Environmental DNA as a tool for single species detection.

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Key Words: eDNA, qPCR, ddPCR, LAMP, RPA, CRISPR-Cas, Sensor, Review

Abstract

Environmental DNA (eDNA) based biosensing has significant potential and offers many advantages over current methodologies for marine species monitoring. It is now possible to isolate a given species' DNA from water samples and use this as a proxy for their detection. While there are many challenges to be addressed in terms of relating DNA based data back to actual species biomass, the sensitivity, specificity and potential to transition to on-site testing is driving innovation in this area. We describe the current advances in environmental DNA based testing with a focus on the three steps or challenges that are being considered for on-site monitoring: 1. DNA acquisition, 2. Molecular Assay development, and 3. Detection mode. We describe the evolution of methods within each of these areas, and highlight which techniques hold the most promise to realise a vision of eDNA based marine biosensing. We conclude with an outline of recent innovations in eDNA based biosensor devices including portable, remote and autonomous systems that, with further development, will facilitate the collection of real-time data on any species of interest.

Introduction

Environmental DNA (eDNA) is emerging as a useful, non-invasive tool to support the monitoring of a range of species of interest particularly in aquatic environments. eDNA based detection essentially involves using a species' own DNA as a proxy for their detection [1]. The source of DNA is generally derived through the natural cell shedding and secretions of the organism during its lifecycle [2]. While eDNA can be isolated from many environmental sample types including sediment and soil, this chapter will focus on the detection of eDNA in marine water samples. Monitoring marine taxa through eDNA approaches broadly involves the collection of a water sample, the concentration of the biomaterial within the sample, and ultimately the extraction of the DNA from the species contained within it. The isolated eDNA can then be used to detect either a single species or provide a snapshot of numerous taxa that are present in that water sample (referred to as metabarcoding or metagenomics). As outlined in a review by Díaz-Ferguson and Moyer (2014) [3], the concept of eDNA began in the 1980s with the isolation of bacterial communities from marine sediments. This approach has since been successfully applied to detect a range of organisms including plants and animals [1]. However, many challenges still remain in this fast emerging field relating back the origin, state and fate of eDNA detection including how best to capture, purify and assay target species [4].

Successful eDNA monitoring also requires familiarity with the ecology and life-history traits of your target species. For example, taking eDNA samples in an estuary or shallow coastal area whilst searching for a large pelagic species is unlikely to be fruitful. Similarly, ecological knowledge can increase the likelihood of detection by knowing where and when to take samples. The concentration of eDNA at any point in time is dependent on both the rate of production (shedding) of eDNA (influenced by the level of activity of individuals, their metabolic rate, and behaviour such as spawning, fighting etc.) as well as the density of the species within a system. Likewise, the amount of eDNA in an environment can vary seasonally in response to environmental changes (i.e. temperature, hydrology etc.) and the behavioural ecology (e.g., migration, increased activity etc.) of a given species [5–7]. For example, a large increase in the amount of free DNA within an environment may be caused by a gathering of individuals during their spawning or breeding season, whereby, a combination of the release of gametes and an increased DNA shedding rate due to an escalation in activity, results in a substantial increase in the detectability of the species [2, 7–11].

The reliability and resolution of environmental DNA (eDNA) monitoring is also contingent upon the ability to detect the presence of a target species contemporaneously. This means taking in to account the occurrence of a false negative where species that are present are not detected (i.e., low-organism density or activity), or a false positive where species not present are detected due to eDNA being transported in from other areas or even through the faeces of predators [12]. Andruszkiewicz *et al.,* (2019) [13] found that eDNA in marine systems can be transported in the order of tens of kilometres in a few days. Knowledge of how long eDNA is likely to persist in a given system is therefore of importance in understanding both of these scenarios as well as the overall spatiotemporal scales of eDNA signals, but it is a particularly pertinent problem for estimating species distribution in marine ecosystems due to the influence of tidal currents which may transport eDNA [14, 15].

Despite such challenges, eDNA based detection offers significant advantages over other monitoring methodologies. Numerous publications have used this approach for the detection of a range of both freshwater and marine species, with 90% of studies demonstrating a positive relationship between eDNA detection and species' biomass abundance [16]. This bodes well for eDNA based species detection with further research in this area likely to be valuable and result in its even wider applicability. Traditional approaches for the detection of aquatic species can be harmful, time-consuming and costly [17], and often involve intensive manual implementation using rods, nets or traps [18, 19] or electricity [20]. Although such approaches can provide important information relating to species abundance and demography, there are significant challenges in the collection of fine-scale temporal data as well as an increased likelihood that low density, elusive or rare species are overlooked. Biosensing using eDNA offers an alternative approach that has the potential to be cheaper, non-invasive, sensitive, and capable of performing high frequency sampling. The long-term goal of eDNA based biosensing is the development of both a manual device which can be implemented for fast on-site detection or an *in-situ* autonomous sensing system which can detect eDNA and send results directly to the practitioner. This Chapter outlines the basis of eDNA detection, current advances in the development of eDNA based biosensors and some of the technical challenges that must be addressed to develop eDNA biosensors (Figure 1).

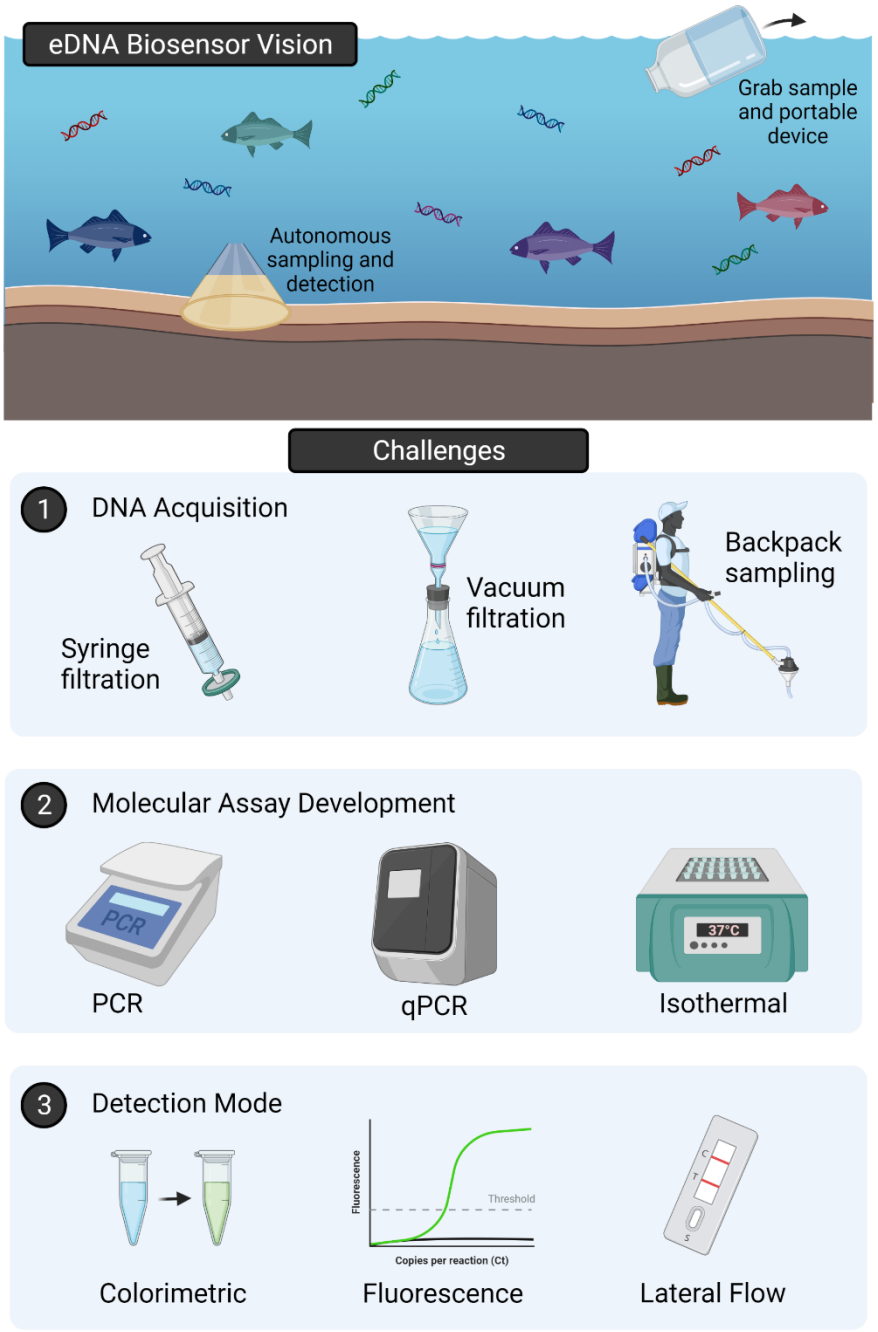

Figure 1: Overview of eDNA biosensor vision and an outline of the technical challenges to be addressed to achieve this (as outlined in this chapter). The vision for eDNA based biosensing incorporates the development of both portable/manual devices which can be implemented for fast on-site detection in combination with manual/grab sampling, and *in-situ* autonomous sensing system. An eDNA biosensor must achieve a series of complex functions, each with their own challenges which can be broadly broken down into the categories of; 1. DNA acquisition, 2. Molecular assay development, and 3. Detection mode. The panels above give a graphic overview of current methods applied to eDNA monitoring within each of the latter categories which are discussed in detail below. Created with BioRender.com.

For standard eDNA monitoring, discrete samples are generally collected manually, preserved in the field and then returned to a laboratory where they are processed many hours, days, or months later depending on resource availability. Some of the current limitations associated with this approach to eDNA monitoring include; transport of water samples and preservation of DNA/RNA during this time; filtration of samples on site and prevention of cross contamination; time spent in analysing samples in a laboratory; and technical expertise needed for laboratory analysis of samples. This may be an acceptable situation for many eDNA applications, however, this can be a significant constraint for eDNA based marine monitoring in remote and offshore areas due to the associated costs and logistics [21]. Consequently, the development of new technologies to overcome some of these challenges has been moving rapidly over the past few years and research into the development of more automated biosensing systems for eDNA monitoring in the field have been escalating. However, an integrated system for eDNA monitoring must achieve a series of complex functions including; sample concentration and lysis; DNA extraction and purification; followed by amplification and specific target detection. Each of these steps present their own challenges when considering adaptation to a portable or *in-situ* device. Regardless of which endpoint is required, there are three distinct steps or challenges that need to be addressed for marine biosensor development (Figure 1) and include 1. DNA acquisition, 2. Molecular assay development, and 3. Detection mode. We describe each of these in turn.

1.0 eDNA Acquisition

The source of eDNA suspended in a given water column is either intra- or extracellular in nature and the length of time for which it may be detected can vary significantly [2, 22, 23]. eDNA persistence in a given system is a parameter of relevance in understanding the spatio-temporal scales of eDNA signals, but it is a particularly pertinent problem for estimating species distribution in marine ecosystems (and other lotic systems) due to the influence of tidal currents which may transport eDNA [14, 15]. For example, eDNA degrades 1.6 times faster in the inshore environment than the offshore environment [14]. Aqueous eDNA has been found to persist in aquatic systems from anything between days to years [14, 24, 25] and is significantly influenced by abiotic factors including moderately high temperature [23, 26–28], exposure to UV light [23], and low pH [23, 27, 29]. Consequently, its' usefulness as a detection tool, relies on the ability to collect and concentrate the eDNA which can be achieved through various methods. Given that eDNA for species detection is still an evolving field, a range of methods and materials have been employed to acquire and purify the eDNA with varying results. The range of methodologies involved for eDNA acquisition has recently been reviewed by Tsuji *et al.,* (2019) [30]. In the main, most protocols involve an initial filtration step of the collected water sample, with the most popular approach (based on 438 published eDNA studies detecting aquatic macro-organisms) to couple filtration with DNA extraction from the filter using a commercial kit such as Qiagen's Blood and Tissue kit or their PowerWater DNA extraction kit [30]. There is variation in the types and pore size of filters that are used, with some studies showing superior performance of open cellulose nitrate filters (0.8 μm) [31] and Sterivex GP enclosed filters (polyethersulfone 0.22 μm) [32]. Other studies have successfully used cellulose nitrate filters with a smaller pore size (0.45 μm) [4] or glass microfiber filters (0.7 – 1.2 μm) [30]. Choice of pore size must take into account the turbidity within the sampling site. High turbidity can reduce the volume of water filtered due to issues with clogging and consequently may result in an increase of substances (e.g., humic, phytic, and tannic acids) that can inhibit downstream applications such as polymerase chain reaction (PCR). Inhibitors

can have a significant effect on qPCR reactions, and the choice of filter, DNA extraction methodology, and qPCR reagents all have a significant impact on detectability in the presence of inhibitors [33]. There are, however, methods to mitigate these impacts such as pre-filtering, using filters with a larger pore size, and the utilisation of specialised lysis buffers such as Longmire's buffer and cetyltrimethylammonium bromide (CTAB) which can significantly reduce inhibition [34, 35].

