-based approaches being less likely to produce false negatives, they can be more prone to producing false positives (in comparison with eRNA-based studies and traditional studies) because of increased efficacy (detection of eDNA that does not come directly from a present or alive target species or pathogen; table 2; Roussel et al. 2015, Goldberg et al. 2016, Evans and Lamberti 2018, Trujillo-Gonzalez et al. 2020). False positives can occur for several reasons, including as a result of contamination either in the field or the lab (Goldberg et al. 2016). Standard procedures to prevent such contamination and the false positives that may occur include the use of negative field controls, the sterilization of equipment, single-use disposables where necessary, enhanced personal protective equipment use, and separate cleanrooms and equipment for the different stages of sample processing (i.e., storage, filtration, extraction, qPCR, sequencing; Goldberg et al. 2016, Armbrect 2019). Detection assays (qPCR assays or metabarcodes) may also amplify or detect a nontarget organism or pathogen because of inadequately designed primers or incomplete knowledge about the sequence diversity within a sample (Goldberg et al. 2016). For qPCR, primer-only-based assays can be more prone to false positive detection (amplification of nontarget fragments) than primer–probe combinations that only produce signal in the presence of the intended amplicon. In addition, qPCR-based assays can be more prone to false positives than LAMP-based assays, which routinely use four to six primers (as opposed to just one pair), diminishing the occurrence of false-positive results (Soliman et al. 2015, Winkworth et al. 2020). The importance of DNA marker selection further affects the efficacy of a metabarcoding approach; marker selection has been proven to be crucial in determining the ecological composition from eDNA, despite there being no true single universal marker (Riaz et al. 2011, Goldberg et al. 2016). A study comparing two differing markers for detecting teleost fish species from eDNA showed that one identified only 15 species, whereas the other identified 24 species (Thomsen et al. 2012, Valentini et al. 2016). In addition, false positives can occur as a result of genetic mutation or unknown variation in closely related species or pathogens (Goldberg et al. 2013). However, in the future, many of these limitations may be overcome, as the current initiatives to generate reference genomes covering a significant proportion of animal and pathogen species come to fruition and as the routine development of pangenomic reference genomes are extended or as nonbarcoded sequencing (shotgun sequencing) becomes increasingly used as costs and analysis time falls. For shotgun sequencing, the gains in detection of unknown variants may be offset somewhat by reduced specificity. However, we recently reported the successful detection of the ChHV5 viral pathogen in tank sea water using nonbarcoded next generation sequencing approaches, which was confirmed with pathogen-specific qPCR (Yetsko et al. 2021).

An alternative source of false positives may occur when the correct target species or pathogen is detected, but the recovered eDNA originated from a distant site. This is much less common with eRNA because of its rapid environmental degradation. However, in such cases, positive species-specific DNA detection may not confirm target species or pathogen presence in the sampling locality but, instead, could occur as a result of transported (via river flow or ocean currents) or preserved DNA after the organism's mortality (predator consumption and defecation; Roussel et al. 2015, Goldberg et al. 2016, Evans and Lamberti 2018, Trujillo-Gonzalez et al. 2020). Such false positives, although potentially informative about unique trophic interactions in the case of predator or prey eDNA, can be misleading when attempting to determine current presence, absence, or distribution and range data for a particular endangered species or harmful invasive species or pathogen (Diaz-Ferguson and Moyer 2014, Roussel et al. 2015). Because RNA is a less stable molecule than DNA (because of the hydroxyl groups), eRNA does not persist in the environment as long as eDNA, mitigating against false positive information that can more readily occur from collecting preserved or transported DNA (Goldberg et al. 2016, Cristescu 2019, Broman et al. 2020, Merou et al. 2020). False positives are also more likely to occur in freshwater systems compared with the marine environment, but the addition of complementary eRNA analysis that only detects present or living organisms, as for SARS-CoV-2, can somewhat overcome this limitation, being a more sensitive or real-time detector of species and their pathogens (Broman et al. 2020).