The preservation of the filter can also be facilitated in either Longmire's buffer or ethanol after filtration and is a key step to ensure integrity of the eDNA for further analyses [30, 32]. Filters preserved in ethanol have been stored at room temperature [31], but in the main, filters are stored at -20°C with or without a preservative solution. Some studies have also frozen the water sample itself at -20°C prior to filtration as a means of preservation of the eDNA, but this appears to lead to suboptimal yields [16, 31]. Alternative approaches for eDNA isolation have included ethanol/isopropanol precipitation [4, 36, 37], but the limited ability to centrifuge large volumes restricts this as a routine method for every sampling situation.

The most optimal protocols reported involve the timely filtration of the water sample within 24 hours to prevent eDNA degradation, which is often exasperated by higher water temperatures and length of time since the deposition of the eDNA from the original organism(s) [30]. Capturing of the eDNA on filters with inclusion of a preservative ensures longer stability. The volume of water sampled tends to be in the 0.5 – 2 L range [30]. As eluded to already, commercial DNA extractions are the most popular, safest and potentially most efficient method to purify filter captured eDNA. However, liquid phase separation using either the traditional DNA extraction protocol of phenol-chloroform isoamyl alcohol (PCI) [38] or CTAB have been successfully employed [4, 39]. Nevertheless, such methods are not likely to be a popular choice for the newly initiated given that they can be technically more challenging and use harmful substances [30] unlike with commercial DNA extraction kits.

1.1 eDNA Acquisition Devices

Current protocols for the acquisition and purification of eDNA are primarily laboratory based methods. The move to develop eDNA based biosensors (Figure 1) requires the eDNA acquisition step to be further developed to allow it to be performed on-site and/or autonomously. A number of eDNA extraction devices have been developed that allow on-site filtering and extraction, and are now becoming commercially available. These include the Aqualytical C.L.A.M device (https://aqualytical.com/) and the Smith-Root eDNA-Sampler. The C.L.A.M device (continuous low level aquatic monitoring) is a submersible, time-integrated, filtration sampler (up to 36 h) initially designed for trace organic analysis but repurposed for eDNA sampling of up to 100 litres of water, using encapsulated lofted micron rated filters. It can capture spatial coverage by continuous large volume filtration whilst submerged, floating, drifting or being towed in the sample itself. The Smith-Root eDNA sampler consists of a backpack portable pump that integrates sensor feedback, a pole extension with remote pump controller, custom-made filter housings in single-use packets for each sampling site and on board sample storage [40]. The eDNA water sampler technology monitors flow rate, pressure, and volume filtered that can help the user adapt to the environmental conditions to receive optimal filtration results [40]. The ultimate goal of an eDNA based biosensor however, is to have a single device that can incorporate all the steps required for species detection, including eDNA acquisition. Such a biosensor is still at the development stage and current progress in 'all-in-one' systems are briefly described in Section 4.

Once the eDNA is successfully isolated and purified, it is ready for species detection by applying bespoke molecular assays. The most widely applied methods include those that are PCR-based with the more recent introduction of isothermal methods and these are described below.

2.0 Molecular Assay Development

The choice of molecular assays that can be applied to eDNA has expanded over recent years. The first challenge with eDNA assay development is to choose an appropriate and highly specific DNA sequence target (usually 80-400bp) that can discern target species DNA in a sample containing the DNA of many other organisms. This requires finding a DNA sequence that is unique to a target but also highly conserved within the species. Consequently, the main constraint for robust assay design is the availability of reliably curated DNA sequence data with adequate geographic representation for a target organism. Consequently, a good understanding of the target species life history and breeding ecology is vital to design a robust assay that incorporates population differences. Mitochondrial DNA (mtDNA) is circular and small (approx. 16kb) relative to the nuclear genome, and is shared across Eukaryotic species [41]. For these reasons, it has tended to be the focus of early DNA sequencing projects in a bid to generate sequence information across all species. Indeed, projects such as the Barcode of Life Database (https://ibol.org/) rely on sequencing a single mitochondrial gene (cytochrome c oxidase subunit I (COI)) as a means for DNA based species identification. Currently, mtDNA is the target region of choice for eDNA studies, due to the availability of sequence data, its perceived resistance to degradation and its multi-copy nature. The challenge is identifying a target sequence that are conserved within species but contain interspecific differences, particularly with sympatric taxa. Ultimately, the reliance on mtDNA sequences for eDNA single species detection will diminish over time as the nuclear DNA sequence information expands through projects such as the Earth Biogenome (https://www.earthbiogenome.org/), and further advances in molecular technologies.

2.1 PCR-based Detection

PCR-based methods are the most commonly used for single species eDNA detection. These techniques allow selective amplification of target DNA that is often low in abundance in environmental samples [42]. Over time, there has been a transition from conventional PCR (cnPCR) [22, 43] to quantitative PCR (qPCR) [44–49] and droplet digital PCR (ddPCR) [50, 51]. This is primarily due to the increased need for highly sensitive and specific assays.

2.1.1 Conventional PCR (cnPCR)

Conventional PCR was the first method used for single species detection from eDNA [52] due to its ability to increase the quantity of target DNA in a sample to a visible amount. The method refers to PCR amplification using a thermal cycler with product visualisation via gel electrophoresis and utilises specific primers to verify the presence or absence of a species [53, 54]. The exact genomic region targeted varies throughout the literature but focus is on high-copy number regions such as the mitochondrial COI gene [54, 55], 12S rRNA gene [55], mitochondrial cytochrome b gene [56] and mitochondrial d-loop region [57]. Examples of the use of cnPCR in the marine environment include for biosecurity assessments of invasive species such as Atlantic wedge clam *Rangia cuneata* [58] and the Australian tubeworm *Ficopomatus enigmaticus* [59].

As with all PCR-based assays, cnPCR requires careful design of highly specific primers and optimisation of conditions to maximise detection, to prevent cross reactivity and reduce the risk of false positives and negatives. It is the least sensitive of the PCR-based methods and has been shown to have a higher limit of detection (LOD) and lower detection rate for field samples than quantitative PCR methods (2.1.2) [60, 61]. Overall, cnPCR has a definite place in eDNA studies, particularly if surveys require low cost, qualitative assessment of highly abundant species. It however, does not offer quantitative measurements and lacks the sensitivity for organisms of low abundance in the environment. Visualisation via gel electrophoresis is also not suitable for a biosensor device and alternative PCR based methods are favoured if moving towards this vision.

2.1.2 Quantitative PCR (qPCR)

The need for increased specificity, sensitivity and quantitative data from eDNA assays resulted in a shift from cnPCR approaches towards quantitative methods, primarily quantitative PCR. Unlike cnPCR, qPCR collects data during the exponential phase of amplification allowing an assessment of reaction efficiency before depletion of reagents begins [62]. Data collection occurs due to the incorporation of fluorescent-based molecules; primarily a sequence specific probe or SYBR® Green dye (discussed in detailed in section 3.2 below). This enables quantification of starting DNA concentration, as, during the exponential amplification phase, the amount of fluorescence is directly proportional to the amount of product [62]. To calculate qPCR detection limits, a standard curve made up of a dilution series of known DNA concentration is used. This commonly consists of synthetic DNA [63], plasmid DNA [64, 65] or DNA extracted from tissue [48, 66]. Comparison of samples to a standard curve allows DNA quantity to be calculated. However, the simplicity of the standards does not reflect the complexity of an eDNA sample and thus direct comparison of lab-based LODs to eDNA sensitivity should be avoided.

The quantitative element, on top of the increased speed, sensitivity and reproducibility [67], has led to qPCR being the most widely used technique for single taxa detection from environmental samples. It has been adapted for single species detection from a wide range of aquatic [44, 68–70] and terrestrial organisms [71], with examples in the marine environment including detection of Atlantic cod (*Gadus morhua*) [72], Maugean skate (*Zearaja maugeana*) [73] and the marine pest *Styela clava* [74]. In addition to increasing specificity, the move to qPCR allows an increase in sensitivity and thus raises the probability of detecting organisms of low abundance [75]. This improved sensitivity has enhanced monitoring of invasive species and measuring the decline in species following eradication events [42]. Furthermore, the mechanism of qPCR allows reactions to be multiplexed enabling detection of multiple species from the same sample, simultaneously [76, 77]. However, it should be noted that qPCR is only quantitative in the sense of quantifying the amount of PCR product that is produced and in line with all other molecular assays (including ddPCR; Section 2.1.3), it does not necessarily relate back to species biomass.

Overall, qPCR is a well-established technique throughout Molecular Biology and robust guidelines, known as the MIQE guidelines [78] have been developed to ensure the integrity of the scientific literature and consistency between laboratories. These guidelines call for full disclosure of experimental conditions and assay characteristics to allow the validity of protocols to be assessed. Whilst primarily designed for use in clinical diagnostics, the MIQE guidelines are often referenced in eDNA studies [79, 80] and offer guidance when designing new eDNA qPCR assays. Further, more

specific guidelines have been published by Bruce *et al.*, (2021) [81] which cover development of the full eDNA workflow with specific recommendations for qPCR based assays.

2.1.3 Droplet digital PCR (ddPCR)

Droplet digital PCR is the most recent PCR-based technique to be applied to eDNA studies and is currently the most quantitative molecular method available [82]. In the marine environment, ddPCR has been used for detection of, for example, *Cryptocaryon irritans* parasite [83], killer whales (*Orcinus* orca) [50] and corallivorous seastar (*Acanthaster cf. solaris*) [51]. Compared to qPCR, it offers superior quantification of DNA samples by splitting molecules into individual droplets and reactions. This means that rather than requiring a standard curve, as in qPCR, ddPCR offers absolute quantification directly from the sample [82]. This absolute quantification, not only reduces time associated with setting up a standard curve, it also reduces the variances associated with the assay [84] and negates the differing effect of sample matrix between standards (often prepared using synthetic DNA) and eDNA samples. Several studies have compared single species detection using both qPCR and ddPCR and show that ddPCR has greater sensitivity [85] with reduced variability at low concentrations [60]. The nature of partitioning the sample into droplets also reduces the effect of PCR inhibitors on the reaction [86]. This could be favourable when working with eDNA samples with high levels of inhibition such as those with a high organic matter content [87]. Despite its promise, ddPCR still has limited use in the eDNA community. Assays are easily adapted from a qPCR platform as the molecular mechanism and assay design are the same, however use is likely stunted by the expense, reportedly two times higher than qPCR [88] and limited availability of equipment.

In general, PCR assays offer more rapid detection and are more sensitive when compared with traditional methods [56]. However, the three different types of PCR assays that are described each have their own merits and disadvantages. The method chosen should therefore suit the purpose and scale of study being performed [85]. cnPCR is perfectly viable for simple eDNA detection of highly abundant organisms, but in the case of a rare target species, qPCR and ddPCR are more appropriate due to their increased sensitivity and specificity. Although suitable for laboratory-based experiments, these techniques pose multiple challenges regarding adaptation to a biosensor device, primarily due to the high temperatures and requirement of thermal cycling. The increased need for simplified, rapid, on-site DNA testing, and the limitations of PCR-based methodology, is driving the development of new DNA amplification technologies.

2.2 Isothermal Detection

Isothermal methods of nucleic acid amplification have been developed to overcome the reliance on thermal cyclic technology. Temperatures are kept constant and different molecular mechanisms are used to maintain the DNA amplification efficiency [89]. Several methods have been developed including Recombinase Polymerase Amplification (RPA) [90], transcription mediated amplification (TMA) [91], strand displacement amplification (SDA) [92], rolling circle amplification (RCA) [93] and Loop-Mediated Isothermal Amplification (LAMP) [94]. Here, we focus on LAMP and RPA, which have been the most used methodologies for environmental monitoring to date.

2.2.1 Loop Mediated Isothermal Amplification

LAMP was first described by Notomi *et al.,* (2000) [94]. It typically uses a *Bacillus stearothermophilus (Bst)* DNA polymerase, which displays strand displacement activity whilst elongating the target sequence, and user-designed primers that recognise six distinct sequences allowing high levels of target specificity. The use of a *Bst* DNA polymerase allows the whole process to occur at a single temperature between 60 °C and 65 °C. In addition to being highly specific, LAMP has the benefit of being readily adaptable to visualisation with the naked eye. For example, incorporation of magnesium pyrophosphate causes increased turbidity as a by-product of DNA amplification [95]. Furthermore, dyes such as SYBR® Green can be added to the amplified product, producing a visible colour change [96]. The simple visualisation, and LAMP's potential to resist inhibitors in complex samples such as blood [97], makes this method a viable tool for disease detection in low resource settings [96, 98, 99].

However, despite the promise of this technique, use of LAMP for environmental monitoring is limited, with applications of specific taxa detection currently reported in freshwater environments only. Examples of use include monitoring of *Dreissena* sp. in the Great Lakes [100], detection of the freshwater snail *Galba truncatula* in Welsh pastureland [101] and surveillance of Trispot Darter (*Etheostoma trisella*) in freshwater sites across Georgia and Alabama [102]. The limited use is likely due to complexity of assay design and lower sensitivity compared to qPCR [103]. Nevertheless, although these limitations impact the usability of LAMP for eDNA detection, the advantages of LAMP presented in both biomedical and microbiological fields question why LAMP has not been more readily adapted to macro-invertebrate eDNA detection. For example, the increased specificity could greatly improve the ability to distinguish closely related species, which are indistinguishable using PCR-based methods and therefore, are not documented in the literature.

2.2.2 Recombinase Polymerase Amplification

RPA is another isothermal method of nucleic acid amplification. This was developed by Piepenburg *et al.*, (2006) [90] and has been commercialised by TwistDx. Unlike LAMP, RPA relies on only two sequence specific primers, which form complexes with a recombinase protein uvsX from T4-like bacteriophages, in the presence of ATP and a crowding agent [104]. This complex seeks and promotes strand invasion at a site complementary to the primer. The recombinase disassembles and a strand displacing DNA polymerase elongates the primer in the presence of dNTPs [104] achieving exponential amplification. Due to the displacement activity of the recombinase and polymerase enzymes, thermal cycling is not required to denature the double stranded target DNA, allowing the whole process to occur between 37-42 °C [90]. The simplicity of RPA makes it the ideal amplification method for on-site eDNA detection as it is simple to design, two sequence specific primers only, occurs at relatively low temperatures and achieves exponential amplification within 20 min [104].

As with LAMP, RPA has been adapted by the microbial community for the detection of parasites [105, 106] and harmful algae [107] in the environment. These studies have not only shown successful RPA detection from water samples [105, 107] but also from soil [105] and directly from pinewood [106]. Although RPA can be used in isolation, with visualisation on an agarose gel as with cnPCR or through incorporation of SYBR® Green for real time detection [90]; it is often combined with a secondary system to aid visualisation. This has included lateral flow systems [105], a portable optical isothermal device [106] and colourimetric sandwich hybridisation assays [107]. Each of these systems presents a pathway to on-site monitoring of specific species through incorporation of biotin-labelled primers, SYBR® Green dye and capture/reporter probes, respectively (discussed in detail below).

A potential concern with using RPA in isolation is the lack of specificity [108]. Removal of annealing temperature control, may lead to non-specific annealing of primers, particularly if insufficient mismatches are present. This could be highly problematic for eDNA detection, with cross amplification possible due to the complexity of eDNA samples and the common presence of sympatric taxa. Recommended optimisation of primer pairs requires carrying out a primer screen whereby multiple primer pairs are tested to select the optimal set [109]. This can be time consuming and may not eliminate cross amplification. It is therefore no wonder that in the medical space, RPA is being coupled with highly specific detection methods such as CRISPR-Cas [110–112] for pathogen detection.

2.2.3 CRISPR-Cas Detection

Clustered Regularly InterSpaced Palindromic Repeats (CRISPR) is primarily associated with genome editing [113] but latest developments have shown its utility as a highly specific nucleic acid detection system for both DNA [110] and RNA [111], often termed CRISPR-dx. This method relies on four main elements: presence of a protospacer adjacent motif (PAM), a sequence specific guide RNA (gRNA), a Cas12a or Cas13 nuclease and a reporter molecule. Briefly, the gRNA and Cas nuclease form a complex that recognises a target sequence upstream/downstream of a PAM site, depending on the specific Cas used. Upon sequence recognition, the target sequence is cleaved and subsequent indiscriminate cleavage of the reporter molecule occurs, allowing detection [110].

Both LAMP and RPA have been coupled with CRISPR-Cas technology to increase detection specificity [110, 111, 114]. In doing so, these systems utilise the isothermal nature of such amplification techniques whilst removing the limitation of specificity through coupling with the highly specific CRISPR system. Despite the shown potential of this system [115], application to single species detection from environmental samples is scarce. To date, an RPA-CRISPR-Cas system has only been used for identification of Delta Smelt (*Hypomesus transpacificus*), Longfin Smelt (*Spirinchus thaleichthys*) and Wakasagi (*Hypomesus nipponensis*) directly from mucus swabs [116] and mitochondrial DNA detection of Atlantic salmon (*Salmo salar)* from freshwater [117, 118]. More work is required to assess the potential of CRISPR-Cas to distinguish problematic sympatric taxa, resist environmental inhibitors or be adapted to an in-field sensor.

2.3 Molecular Assay Requirements

Regardless of the selected methodology, eDNA studies require robust assay design to reduce the risk of false positive detection. As mentioned already, the identification of a DNA sequence region that is unique to the target species is vital. The sequence specificity must be checked against closely related and co-habiting taxa throughout the design process [44]. This may be limited by insufficient reference sequences and polymorphism data, or inaccessibility to tissue of relevant species; however, it is of upmost importance if this approach is to be a valid one.

Ideally, assays should be designed to contain a high number of base pair differences in all sequence dependent elements such as primers and, if applicable, probes. Mismatches in primer binding regions reduce the affinity of the primers for non-target DNA and consequently reduce amplification of non-target DNA. On the other hand, mismatches in the probe-binding region reduce affinity of the probe for non-targets, ergo reducing or eliminating fluorescence from non-target species. Wilcox *et al.*, (2013) [44] found that assay specificity was most influenced by nucleotide

mismatches in the primers rather than the probe, resulting in reduced or no amplification of non-target sequences. The location of mismatches within primers is also important, with previous literature showing that mismatches near the 3' end of the primer have a much larger impact on specificity than mismatches at the 5' end [119]. Such considerations, of sequence specific mismatches, must also be applied to isothermal methods of amplification, despite variations in the exact assay design. In relation to CRISPR-Cas assays, ensuring that non-target closely related species lack the required PAM sequence is essential even if the gRNA contains mismatches (Williams *et al.,* Unpublished).

In summary, PCR-based methods still dominate the field, with qPCR the most commonly used. This is likely due to it providing higher sensitivity and specificity than cnPCR, being more cost effective and requiring more readily available instrumentation than ddPCR. The developing field of molecular detection for pathogens in low-economic countries is driving research into alternative methods of detection, moving away from PCR and towards point of care tests using isothermal methodology. These methods could benefit the eDNA community by simplifying adaptation to an in-field detection system or simply providing an alternative detection method if PCR fails. Depending on the requirements of the study, careful consideration needs to be made regarding the molecular assay of choice for marine biosensing.

Transitioning these molecular assays from the laboratory to on-site or autonomous eDNA based biosensing requires consideration of their detection modes. We describe a selection of detection methods that have potential to make this transition that are currently being developed.

3.0 Detection Modes

The ability to detect minute concentrations of DNA directly from the aquatic environment is transforming ecological monitoring and environmental management [120]. However, the reliability of eDNA as a tool for monitoring is contingent upon the ability to detect the presence of a target species. Environmental DNA is generally present in the environment in very low concentrations. These concentrations may be even lower for rare, sedentary, or elusive species. Consequently, an appropriate and extremely sensitive method of detection is vital. The two most common methods to improve the sensitivity of a detection methodology are target- and signal-based approaches [121, 122]. Target-based approaches produce copies of a specific target continuously, in a cyclic process (e.g., PCR) as well as isothermal alternatives to PCR, such as LAMP, RPA, and CRISPR-Cas RPA (as discussed above). Signal-based amplification approaches, such as enzyme-assisted target recycling, have also been developed to attain sensitive detection by enhancing the readable signal. These approaches are subsequently coupled with a specific chemical mechanism for detection, which indicate whether a target has been detected (e.g., fluorescence or colourimetry). Below we discuss some of these detection mechanisms relevant to eDNA biosensing, specifically: fluorescence, colourimetry and lateral flow and how they tally with their associated molecular assays in terms of the temperature requirements and incubation times (Table 1).

Table 1: Molecular assays and their detection modes

Assay	Specificity	Temperature	Time (min)	No. of steps	Detection Mode
cnPCR	Medium	Cycles	60-180*	2	Gel visualisation
qPCR	High	Cycles	50-120*	1	Fluor.
ddPCR	High	Cycles	180-300	1	Fluor.
LAMP	Medium	65°C	30-60	1	Colourimetric
RPA-Probe	Low-Medium	37°C	20	1	Fluor./LF
RPA-CRISPR-Cas	High	37°C	50	2	Fluor./LF

*Based on a typical 40-cycle reaction. Time will also vary depending on the ramping rate of the thermal cycler. Fluor. = fluorescence; LF = Lateral Flow

3.1 Colourimetry

Colourimetric eDNA assays utilise reagents that undergo a measurable colour change in the presence of the target DNA. Detection of a positive sample can be either by visual observation or through the use of optical sensors which offer more sensitivity. Optical sensors for colourimetric detection can range from easily accessible low-cost devices (i.e., scanners or camera phones), to more specialized instruments, such as spectrophotometers which also allow real-time DNA quantification via colourimetric change [123, 124]. Target based methods such as LAMP have utilised colourimetric eDNA detection and demonstrated a limit of detection comparable to that of qPCR-based detection (TaqMan chemistry) [125]. Similarly, Toldrà *et al.,* (2019) [107] used a colourimetric approach for the detection of two species of *Ostreopsis* dinoflagellates which are associated with toxic algal blooms. RPA amplification was combined with a colourimetric sandwich hybridisation assay (i.e. enzyme-linked oligonucleotide assay, ELONA) again to produce results comparable to qPCR in both sensitivity (LOD) and in the quantification of *O.* cf. *ovata* and *O.* cf. *siamensis* in environmental samples [107]. Elumalai *et al.,* (2021) [126] recently reported a novel strategy for the detection of the invasive zebra mussel (*Dreissena polymorpha*) which is based on nicking endonuclease (NEase) signal amplification combined with gold nanoparticles (AuNPS) for colourimetric readout. NEase signal amplification can enhance the specificity and sensitivity of conventional colourimetric sandwich assays due to its high efficiency in single-strand cleavage [127]. The isothermal properties of the eDNA detection approaches employed by the former studies [107, 125], make these assays highly adaptable to biosensing devices. However, the latter application by Elumalai *et al.,* (2021) [126], which exhibits significant sensitivity, could present more of a challenge for adaptation to biosensing due the range of temperatures employed (58°C, 37°C, 80°C).