Limited abundance or biomass detection and correlation. As current technology stands, eDNA and eRNA-based approaches are potentially subject to important limitations in discerning in-depth data concerning species diversity, population structure, size, or sex; however, advancements are being made, as in the case of ungulate sex identification via eDNA-based SNP assays (Nichols and Spong 2017, Barnes and Turner 2016, Holdaway et al. 2017, Adams et al. 2019, Stat et al. 2019). Yates and colleagues (2019) suggested that current qPCR- and sequencing-based eDNA and eRNA methods may be unable to correlate species-specific eDNA

or eRNA concentrations with target species abundance or biomass. Instead, eDNA concentrations more often appear to increase as a result of decreasing waterbody volume (Yates et al. 2019), as has been illustrated by eDNA detection involving European turtles and Yangtze finless porpoises (*Neophocaena asiaeorientalis;* Roussel et al. 2015, Raemy and Ursenbacher 2018, Tang et al. 2019). However, such limitations do not appear to be as prevalent for pathogen-focused studies in which qPCR C_t values can be used as predictors for pathogenic parasite infection abundance and viral load (*Ranaviruses* and chelonid herpesviruses; Huver et al. 2015, Miaud et al. 2019, Yetsko et al. 2021). Nor are they as prevalent in manmade settings, such as wastewater treatment facilities (figure 3), in which volume and flow rates are more controlled and consistent and in which concerted effort and prioritized funding have enabled rapid optimization of applicable eRNA-based technology to improve human healthcare, particularly in response to the current viral (COVID-19) pandemic (Ahmed et al. 2020, Kumar et al. 2020, Naujokaityte 2020, Randazzo et al. 2020, Street et al. 2020). The inclusion of PCR replication, which is often used to increase sequencing reads, can increase the variation in linking species abundance or biomass to sequence reads (Bista et al. 2018). However, PCR-free library preparation approaches or shotgun sequencing can overcome this, providing highly significant correlations between read numbers and biomass (Bista et al. 2018). Rank abundance and proportional read counts across samples can also give reliable indications of population dynamics via metabarcoding (Hanfling et al. 2016). Evidently, not all technologies have equivalent quantification capabilities. Metabarcoding, although it is able to describe species communities (fish) in large aquatic waterbodies (lakes), is particularly susceptible to inadequate primer design, affecting sequencing depth and biomass estimates (Hanfling et al. 2016).

Targeted eDNA- or eRNA-based approaches (qPCR and sequencing) require prior knowledge of specific species presence (as do traditional methods), which can result in sample bias (Creer et al. 2016, Broman et al. 2020). However, for pathogen monitoring, the pathogen of interest is usually known, so this is also less of a limitation than for species conservation applications. In addition, advancements in deep sequencing and the associated bioinformatics analyses are streamlining novel pathogen detection. Although traditional aquatic detection methods are typically aimed at macrofauna (and therefore biased toward such species), nontargeted eDNA and eRNA high-throughput sequencing can just as easily collect information on the microscopic fauna and pathogens present in natural and manmade aquatic systems (Fonseca et al. 2010, Creer et al. 2016, Broman et al. 2020). The importance of being able to detect microfauna is highlighted by the fact that 60% of animal phyla in marine systems are vital microscopic benthic metazoans (primarily nematodes 45–500 micrometers in size) that perform crucial roles in the marine environment and would typically go undocumented during ecological surveys or a targeted species approach (Fonseca et al. 2010). Furthermore, shotgun eDNA or eRNA sequencing or eDNA or eRNA metabarcoding is capable of simultaneously detecting macro-, meso-, and microfauna all from the same sample (Ficetola et al. 2008, Djurhuus et al. 2020). By using less targeted approaches in the context of pathogen detection, users are able to detect known and unknown pathogens alike, which could aid in the treatment and management strategies for species conservation and for endemic and emerging human pathogens.

Future directions

In the future, improved methodological and technological applications may overcome many of these limitations and enable more sensitive detection of human and animal pathogens. Although eRNA-based approaches currently require specialized sample storage and significant time or costs, the ever-decreasing costs of molecular technologies will gradually phase out many of these constraints (Broman et al. 2020). Adaptation of current standard practices for more specific or temperamental eDNA- or eRNA-specific research will result in relatively rapid improvements of the field. For example, PCR-free library preparation approaches for high-throughput sequencing are well established outside of the eDNA field, although conventional library preparation involving a PCR amplification step still predominates, and in our experience such PCR-free library preparation approaches can be successfully applied to the sequencing of eDNA samples. This can reduce bias introduced by the PCR step because of unequal gene amplification and will be informative for future work examining species or pathogen abundance and biomass. In addition, advancement and increased accessibility of current molecular technologies, such as ddPCR and high-throughput sequencing, will be able to provide near-term improvements in eDNA detection sensitivity, all while adapting to enable low-cost nonexpert citizen scientists to evaluate and monitor the health of their own ecosystems (Goldberg et al. 2016, AIMS 2018, 2020, Doyle and Uthicke 2020). For example, Doyle and Uthicke (2020) have successfully developed a sensitive eDNA detection device that uses a lateral flow assay to detect the highly invasive crown-of-thorns sea star (*Acanthaster* cf. *solaris*) in the form of a handheld dipstick, similar to a home glucose or pregnancy test (AIMS 2018, 2020). The adaptation of eDNA detection from a solely laboratory environment to a technology that is applicable and accessible to the general public has the potential to vastly improve the field of eDNA (and eRNA) analysis (AIMS 2018, 2020, Doyle and Uthicke 2020). In addition, portable high-throughput sequencing machines are being used to detect pathogens in both field and laboratory conditions (Urban et al. 2021) and hold the promise of significantly improving pathogen detection in clinical and clean manufacturing settings, especially for unculturable species. The increasingly high specificity of eDNA- or eRNA-based methodologies has also begun to open entirely new avenues of research, by elucidating the