3.2 Lateral Flow

Lateral flow biosensors (LFBs) are paper-based devices that permit low-cost and fast diagnostics. This application is commonly associated with home pregnancy tests [128] and more recently COVID-19 antigen testing [129]. The method is based on the separation of molecules flowing through test strip membranes followed by the detection of target molecules in a test zone using coloured labels [130]. The detection label in the test zone most often utilises nanoparticles such as colloidal gold, coloured latex, fluorescent particles (e.g., quantum dots), and carbon particles [131]. In LFBs, the specific recognition of a target is owing to receptors (most often antibodies – termed lateral flow immunoassays) being immobilized on the nanoparticle surface concentrated in the binding (test and

control) zones and the subsequent formation of specific complexes, resulting in colourisation in these zones [130] (Figure 2). Two approaches are generally used for the detection of nucleic acid targets with LFBs: direct detection of DNA sequences and nucleic acid lateral flow immunoassays (NALFIAs). Direct detection involves exploiting capture of labelled reporter oligonucleotide probes [132, 133], whereas in NAFLIAs labelled oligonucleotides are captured via antibodies or streptavidin. For example, the sample is loaded at one end of the strip and migrates, via capillary action, through the conjugate release pad, which contains antibodies that are specific to the target analyte and are conjugated to coloured or fluorescent nanoparticles. The sample, together with the conjugated antibody bound to the target analyte, migrates along the strip into the detection zone, which will react and cause a colour change [134] (Figure 2).

Target based isothermal amplification methods such as RPA and LAMP are also commonly used with LFBs [130, 135]. There are a number of advantages to using LFBs for eDNA detection: components of LFB strips can be modified and optimized for signal increase in the test zone; LFB strips are commercially available for detecting biotin-/FAM-labelled amplicons, including those by Millenia (Germany), BioUSTAR (China), Agdia (USA), and TwistDX (USA); and portable devices to register colourisation in the test zone of LFBs are commercially available e.g., (Abingdon Health, PCRD^{TM}). Doyle and Uthicke (2021) [136] used the PCRD^{TM} as a detection method for eDNA monitoring of a sea star (*Acanthaster cf. solaris*) and found their combined PCR and lateral flow assay to be as sensitive as ddPCR and able to detect < 10 copies of target DNA, per PCR. However, without the use of a detection device to enhance the ability to detect colour change in a LFB, sensitivity can be a limitation when applying this method of detection.

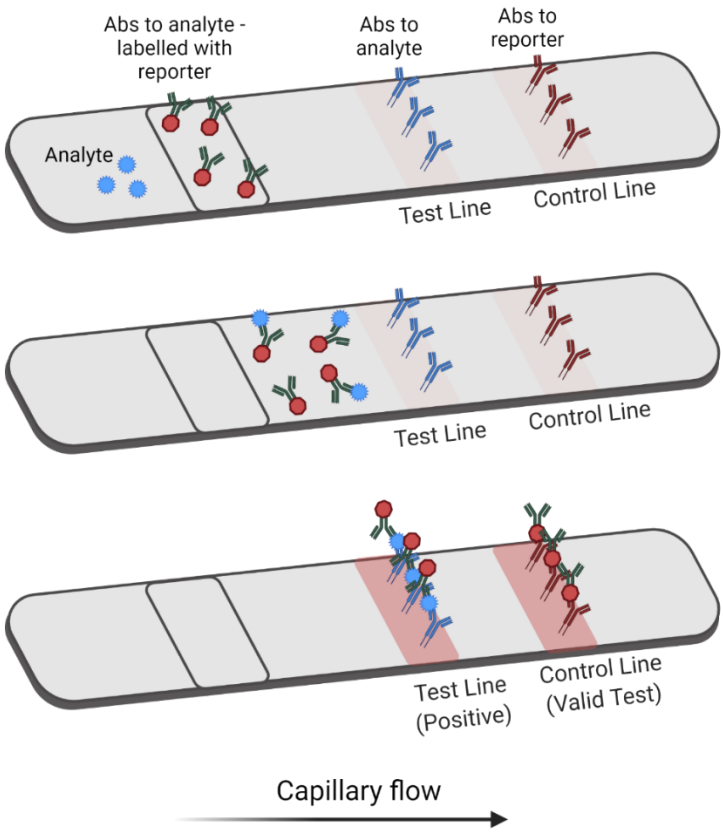

Figure 2: Mechanism of a Lateral Flow Immunoassay platform. An antibody pair is required for this sandwich immunoassay; one antibody to one epitope of the analyte is labelled with a reporter, a secondary capture antibody to a different epitope of the same analyte is immobilised to the platform (test line). The control line consists of an antibody to the reporter and ensures the test is valid. Adapted from Lee *et al.,* (2013) [137], created with BioRender.com.

3.2 Fluorescence

Fluorescence based bioanalysis and imaging techniques, using uniquely tailored probes, have attracted increasing attention in the detection of environmental DNA molecules *in-situ.* This approach to detection has been widely adopted in the fields of life science, biomedicine and pre(clinical) diagnostics owing to its high resolution, excellent selectivity and sensitivity, simplicity, and capability of real-time monitoring [138]. As outlined in Table 1 and Section 2, fluorescence detection is an option for a number of molecular assays including qPCR, ddPCR, RPA-probe and RPA-CRISPR-Cas. Fluorescence arises from a probe, a reporter or an intercalating dye, and is bespoke for each assay. One group of probes frequently used in qPCR and ddPCR are hydrolysis (eg. TaqMan®) probes. These probes consist of a short DNA fragment, complementary to the target, labelled with a reporter dye on one end (i.e., FAM, TAMRA, Hex etc.) and a quencher molecule on the other. The quencher absorbs reporter fluorescence when the probe is intact *via* fluorescent resonance energy transfer (FRET). During each PCR cycle, the 5'-3' nuclease activity of the DNA polymerase cleaves the probe during

target elongation [139]. This cleavage separates the quencher and results in an amplification-dependent increase in fluorescence. TaqMan® MGB probes are an advanced type of hydrolysis probe commonly used in eDNA assays [46, 48, 140]. They have the advantage of having a 3' modification allowing the formation of extremely stable duplexes with the target strand and allowing construction of short probes highly sensitive to nucleotide differences [141].

Fluorescence detection can also arise from the use of non-sequence specific intercalating dyes, such as SYBR® Green. This dye exhibits little fluorescence when free in solution, but once bound to the minor groove of double-stranded DNA (dsDNA), its fluorescence increases up to 1,000-fold [142]. As more dsDNA is produced during DNA amplification, more dye binds to the DNA and the fluorescence increases. Although SYBR® Green lacks the specificity of hydrolysis probes; it provides a cheaper method of detection and has been shown to allow reliable detection of single species from eDNA [54]. Additionally, it may reduce the susceptibility of qPCR to inhibitors often present in the environment [143]. The major drawback, however, of DNA-binding dyes is their lack of specificity, as they will bind to all dsDNA. As a result, the presence of nonspecific products in a real-time PCR reaction, such as PCR primer-dimers, contributes to the overall fluorescence and reduces the accuracy of quantification [144]. For the latter reasons, eDNA detection using probe-based qPCR assays is preferable to dye-based approaches for species-specific eDNA analysis.

CRISPR-Cas coupled with RPA utilises a fluorescently labelled reporter molecule (such as FAM). The collateral cleavage activity of a Cas12a nuclease results in release of the fluorescent label from the quencher (ssDNA-FQ) molecule only upon recognition of the target DNA sequence [117]. A more recent advance on this mode of detection is to use CRISPR-Cas12a based nucleic acid amplification-free fluorescent detection using metal-enhanced fluorescence (MEF) by DNA-functionalized gold nanoparticles (AuNP). Upon activating the CRISPR-Cas12a complex by the target DNA and subsequent single-strand DNA (ssDNA) degradation between AuNP and fluorophore, MEF occurred with colour changes from purple to red-purple [145].

4.0 Biosensor Devices

Although there is considerable momentum in the field of eDNA biosensing over recent years, there are a very limited number of devices with the capabilities to carry out all of the functions necessary to conduct autonomous eDNA monitoring *in-situ*. However, several large-scale projects are in progress that are developing *in-situ* autonomous biosensors (e.g., TechOceanS and iATlantic). Below, is a summary of published and /or commercially available devices that have been applied to eDNA monitoring. These are divided into portable (i.e., manual) devices and autonomous systems.

4.1 Portable devices

To create a complete field eDNA sampling and detection process, Thomas *et al.,* (2019) [146] combined the Smith-Root eDNA Sampler filtration system with a rapid (30-60 minutes) field DNA extraction and qPCR analysis platform (Biomeme™) and encountered similar sensitivity to benchtop qPCR applications. Using the same Biomeme™ qPCR platform, Ponce *et al.,* (2021) [147] found a 10% increase in positive eDNA detection relative to visual surveys, however this is contrary to other studies that have found this portable device to have lower detection probabilities than laboratory-based methods [148]. The Biomeme™ system combines a quick sample preparation column to extract DNA,

'Go-Strips' which contain lyophilised qPCR reagents, and a handheld real-time thermal cycler connected to a phone, for on-site monitoring [149]. Although it offers potential for eDNA monitoring, the Biomeme™ system is expensive and susceptible to greater influence from inhibitors than laboratory-based approaches, with inhibition rates as high as 28% for field samples [148]. Doi *et al.,* (2021) [150] also utilised a commercially available mobile qPCR platform (PicoGene PCR1100 Nippon Sheet Glass, Sagamihara, Japan) for eDNA detection on site. In this study, a water sample was syringe filtered using a Sterivex cartridge filter (Merck Millipore) and DNA extraction was performed using a Kaneka Simple DNA Extraction Kit v.2 (Kaneka, Tokyo, Japan). Via the real-time fluorescence detection, PicoGene (binds similarly to SYBR® Green) successfully identified target DNA from the invasive silver carp (*Hypophthalmichthys molitrix*) in 30 minutes using a custom Taqman® qPCR assay [150].

Another commercially available biosensing device is the Pebble (biopix-t). Pebble was developed for performing real-time colourimetric LAMP in a single reaction-pot (Eppendorf-tube) for simultaneous amplification and quantitative detection. The device uses a plastic tube anchored vertically on a hot surface while the sidewalls are exposed to a mini camera able to take snapshots of the colour change in real time during LAMP amplification [124]. Detection can occur from purified nucleic acids or directly from crude samples (swabs, saliva, filters and biopsy tissue) and has so far been applied to point of care applications such as the detection of SARS-CoV-2 and cancer mutations [124], as well as the detection of invasive lionfish (*Pterois miles*) from marine environmental samples (M. Vivaldi pers comm).

Heery *et al.,* (2016) [151] developed a field portable detection system (ColiSense) using a β-d-Glucuronidase (GUS) enzyme assay. The ColiSense system is an optical biosensor, which utilises the enzymatic activity of *Escherichia coli* to monitor levels in bathing waters and has successfully detected *E. coli* in freshwater and seawater, providing results in 75 min from sample collection [152]. ColiSense was further adapted to enable eDNA detection (known as the SensEDNA) using an RPA-CRISPR-Cas approach and has been demonstrated as a proof-of-concept (Williams *et al.,* Unpublished). Whilst promising, this method currently requires lab-based extraction and purification of eDNA samples, prior to molecular detection and is not yet suitable for *in-situ* eDNA applications.

4.2 Remote and autonomous systems

A fundamental limitation to the application of eDNA monitoring techniques in a marine setting is the acquisition of the physical sample for analysis. Manual sample collection can be expensive and logistically very challenging especially when ship time is required. Similarly, the handling and transport of samples introduces the potential for contamination and degradation of the integrity of samples, as well as significant time delays in attaining results. Such limitations also prevent the acquisition of time series data, which is critical in the detection of temporal and spatial changes in species occurrence and abundance. This is of particular relevance for rare and migratory species [25]. As discussed above, applying real-time molecular analytical techniques simultaneously with sample collection can enhance the effectiveness and efficiency of the monitoring process. This has prompted an impetus for *in-situ* sample processing and real-time molecular detection to be implemented using deployable autonomous systems that can be operated in diverse ocean environments. Such devices have been termed "ecogenomic sensors" [153–155].