presence of different haplotypes of certain species at different times of year, highlighting its ever-advancing ability to investigate elusive and endangered aquatic population dynamics (Deiner and Altermatt 2014, Diaz-Ferguson and Moyer 2014, Barnes and Turner 2016). In addition, studies have demonstrated how combining eDNA-based techniques with supportive techniques such as those that are eRNA-based can greatly improve the results of imperfect detection. As current science stands, the promise of eDNA- and eRNA-based approaches greatly outweighs the ever-diminishing limitations (table 2), many of which are expected to be overcome in subsequent years. Sample collection, often simply requiring the collection of several bottles of water or sediment, can be straightforward enough to be performed by most field technicians (and citizen scientists) and substantially more efficient and cost effective than traditional alternatives (Valentini et al. 2016, Miaud et al. 2019). No macroorganisms are handled, manipulated, stressed, or harmed in the process, and the data obtained are objective.

Taken together, eDNA- or eRNA-based aquatic pathogen monitoring is redefining our ability to monitor disease-inducing microorganisms in settings as diverse as drinking water, wastewater, freshwater, and marine environments. The innovative application of this novel technology is enabling us to better understand the dynamics and origins of human and animal pandemics and track disease outbreaks from the global to local scale. The impressive capacity of eDNA- or eRNA-based approaches to provide advanced warning of human and wildlife disease outbreaks has been demonstrated across a number of pioneering studies (Miaud et al. 2019, Ahmed et al. 2020, Kumar et al. 2020, Naujokaityte 2020, Randazzo et al. 2020, Street et al. 2020). This strongly suggests that continuous eDNA- or eRNA-based monitoring programs promise to provide improvements to human and animal health by readily predicting disease outbreaks in advance, thereby facilitating proactive early responses rather than our previously reactive measures.

Conclusions

Conventional methods for endangered species and pathogen monitoring can often be invasive to the species that are being protected or to the human undergoing diagnostic testing (Andruszkiewicz et al. 2017). However, integration of conventional and eDNA- or eRNA-based methods in tandem can increase the robustness of detection and monitoring of vulnerable species and pathogens and has already been successfully implemented in places such as New Zealand (biosecurity; Deiner et al. 2017, Holdaway et al. 2017, Adams et al. 2019) and in human medical and public health settings (Wolffs et al. 2011, Ahmed et al. 2020, Kumar et al. 2020, Naujokaityte 2020, Randazzo et al. 2020, Street et al. 2020). Despite the current limitations, eDNA or eRNA analysis is an approach with the potential to vastly exceed traditional detection methods and the capacity to improve the detection and monitoring of aquatic pathogens and their vulnerable host species, including humans (Goldberg et al. 2016, Deiner et al. 2017, Boussarie et al. 2018, Ruppert et al. 2019, Ahmed et al. 2020, Kumar et al. 2020, Naujokaityte 2020, Randazzo et al. 2020, Street et al. 2020). Despite being relatively novel methodologies, eDNA- or eRNA-based approaches have already seen significant improvements as the fields of molecular technology and genetics progress rapidly (Goldberg et al. 2016, Urban et al. 2021) and are quickly becoming leading methodologies capable of providing refined real-time understanding of viral shedding dynamics (Yetsko et al. 2021) and advanced warning of pathogen-induced mass mortality events in both humans and wildlife (Andruszkiewicz et al. 2017, Miaud et al. 2019, Strand et al. 2019, Ahmed et al. 2020, Kumar et al. 2020, Naujokaityte 2020, Randazzo et al. 2020, Street et al. 2020). They show incredible potential to streamline and improve aquatic endangered species and pathogen detection, and wildlife and human health monitoring in the future (Miaud et al. 2019). Monitoring programs incorporating eDNA- or eRNA-based approaches will enable the transition from reactive responses to disease outbreaks to more proactive and preventative medicine and animal and public health management, potentially limiting the worst outcomes of unchecked human and animal pathogens.

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