The initial challenge with creating an autonomous system is developing an effective method to collect and concentrate water samples. To this end, two such robotic samplers have been developed to date: the Environmental Sample Processor (ESP) developed by the Monterey Bay Aquarium Research Institute (MBARI) [156] and the Robotic Cartridge Sampling Instrument (RoCSI) developed by the National Oceanography Centre (NOC; UK) [157]. RoCSI is an autonomous high-frequency sampling device for filtering predetermined volumes of water and preserving the filters *in-situ*. Tang *et al.,* (2020) [157] used RoCSI to collect over 200 samples (varying from ~1.5 L to over 4 L depending on particle concentration) at high frequency (every hour) on a 0.2 µm Sterivex filter (Millipore, MA, USA). The combination of high-resolution N_2-fixation observations and molecular sampling allowed Tang *et al.,* (2020) [157] to successfully capture and characterize episodic diazotrophic blooms and evaluate their environmental controls. High frequency sampling such as this, which is enabled by autonomous systems, such as RoCSI and ESP, facilitate the collection of significantly more data with which to make robust inferences about the presence/absence of a target species. Various iterations of the ESP have been developed over the past 25 years and the ESP (2G) has been utilised to automate water sample filtration and preservation of the captured material, or homogenize it for immediate analyses *in-situ*. Sepulveda *et al.,* (2020) [156] collected eDNA samples at high frequency (e.g., every 3 h) using the ESP system and successfully detected the DNA of human pathogens, fish pathogens and introduced fishes, as well as collecting additional archival samples. When comparing the ESP to traditional sampling methods, no significant differences in eDNA densities were observed between the two sample collection and filtration methods in either marine [158] or freshwater settings [156]. The ESP can be deployed directly on the seabed or in a pelagic configuration, where it can operate for several months depending on power consumption. The newer iterations are also capable of applying DNA probe and protein arrays as well as using qPCR to detect a wide range of organisms, genes and metabolites [159]. The performance and sensitivity of ESP to detect and quantify eDNA in a large mesocosm was assessed by Hansen *et al.,* (2020) [160]. They found the ESP was able to consistently detect and quantify target molecules from the most abundant species (Atlantic mackerel *Scromber scrombus*) both in real-time and from the archived samples. However, the low abundance species were rarely (European eel *Anguilla anguilla*, N = 2) or never (European plaice *Pleuronectes platessa* and European flounder *Platichthys flesus*) detected with the *in-situ* analysis [160]. This lower detection may be a result of disparity in shedding rate (i.e., benthic species may be less active) or behaviour ecology (diel migration activity was not considered when developing the sampling strategy).

The ESP system is based on a modular design consisting of a core sample processor (the ESP), analytical modules and sampling modules. Sampling modules are devices external to the core ESP that can be added to meet specialized needs, such as operating in the deep-sea, whilst analytical modules are conceived to apply different suites of analytical functions downstream of common sample processing operations [161]. The ESPs' analytical 'pucks' are custom designed reaction chambers that support a wide variety of filters or chemically adsorptive media depending upon protocol requirements [162]. This modular design has enabled effective deployment of the ESP in a variety of environments. For example, the shallow water configuration of the ESP [155] can be modified for ecogenomic sensing at depths up to 4000 m [163]. These unique features make the ESP a candidate for offshore monitoring of marine biodiversity, as well as a potential early warning/detection system e.g. for invasive species.

5.0 Concluding remarks

Rapid monitoring of aquatic organisms is essential for preserving the Earth's biodiversity, which is currently at risk due to the increasing threat of climate change [164, 165]. Management and conservation of aquatic species requires knowledge of distribution, traditionally gained through visual detection. However, such methodologies are expensive, labour intensive and can cause harm to the target organisms [17]. The use of eDNA offers a solution, using non-invasive molecular techniques to detect DNA shed into the environment [166]. Whilst the majority of eDNA studies to date are limited to the laboratory and require highly skilled individuals to perform experimentation, progress is being made to develop portable and autonomous biosensors, which would enable rapid, on-site detection of target organisms. As highlighted in this chapter, such sensors must overcome three main challenges; eDNA acquisition, sensitive and specific molecular assay design, and choice of detection mode. When focusing on autonomous systems, there are also additional challenges, not discussed in this chapter, such as the preservation of reagents and the ability to transmit results in real-time. To overcome such limitations, it is important that the field takes guidance from other disciplines by observing developments made in, for example, point of care testing based on the World Health Organisation ASSURED guidelines (Affordable, Sensitive, Specific, User-friendly, Robust and rapid, Equipment-free, Deliverable to all people who need the test) and environmental sensing of other data (e.g. physio-chemical).

The full potential of robotic technologies like the ESP will be achieved when *in-situ* analyses of water samples can transmit real-time results. 'Near' real-time analysis has been demonstrated using *in-situ* qPCR [159, 163], however, these *in-situ* modules are still in development and there are significant limitations regarding expedient data processing and the integration of eDNA results relative to other environmental (e.g. physio-chemical) data [160]. The generic nature of water sampling, however, also makes eDNA sampling highly amenable to automation via the adaptation of other remote sensing technologies that traditionally focus on electromagnetic, acoustic, or other data [25]. Improving the technology to enable fully remote and autonomous eDNA methodologies will greatly expand the future potential of eDNA applications to collect robust temporal and spatial data relating to species that, in the past, may have been overlooked due to sampling constraints.

Bibliography

1. Taberlet P, Coissac E, Hajibabaei M, Riesberg L (2012) Environmental DNA. Mol Ecol 21:1789–1793. https://doi.org/10.1111/j.1365-294X.2012.05542.x

2. Thomsen PF, Kielgast J, Iversen LL, Wiuf C, Rasmussen M, Gilbert MTP, Orlando L, Willerslev E (2012) Monitoring endangered freshwater biodiversity using environmental DNA. Mol Ecol 21:2565–2573. https://doi.org/10.1111/j.1365-294X.2011.05418.x

3. Díaz-Ferguson EE, Moyer GR (2014) History, applications, methodological issues and perspectives for the use of environmental DNA (eDNA) in marine and freshwater environments. Rev Biol Trop 62:1273–1284. https://doi.org/10.15517/RBT.V62I4.13231

4. Turner CR, Barnes MA, Xu CCY, Jones SE, Jerde CL, Lodge DM (2014) Particle size distribution and optimal capture of aqueous macrobial eDNA. Methods Ecol Evol 5:676–684. https://doi.org/10.1111/2041-210X.12206

5. Goldberg CS, Pilliod DS, Arkle RS, Waits LP (2011) Molecular Detection of Vertebrates in Stream Water: A Demonstration Using Rocky Mountain Tailed Frogs and Idaho Giant Salamanders. PLoS One 6:e22746. https://doi.org/10.1371/journal.pone.0022746

6. Barnes MA, Turner CR, Jerde CL, Renshaw MA, Chadderton WL, Lodge DM (2014) Environmental Conditions Influence eDNA Persistence in Aquatic Systems. Environ Sci Technol 48:1819–1827. https://doi.org/10.1021/ES404734P

7. Lacoursière-Roussel A, Côté G, Leclerc V, Bernatchez L (2016) Quantifying relative fish abundance with eDNA: a promising tool for fisheries management. J Appl Ecol 53:1148–1157. https://doi.org/10.1111/1365-2664.12598

8. Takahara T, Minamoto T, Yamanaka H, Doi H, Kawabata Z (2012) Estimation of Fish Biomass Using Environmental DNA. PLoS One 7:e35868. https://doi.org/10.1371/journal.pone.0035868

9. Pilliod DS, Goldberg CS, Arkle RS, Waits LP (2013) Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. Can J Fish Aquat Sci 70:1123–1130. https://doi.org/10.1139/cjfas-2013-0047

10. Bracken FSA, Rooney SM, Kelly-Quinn M, King JJ, Carlsson J (2019) Identifying spawning sites and other critical habitat in lotic systems using eDNA “snapshots”: A case study using the sea lamprey Petromyzon marinus L. Ecol Evol 9:553–567. https://doi.org/10.1002/ECE3.4777

11. Mahon AR, Jerde CL, Galaska M, Bergner JL, Chadderton WL, Lodge DM, Hunter ME, Nico LG (2013) Validation of eDNA Surveillance Sensitivity for Detection of Asian Carps in Controlled and Field Experiments. PLoS One 8:e58316. https://doi.org/10.1371/JOURNAL.PONE.0058316

12. Evans N, Shirey P, Wieringa J, Mahon A, Lambertia G (2017) Comparitive cost and effort of fish distribution detection via environmental DNA analysis and electrofishing. Fisheries 42:90–99

13. Andruszkiewicz EA, Koseff JR, Fringer OB, Ouellette NT, Lowe AB, Edwards CA, Boehm AB (2019) Modeling environmental DNA transport in the coastal ocean using Lagrangian particle tracking. Front Mar Sci 6:477. https://doi.org/10.3389/FMARS.2019.00477/BIBTEX

14. Collins RA, Wangensteen OS, O'Gorman EJ, Mariani S, Sims DW, Genner MJ (2018) Persistence of environmental DNA in marine systems. Commun Biol 1:185. https://doi.org/10.1038/s42003-018-0192-6

15. Jo T, Minamoto T (2021) Complex interactions between environmental DNA (eDNA) state and water chemistries on eDNA persistence suggested by meta-analyses. Mol Ecol Resour 21:1490–1503. https://doi.org/10.1111/1755-0998.13354

16. Rourke ML, Fowler AM, Hughes JM, Broadhurst MK, DiBattista JD, Fielder S, Walburn JW, Furlan EM (2021) Environmental DNA (eDNA) as a tool for assessing fish biomass: A review of approaches and future considerations for resource surveys. Environ DNA 00:1–25. https://doi.org/10.1002/EDN3.185

17. Snyder D (2003) Invite overview: conclusions from a review of electrofishing and its harmful effects on fish. Rev Fish Biol Fish 13:445–453

18. Browne CL, Hecnar SJ (2007) Species loss and shifting population structure of freshwater turtles despite habitat protection. Biol Conserv 138:421–429. https://doi.org/10.1016/j.biocon.2007.05.008

19. Dodd CK, Griffey ML, Corser JD (2001) The Cave Associated Amphibians of Great Smoke Mountains National Park: Review and Monitoring. J Elisha Mitchell Sci Soc 117:139–149

20. Bohlin T, Hamrin S, Heggberget TG, Rasmussen G, Saltveit SJ (1989) Electrofishing — Theory and practice with special emphasis on salmonids. Hydrobiologia 173:9–43. https://doi.org/10.1007/BF00008596

21. Kelly RP (2014) Will more, better, cheaper and faster monitoring improve environmental management? Environ Law 44:1111–1147

22. Dejean T, Valentini A, Duparc A, Pellier-Cuit S, Pompanon F, Taberlet P, Miaud C (2011) Persistence of Environmental DNA in Freshwater Ecosystems. PLoS One 6:e23398. https://doi.org/10.1371/journal.pone.0023398

23. Strickler KM, Fremier AK, Goldberg CS (2015) Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. Biol Conserv 183:85–92. https://doi.org/10.1016/j.biocon.2014.11.038

24. Ficetola GF, Poulenard J, Sabatier P, Messager E, Gielly L, Leloup A, Etienne D, Bakke J, Malet E, Fanget B, Støren E, Reyss JL, Taberlet P, Arnaud F (2018) DNA from lake sediments reveals long-term ecosystem changes after a biological invasion. Sci Adv 4:. https://doi.org/10.1126/SCIADV.AAR4292/SUPPL_FILE/AAR4292_SM.PDF

25. Barnes MA, Turner CR (2015) The ecology of environmental DNA and implications for conservation genetics. Conserv Genet 2015 171 17:1–17. https://doi.org/10.1007/S10592-015-0775-4

26. Eichmiller JJ, Best SE, Sorensen PW (2016) Effects of Temperature and Trophic State on Degradation of Environmental DNA in Lake Water. Environ Sci Technol 50:1859–1867. https://doi.org/10.1021/ACS.EST.5B05672/SUPPL_FILE/ES5B05672_SI_001.PDF

27. Lance RF, Klymus KE, Richter CA, Guan X, Farrington HL, Carr MR, Thompson N, Chapman DC, Baerwaldt KL (2017) Experimental observations on the decay of environmental DNA from

bighead and silver carps. Manag Biol Invasions 8:343–359. https://doi.org/10.3391/mbi.2017.8.3.08

28. Jo T, Arimoto M, Murakami H, Masuda R, Minamoto T (2020) Estimating shedding and decay rates of environmental nuclear DNA with relation to water temperature and biomass. Environ DNA 2:140–151. https://doi.org/10.1002/EDN3.51

29. Seymour M, Durance I, Cosby BJ, Ransom-Jones E, Deiner K, Ormerod SJ, Colbourne JK, Wilgar G, Carvalho GR, de Bruyn M, Edwards F, Emmett BA, Bik HM, Creer S (2018) Acidity promotes degradation of multi-species environmental DNA in lotic mesocosms. Commun Biol 2018 11 1:1–8. https://doi.org/10.1038/s42003-017-0005-3

30. Tsuji S, Takahara T, Doi H, Shibata N, Yamanaka H (2019) The detection of aquatic macroorganisms using environmental DNA analysis—A review of methods for collection, extraction, and detection. Environ DNA 1:99–108. https://doi.org/10.1002/edn3.21

31. Hinlo R, Gleeson D, Lintermans M, Furlan E (2017) Methods to maximise recovery of environmental DNA from water samples. PLoS One 12:e0179251. https://doi.org/10.1371/journal.pone.0179251

32. Spens J, Evans AR, Halfmaerten D, Knudsen SW, Sengupta ME, Mak SST, Sigsgaard EE, Hellström M (2017) Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. Methods Ecol Evol 8:635–645. https://doi.org/10.1111/2041-210X.12683

33. Eichmiller JJ, Miller LM, Sorensen PW (2016) Optimizing techniques to capture and extract environmental DNA for detection and quantification of fish. Mol Ecol Resour 16:56–68. https://doi.org/10.1111/1755-0998.12421

34. Hunter ME, Ferrante JA, Meigs-Friend G, Ulmer A (2019) Improving eDNA yield and inhibitor reduction through increased water volumes and multi-filter isolation techniques. Sci Reports 2019 91 9:1–9. https://doi.org/10.1038/s41598-019-40977-w

35. Robson HLA, Noble TH, Saunders RJ, Robson SKA, Burrows DW, Jerry DR (2016) Fine-tuning for the tropics: application of eDNA technology for invasive fish detection in tropical freshwater ecosystems. Mol Ecol Resour 16:922–932. https://doi.org/10.1111/1755-0998.12505

36. Doi H, Uchii K, Matsuhashi S, Takahara T, Yamanaka H, Minamoto T (2017) Isopropanol precipitation method for collecting fish environmental DNA. Limnol Oceanogr Methods 15:212–218. https://doi.org/10.1002/LOM3.10161

37. Deiner K, Walser JC, Mächler E, Altermatt F (2015) Choice of capture and extraction methods affect detection of freshwater biodiversity from environmental DNA. Biol Conserv 183:53–63. https://doi.org/10.1016/J.BIOCON.2014.11.018

38. Sambrook J., Russell D. (2001) Molecular Cloning: A Laboratory Manual, 3rd ed., Vols 1, 2 and 3. Cold Spring Harbor Laboratory Press

39. Renshaw MA, Olds BP, Jerde CL, McVeigh MM, Lodge DM (2015) The room temperature preservation of filtered environmental DNA samples and assimilation into a phenol-chloroform-isoamyl alcohol DNA extraction. Mol Ecol Resour 15:168–176. https://doi.org/10.1111/1755-0998.12281

40. Thomas AC, Howard J, Nguyen PL, Seimon TA, Goldberg CS (2018) eDNA Sampler: A fully integrated environmental DNA sampling system. Methods Ecol Evol 9:1379–1385. https://doi.org/10.1111/2041-210X.12994

41. Bogenhagen DF (2012) Mitochondrial DNA nucleoid structure. Biochim Biophys Acta - Gene Regul Mech 1819:914–920. https://doi.org/10.1016/J.BBAGRM.2011.11.005

42. Robinson CV, Garcia de Leaniz C, Rolla M, Consuegra S (2019) Monitoring the eradication of the highly invasive topmouth gudgeon (Pseudorasbora parva) using a novel eDNA assay. Environ DNA 1:74–85. https://doi.org/10.1002/edn3.12

43. Thomsen PF, Kielgast J, Iversen LL, Møller PR, Rasmussen M, Willerslev E (2012) Detection of a Diverse Marine Fish Fauna Using Environmental DNA from Seawater Samples. PLoS One 7:e41732. https://doi.org/10.1371/journal.pone.0041732

44. Wilcox TM, McKelvey KS, Young MK, Jane SF, Lowe WH, Whiteley AR, Schwartz MK (2013) Robust Detection of Rare Species Using Environmental DNA: The Importance of Primer Specificity. PLoS One 8:e59520. https://doi.org/10.1371/journal.pone.0059520

45. Boothroyd M, Mandrak NE, Fox M, Wilson CC (2016) Environmental DNA (eDNA) detection and habitat occupancy of threatened spotted gar (Lepisosteus oculatus). Aquat Conserv Mar Freshw Ecosyst 26:1107–1119. https://doi.org/10.1002/aqc.2617

46. Carlsson JEL, Egan D, Collins PC, Farrell ED, Igoe F, Carlsson J (2017) A qPCR MGB probe based eDNA assay for European freshwater pearl mussel (*Margaritifera margaritifera* L.). Aquat Conserv Mar Freshw Ecosyst 27:1341–1344. https://doi.org/10.1002/aqc.2788

47. Gargan LM, Morato T, Pham CK, Finarelli JA, Carlsson JEL, Carlsson J (2017) Development of a sensitive detection method to survey pelagic biodiversity using eDNA and quantitative PCR: a case study of devil ray at seamounts. Mar Biol 164:112. https://doi.org/10.1007/s00227-017-3141-x

48. Atkinson S, Carlsson JEL, Ball B, Egan D, Kelly-Quinn M, Whelan K, Carlsson J (2018) A quantitative PCR-based environmental DNA assay for detecting Atlantic salmon (*Salmo salar* L.). Aquat Conserv Mar Freshw Ecosyst 28:1238–1243. https://doi.org/10.1002/aqc.2931

49. Rusch JC, Hansen H, Strand DA, Markussen T, Hytterød S, Vrålstad T (2018) Catching the fish with the worm: a case study on eDNA detection of the monogenean parasite Gyrodactylus salaris and two of its hosts, Atlantic salmon (Salmo salar) and rainbow trout (Oncorhynchus mykiss). Parasit Vectors 11:333. https://doi.org/10.1186/s13071-018-2916-3

50. Baker CS, Steel D, Nieukirk S, Klinck H (2018) Environmental DNA (eDNA) From the Wake of the Whales: Droplet Digital PCR for Detection and Species Identification. Front Mar Sci 5:133. https://doi.org/10.3389/fmars.2018.00133

51. Uthicke S, Lamare M, Doyle JR (2018) eDNA detection of corallivorous seastar (Acanthaster cf. solaris) outbreaks on the Great Barrier Reef using digital droplet PCR. Coral Reefs 37:1229–1239. https://doi.org/10.1007/s00338-018-1734-6

52. Ficetola GF, Miaud C, Pompanon F, Taberlet P (2008) Species detection using environmental DNA from water samples. Biol Lett 4:423–425. https://doi.org/10.1098/rsbl.2008.0118

53. Dejean T, Valentini A, Miquel C, Taberlet P, Bellemain E, Miaud C (2012) Improved detection of

alien invasive species through environmental DNA barcoding: the example of the American bullfrog Lithobates catesbeianus. J Appl Ecol 49:953–959

54. Davy CM, Kidd AG, Wilson CC (2015) Development and Validation of Environmental DNA (eDNA) Markers for Detection of Freshwater Turtles. PLoS One 10:e0130965. https://doi.org/10.1371/journal.pone.0130965

55. Deiner K, Altermatt F (2014) Transport Distance of Invertebrate Environmental DNA in a Natural River. PLoS One 9:e88786. https://doi.org/10.1371/journal.pone.0088786

56. Janosik AM, Johnston CE (2015) Environmental DNA as an effective tool for detection of imperiled fishes. Environ Biol Fishes 98:1889–1893. https://doi.org/10.1007/s10641-015-0405-5

57. Jerde CL, Mahon AR, Chadderton WL, Lodge DM (2011) "Sight-unseen" detection of rare aquatic species using environmental DNA. Conserv Lett 4:150–157. https://doi.org/10.1111/j.1755-263X.2010.00158.x

58. Ardura A, Zaiko A, Martinez JL, Samulioviene A, Semenova A, Garcia-Vazquez E (2015) eDNA and specific primers for early detection of invasive species – A case study on the bivalve Rangia cuneata, currently spreading in Europe. Mar Environ Res 112:48–55. https://doi.org/10.1016/J.MARENVRES.2015.09.013

59. Muñoz-Colmenero M, Ardura A, Clusa L, Miralles L, Gower F, Zaiko A, Garcia-Vazquez E (2018) New specific molecular marker detects Ficopomatus enigmaticus from water eDNA before positive results of conventional sampling. J Nat Conserv 43:173–178. https://doi.org/10.1016/J.JNC.2017.12.004

60. Nathan LM, Simmons M, Wegleitner BJ, Jerde CL, Mahon AR (2014) Quantifying Environmental DNA Signals for Aquatic Invasive Species Across Multiple Detection Platforms. Environ Sci Technol 48:12800–12806. https://doi.org/10.1021/es5034052

61. Xia Z, Johansson ML, Gao Y, Zhang L, Haffner GD, MacIsaac HJ, Zhan A (2018) Conventional versus real-time quantitative PCR for rare species detection. Ecol Evol 8:11799–11807. https://doi.org/10.1002/ece3.4636

62. Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonák J, Lind K, Sindelka R, Sjöback R, Sjögreen B, Strömbom L, Ståhlberg A, Zoric N (2006) The real-time polymerase chain reaction. Mol Aspects Med 27:95–125. https://doi.org/10.1016/j.mam.2005.12.007

63. Hernandez CC, Bougas B, Perreault-Payette A, Simard A, Côté G, Bernatchez L, Perreault A, Simard A, Cote G, Bernatchez L (2020) 60 specific eDNA qPCR assays to detect invasive, threatened and exploited freshwater vertebrates and invertebrates in Eastern Canada. Environ DNA 2:373–386. https://doi.org/10.1002/edn3.89

64. Jane SF, Wilcox TM, McKelvey KS, Young MK, Schwartz MK, Lowe WH, Letcher BH, Whiteley AR (2015) Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. Mol Ecol Resour 15:216–227. https://doi.org/10.1111/1755-0998.12285

65. Minamoto T, Uchii K, Takahara T, Kitayoshi T, Tsuji S, Yamanaka H, Doi H (2017) Nuclear internal transcribed spacer-1 as a sensitive genetic marker for environmental DNA studies in common carp Cyprinus carpio. Mol Ecol Resour 17:324–333. https://doi.org/10.1111/1755-0998.12586

66. Wood SA, Pochon X, Laroche O, Ammon U, Adamson J, Zaiko A (2019) A comparison of droplet digital polymerase chain reaction (PCR), quantitative PCR and metabarcoding for species-specific detection in environmental DNA. Mol Ecol Resour 19:1407–1419. https://doi.org/10.1111/1755-0998.13055

67. Yang S, Rothman RE (2004) PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. Lancet Infect Dis 4:337–348. https://doi.org/10.1016/S1473-3099(04)01044-8

68. Goldberg CS, Sepulveda A, Ray A, Baumgardt J, Waits LP (2013) Environmental DNA as a new method for early detection of New Zealand mudsnails (Potamopyrgus antipodarum). Freshw Sci 32:792–800. https://doi.org/10.1899/13-046.1

69. Lugg WH, Griffiths J, Rooyen AR, Weeks AR, Tingley R (2018) Optimal survey designs for environmental DNA sampling. Methods Ecol Evol 9:1049–1059. https://doi.org/10.1111/2041-210X.12951

70. O'Sullivan AM, Samways KM, Perreault A, Hernandez C, Gautreau MD, Curry RA, Bernatchez L (2020) Space invaders: Searching for invasive Smallmouth Bass (Micropterus dolomieu) in a renowned Atlantic Salmon (Salmo salar) river. Ecol Evol 10:2588–2596. https://doi.org/10.1002/ece3.6088

71. Franklin TW, McKelvey KS, Golding JD, Mason DH, Dysthe JC, Pilgrim KL, Squires JR, Aubry KB, Long RA, Greaves SE, Raley CM, Jackson S, MacKay P, Lisbon J, Sauder JD, Pruss MT, Heffington D, Schwartz MK (2019) Using environmental DNA methods to improve winter surveys for rare carnivores: DNA from snow and improved noninvasive techniques. Biol Conserv 229:50–58. https://doi.org/10.1016/j.biocon.2018.11.006

72. Salter I, Joensen M, Kristiansen R, Steingrund P, Vestergaard P (2019) Environmental DNA concentrations are correlated with regional biomass of Atlantic cod in oceanic waters. Commun Biol 2019 21 2:1–9. https://doi.org/10.1038/s42003-019-0696-8

73. Weltz K, Lyle JM, Ovenden J, Morgan JAT, Moreno DA, Semmens JM (2017) Application of environmental DNA to detect an endangered marine skate species in the wild. PLoS One 12:e0178124. https://doi.org/10.1371/journal.pone.0178124

74. Gillum JE, Jimenez L, White DJ, Goldstien SJ, Gemmell NJ (2014) Development and application of a quantitative real-time PCR assay for the globally invasive tunicate Styela clava. Manag Biol Invasions 5:133–142. https://doi.org/10.3391/mbi.2014.5.2.06

75. Piggott MP (2016) Evaluating the effects of laboratory protocols on eDNA detection probability for an endangered freshwater fish. Ecol Evol 6:2739–2750. https://doi.org/10.1002/ece3.2083

76. Tsuji S, Iguchi Y, Shibata N, Teramura I, Kitagawa T, Yamanaka H (2018) Real-time multiplex PCR for simultaneous detection of multiple species from environmental DNA: an application on two Japanese medaka species. Sci Rep 8:9138. https://doi.org/10.1038/s41598-018-27434-w

77. Hulley EN, Tharmalingam S, Zarnke A, Boreham DR (2019) Development and validation of probe-based multiplex real-time PCR assays for the rapid and accurate detection of freshwater fish species. PLoS One 14:e0210165. https://doi.org/10.1371/journal.pone.0210165

78. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE Guidelines: Minimum

Information for Publication of Quantitative Real-Time PCR Experiments. Clin Chem 55:611–622. https://doi.org/10.1373/CLINCHEM.2008.112797

79. Klymus KE, Merkes CM, Allison MJ, Goldberg CS, Helbing CC, Hunter ME, Jackson CA, Lance RF, Mangan AM, Monroe EM, Piaggio AJ, Stokdyk JP, Wilson CC, Richter CA (2020) Reporting the limits of detection and quantification for environmental DNA assays. Environ DNA 2:271–282. https://doi.org/10.1002/edn3.29

80. Langlois VS, Allison MJ, Bergman LC, To TA, Helbing CC (2020) The need for robust qPCR-based eDNA detection assays in environmental monitoring and species inventories. Environ DNA. https://doi.org/10.1002/edn3.164

81. Bruce K, Blackman RC, Bourlat SJ, Hellström M, Bakker J, Bista I, Bohmann K, Bouchez A, Brys R, Clark K, Elbrecht V, Fazi S, Fonseca VG, Hänfling B, Leese F, Mächler E, Mahon AR, Meissner K, Panksep K, Pawlowski J, Luis P, Yáñez S, Seymour M, Thalinger B, Valentini A, Woodcock P, Traugott M, Vasselon V, Deiner K (2021) A practical guide to DNA-based methods for biodiversity assessment. Adv Books 1:e68634-. https://doi.org/10.3897/AB.E68634

82. Hindson CM, Chevillet JR, Briggs HA, Gallichotte EN, Ruf IK, Hindson BJ, Vessella RL, Tewari M (2013) Absolute quantification by droplet digital PCR versus analog real-time PCR. Nat Methods 10:1003–1005. https://doi.org/10.1038/nmeth.2633

83. Tsang HH, Domingos JA, Westaway JA, Kam MH, Huerlimann R, Gomes GB (2021) Digital droplet PCR-based environmental DNA tool for monitoring Cryptocaryon irritans in a marine fish from from Hong Kong. Diversity 13:350

84. Cao Y, Griffith JF, Weisberg SB (2016) The Next-Generation PCR-Based Quantification Method for Ambient Waters: Digital PCR. In: Bourlat SJ (ed) Marine Genomics: Methods and Protocols. Springer, pp 113–130

85. Doi H, Takahara T, Minamoto T, Matsuhashi S, Uchii K, Yamanaka H (2015) Droplet Digital Polymerase Chain Reaction (PCR) Outperforms Real-Time PCR in the Detection of Environmental DNA from an Invasive Fish Species. Environ Sci Technol 49:5601–5608. https://doi.org/10.1021/acs.est.5b00253

86. Capo E, Spong G, Norman S, Königsson H, Bartels P, Byström P (2019) Droplet digital PCR assays for the quantification of brown trout (Salmo trutta) and Arctic char (Salvelinus alpinus) from environmental DNA collected in the water of mountain lakes. PLoS One 14:e0226638. https://doi.org/10.1371/journal.pone.0226638

87. McKee AM, Spear SF, Pierson TW (2015) The effect of dilution and the use of a post-extraction nucleic acid purification column on the accuracy, precision, and inhibition of environmental DNA samples. Biol Conserv 183:70–76. https://doi.org/10.1016/j.biocon.2014.11.031

88. Yang R, Paparini A, Monis P, Ryan U (2014) Comparison of next-generation droplet digital PCR (ddPCR) with quantitative PCR (qPCR) for enumeration of Cryptosporidium oocysts in faecal samples. https://doi.org/10.1016/j.ijpara.2014.08.004

89. Gill P, Ghaemi A (2008) Nucleic acid isothermal amplification technologies - a review. Nucleosides, Nucleotides and Nucleic Acids 27:224–243

90. Piepenburg O, Williams CH, Stemple DL, Armes NA (2006) DNA detection using recombination proteins. PLoS Biol 4:e204. https://doi.org/10.1371/journal.pbio.0040204

91. Gonzales F, McDonough S (1998) Applications of Transcription-Mediated Amplification to Quantification of Gene Sequences. In: Francois F (ed) Gene Amplification. Birkhauser, Boston, pp 189–204

92. Walker GT, Fraiser MS, Schram JL, Little MC, Nadeau JG, Malinowski DP (1992) Strand displacement amplification--an isothermal, in vitro DNA amplification technique. Nucleic Acids Res 20:1691–6. https://doi.org/10.1093/nar/20.7.1691

93. Lizardi PM, Huang X, Zhu Z, Bray-Ward P, Thomas DC, Ward DC (1998) Mutation detection and single-molecule counting using isothermal rolling-circle amplification. Nat Genet 19:225–232. https://doi.org/10.1038/898

94. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28:63e – 63

95. Mori Y, Nagamine K, Tomita N, Notomi T (2001) Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem Biophys Res Commun 289:150–154. https://doi.org/10.1006/bbrc.2001.5921

96. Njiru ZK, Mikosza ASJ, Armstrong T, Enyaru JC, Ndung'u JM, Thompson ARC (2008) Loop-mediated isothermal amplification (LAMP) method for rapid detection of Trypanosoma brucei rhodesiense. PLoS Negl Trop Dis 2:. https://doi.org/10.1371/journal.pntd.0000147

97. Kaneko H, Kawana T, Fukushima E, Suzutani T (2007) Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. J Biochem Biophys Methods 70:499–501. https://doi.org/10.1016/j.jbbm.2006.08.008

98. Boehme CC, Nabeta P, Henostroza G, Raqib R, Rahim Z, Gerhardt M, Sanga E, Hoelscher M, Notomi T, Hase T, Perkins MD (2007) Operational feasibility of using loop-mediated isothermal amplification for diagnosis of pulmonary tuberculosis in microscopy centers of developing countries. J Clin Microbiol 45:1936–1940. https://doi.org/10.1128/JCM.02352-06

99. Curtis KA, Rudolph DL, Owen SM (2008) Rapid detection of HIV-1 by reverse-transcription, loop-mediated isothermal amplification (RT-LAMP). J Virol Methods 151:264–270. https://doi.org/10.1016/j.jviromet.2008.04.011

100. Williams MR, Stedtfeld RD, Engle C, Salach P, Fakher U, Stedtfeld T, Dreelin E, Stevenson RJ, Latimore J, Hashsham SA (2017) Isothermal amplification of environmental DNA (eDNA) for direct field-based monitoring and laboratory confirmation of Dreissena sp. PLoS One 12:e0186462. https://doi.org/10.1371/journal.pone.0186462

101. Davis CN, Tyson F, Cutress D, Davies E, Jones DL, Brophy PM, Prescott A, Rose MT, Williams M, Williams HW, Jones RA (2020) Rapid detection of Galba truncatula in water sources on pasture-land using loop-mediated isothermal amplification for control of trematode infections. Parasites and Vectors 13:1–11. https://doi.org/10.1186/S13071-020-04371-0/TABLES/4

102. Fast K, Popp A, O'Neil P, McGregor S, Sandel M (2020) Surveillance of a federally protected freshwater fish using loop-mediated isothermal amplification (LAMP) and eDNA. Authorea Prepr. https://doi.org/10.22541/AU.159586067.74431972

103. Martzy R, Kolm C, Brunner K, Mach RL, Krska R, Sinkovec H, Sommer R, Farnleitner AH, Reischer GH (2017) A loop-mediated isothermal amplification (LAMP) assay for the rapid detection of Enterococcus spp. in water. Water Res 122:62–69

104. Lobato IM, O'Sullivan CK (2018) Recombinase polymerase amplification: Basics, applications and recent advances. TrAC Trends Anal Chem 98:19–35. https://doi.org/10.1016/J.TRAC.2017.10.015

105. Wu YD, Xu MJ, Wang QQ, Zhou CX, Wang M, Zhu XQ, Zhou DH (2017) Recombinase polymerase amplification (RPA) combined with lateral flow (LF) strip for detection of Toxoplasma gondii in the environment. Vet Parasitol 243:199–203. https://doi.org/10.1016/j.vetpar.2017.06.026

106. Cha D, Kim D, Choi W, Park S, Han H (2020) Point-of-care diagnostic (POCD) method for detecting Bursaphelenchus xylophilus in pinewood using recombinase polymerase amplification (RPA) with the portable optical isothermal device (POID). PLoS One 15:. https://doi.org/10.1371/journal.pone.0227476

107. Toldrà A, Alcaraz C, Andree KB, Fernández-Tejedor M, Diogène J, Katakis I, O'Sullivan CK, Campàs M (2019) Colorimetric DNA-based assay for the specific detection and quantification of Ostreopsis cf. ovata and Ostreopsis cf. siamensis in the marine environment. Harmful Algae 84:27–35. https://doi.org/10.1016/j.hal.2019.02.003

108. Wu L, Ye L, Wang Z, Cui Y, Wang J (2019) Utilization of recombinase polymerase amplification combined with a lateral flow strip for detection of Perkinsus beihaiensis in the oyster Crassostrea hongkongensis. Parasit Vectors 12:360. https://doi.org/10.1186/s13071-019-3624-3

109. Li J, Macdonald J, Von Stetten F (2019) Review: a comprehensive summary of a decade development of the recombinase polymerase amplification. Analyst 144:31–67

110. Chen JS, Ma E, Harrington LB, Costa M Da, Tian X, Palefsky JM, Doudna JA (2018) CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. Science (80-) 360:436–439. https://doi.org/10.1126/SCIENCE.AAR6245

111. Gootenberg JS, Abudayyeh OO, Lee JW, Essletzbichler P, Dy AJ, Joung J, Verdine V, Donghia N, Daringer NM, Freije CA, Myhrvold C, Bhattacharyya RP, Livny J, Regev A, Koonin E V., Hung DT, Sabeti PC, Collins JJ, Zhang F (2017) Nucleic acid detection with CRISPR-Cas13a/C2c2. Science (80-) 356:438–442. https://doi.org/10.1126/science.aam9321

112. Li SY, Cheng QX, Li XY, Zhang ZL, Gao S, Cao RB, Zhao GP, Wang JM, Wang JM (2018) CRISPR-Cas12a-assisted nucleic acid detection. Cell Discov. 4:20

113. Barrangou R, Doudna JA (2016) Applications of CRISPR technologies in research and beyond. Nat Biotechnol 34:933–941. https://doi.org/10.1038/nbt.3659

114. Broughton JP, Deng X, Yu G, Fasching CL, Servellita V, Singh J, Miao X, Streithorst JA, Granados A, Sotomayor-Gonzalez A, Zorn K, Gopez A, Hsu E, Gu W, Miller S, Pan CY, Guevara H, Wadford DA, Chen JS, Chiu CY (2020) CRISPR–Cas12-based detection of SARS-CoV-2. Nat Biotechnol 38:870–874. https://doi.org/10.1038/s41587-020-0513-4

115. Adli M (2018) The CRISPR tool kit for genome editing and beyond. Nat Commun 9:1911. https://doi.org/10.1038/s41467-018-04252-2

116. Baerwald MR, Goodbla AM, Nagarajan RP, Gootenberg JS, Abudayyeh OO, Zhang F, Schreier AD (2020) Rapid and accurate species identification for ecological studies and monitoring using CRISPR-based SHERLOCK. Mol Ecol Resour 20:961–970. https://doi.org/10.1111/1755-0998.13186

117. Williams MA, O'Grady J, Ball B, Carlsson J, de Eyto E, McGinnity P, Jennings E, Regan F, Parle-McDermott A (2019) The application of CRISPR-Cas for single species identification from environmental DNA. Mol Ecol Resour 19:1755–0998. https://doi.org/10.1111/1755-0998.13045

118. Williams M-A, Hernandez C, O'Sullivan AM, April J, Regan F, Bernatchez L, Parle-McDermott A (2021) Comparing CRISPR-Cas and qPCR eDNA assays for the detection of Atlantic salmon (Salmo salar L.). Environ DNA 3:297–304. https://doi.org/10.1002/EDN3.174

119. Whiley DM, Sloots TP (2005) Sequence variation in primer targets affects the accuracy of viral quantitative PCR. J Clin Virol 34:104–107. https://doi.org/10.1016/j.jcv.2005.02.010

120. Rees HC, Maddison BC, Middleditch DJ, Patmore JRM, Gough KC (2014) REVIEW: The detection of aquatic animal species using environmental DNA - a review of eDNA as a survey tool in ecology. J Appl Ecol 51:1450–1459. https://doi.org/10.1111/1365-2664.12306

121. Goggins S, Frost CG (2016) Approaches towards molecular amplification for sensing. Analyst 141:3157–3218. https://doi.org/10.1039/C6AN00348F

122. Zhao J, Chen G (2019) Introduction. In: Li G (ed) Nano-inspired biosensors for protein assay with clinical applications. Elsevier, pp 17–47

123. Alberti G, Zanoni C, Magnaghi LR, Biesuz R (2020) Disposable and Low-Cost Colorimetric Sensors for Environmental Analysis. Int J Environ Res Public Health 17:1–23. https://doi.org/10.3390/IJERPH17228331

124. Papadakis G, Pantazis AK, Fikas N, Chatziioannidou S, Tsiakalou V, Michaelidou K, Pogka V, Megariti M, Vardaki M, Giarentis K, Heaney J, Nastouli E, Karamitros T, Mentis A, Zafiropoulos A, Sourvinos G, Agelaki S, Gizeli E (2022) Portable real-time colorimetric LAMP-device for rapid quantitative detection of nucleic acids in crude samples. Sci Reports 2022 121 12:1–15. https://doi.org/10.1038/s41598-022-06632-7

125. Huang B, Montgomery BL, Adamczyk R, Ehlers G, van den Hurk AF, Warrilow D (2020) A LAMP-based colorimetric assay to expedite field surveillance of the invasive mosquito species Aedes aegypti and Aedes albopictus. PLoS Negl Trop Dis 14:e0008130. https://doi.org/10.1371/JOURNAL.PNTD.0008130

126. Elumalai M, Ipatov A, Carvalho J, Guerreiro J, Prado M (2021) Dual colorimetric strategy for specific DNA detection by nicking endonuclease-assisted gold nanoparticle signal amplification. Anal Bioanal Chem. https://doi.org/10.1007/S00216-021-03564-5

127. Xu W, Xue X, Li T, Zeng H, Liu X (2009) Ultrasensitive and Selective Colorimetric DNA Detection by Nicking Endonuclease Assisted Nanoparticle Amplification. Angew Chemie Int Ed 48:6849–6852. https://doi.org/10.1002/ANIE.200901772

128. Butler SA, Khanlian SA, Cole LA (2001) Detection of Early Pregnancy Forms of Human Chorionic Gonadotropin by Home Pregnancy Test Devices. Clin Chem 47:2131–2136. https://doi.org/10.1093/CLINCHEM/47.12.2131

129. Hsiao WWW, Le TN, Pham DM, Ko HH, Chang HC, Lee CC, Sharma N, Lee CK, Chiang WH (2021) Recent Advances in Novel Lateral Flow Technologies for Detection of COVID-19. Biosens 2021, Vol 11, Page 295 11:295. https://doi.org/10.3390/BIOS11090295

130. Ivanov A V., Safenkova I V., Zherdev A V., Dzantiev BB (2021) The Potential Use of Isothermal Amplification Assays for In-Field Diagnostics of Plant Pathogens. Plants (Basel, Switzerland) 10:. https://doi.org/10.3390/PLANTS10112424

131. Huang X, Aguilar ZP, Xu H, Lai W, Xiong Y (2016) Membrane-based lateral flow immunochromatographic strip with nanoparticles as reporters for detection: A review. Biosens Bioelectron 75:166–180. https://doi.org/10.1016/J.BIOS.2015.08.032

132. Mao X, Ma Y, Zhang A, Zhang L, Zeng L, Liu G (2009) Disposable nucleic acid biosensors based on gold nanoparticle probes and lateral flow strip. Anal Chem 81:1660–1668. https://doi.org/10.1021/ac8024653

133. Jahanpeyma F, Forouzandeh M, Rasaee MJ, Shoaie N (2019) An enzymatic paper-based biosensor for ultrasensitive detection of DNA. Front Biosci (Schol Ed) 11:122–135. https://doi.org/10.2741/S530

134. Koczula KMM, Gallotta A (2016) Lateral flow assays. Essays Biochem 60:111. https://doi.org/10.1042/EBC20150012

135. Li J, Ma B, Fang J, Zhi A, Chen E, Xu Y, Yu X, Sun C, Zhang M (2019) Recombinase Polymerase Amplification (RPA) Combined with Lateral Flow Immunoassay for Rapid Detection of Salmonella in Food. Foods (Basel, Switzerland) 9:. https://doi.org/10.3390/FOODS9010027

136. Doyle J, Uthicke S (2021) Sensitive environmental DNA detection via lateral flow assay (dipstick)—A case study on corallivorous crown-of-thorns sea star (Acanthaster cf. solaris) detection. Environ DNA 3:323–342. https://doi.org/10.1002/EDN3.123

137. Lee L, Nordman E, Johnson M, Oldham M (2013) A Low-Cost, High-Performance System for Fluorescence Lateral Flow Assays. Biosensors 3:360–373. https://doi.org/10.3390/bios3040360

138. Kobayashi H, Longmire MR, Ogawa M, Choyke PL (2011) Rational chemical design of the next generation of molecular imaging probes based on physics and biology: mixing modalities, colors and signals. Chem Soc Rev 40:4626–4648. https://doi.org/10.1039/C1CS15077D

139. Holland PM, Abramson RD, Watson R, Gelfand DH (1991) Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. Proc Natl Acad Sci U S A 88:7276–80. https://doi.org/10.1073/pnas.88.16.7276

140. Herrero B, Madriñán M, Vieites JM, Espiñeira M (2010) Authentication of Atlantic Cod (*Gadus morhua*) Using Real Time PCR. J Agric Food Chem 58:4794–4799. https://doi.org/10.1021/jf904018h

141. Kutyavin I V., Afonina IA, Mills A, Gorn V V, Lukhtanov EA, Belousov ES, Singer MJ, Walburger DK, Lokhov SG, Gall AA, Dempcy R, Reed MW, Meyer RB, Hedgpeth J (2000) 3'-Minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Res 28:655–661. https://doi.org/10.1093/nar/28.2.655

142. Marmiroli N, Maestri E (2007) Chapter 6 - Polymerase chain reaction (PCR). In: Picó Y (ed) Food Toxicants Analysis. Elsevier, Amsterdam, pp 147–187

143. Mauvisseau Q, Coignet A, Delaunay C, Pinet F, Bouchon D, Souty-Grosset C (2018) Environmental DNA as an efficient tool for detecting invasive crayfishes in freshwater ponds.

Hydrobiologia 805:163–175. https://doi.org/10.1007/s10750-017-3288-y

144. Dragan AI, Pavlovic R, McGivney JB, Casas-Finet JR, Bishop ES, Strouse RJ, Schenerman MA, Geddes CD (2012) SYBR Green I: fluorescence properties and interaction with DNA. J Fluoresc 22:1189–1199. https://doi.org/10.1007/S10895-012-1059-8

145. Choi JH, Lim J, Shin M, Paek SH, Choi JW (2021) CRISPR-Cas12a-Based Nucleic Acid Amplification-Free DNA Biosensor via Au Nanoparticle-Assisted Metal-Enhanced Fluorescence and Colorimetric Analysis. Nano Lett 21:693–699. https://doi.org/10.1021/ACS.NANOLETT.0C04303/SUPPL_FILE/NL0C04303_SI_001.PDF

146. Thomas AC, Tank S, Nguyen PL, Ponce J, Sinnesael M, Goldberg CS (2019) A system for rapid eDNA detection of aquatic invasive species. Environ DNA. https://doi.org/10.1002/edn3.25

147. Ponce JJ, Arismendi I, Thomas A (2021) Using in-situ environmental DNA sampling to detect the invasive New Zealand Mud Snail (Potamopyrgus antipodarum) in freshwaters. PeerJ 9:e11835. https://doi.org/10.7717/PEERJ.11835/SUPP-1

148. Sepulveda AJ, Hutchins PR, Massengill RL, Dunker KJ (2018) Tradeoffs of a portable, field-based environmental DNA platform for detecting invasive northern pike (Esox lucius) in Alaska. Manag Biol Invasions 9:253–258. https://doi.org/10.3391/mbi.2018.9.3.07

149. Nguyen PL, Sudheesh PS, Thomas AC, Sinnesael M, Haman K, Cain KD (2018) Rapid Detection and Monitoring of *Flavobacterium psychrophilum* in Water by Using a Handheld, Field-Portable Quantitative PCR System. J Aquat Anim Health 30:302–311. https://doi.org/10.1002/aah.10046

150. Doi H, Watanabe T, Nishizawa N, Saito T, Nagata H, Kameda Y, Maki N, Ikeda K, Fukuzawa T (2021) On-site environmental DNA detection of species using ultrarapid mobile PCR. Mol Ecol Resour 21:2364–2368. https://doi.org/10.1111/1755-0998.13448

151. Heery B, Briciu-Burghina C, Zhang D, Duffy G, Brabazon D, O'Connor N, Regan F (2016) ColiSense, today's sample today: A rapid on-site detection of β-d-Glucuronidase activity in surface water as a surrogate for E. coli. Talanta 148:75–83. https://doi.org/10.1016/J.TALANTA.2015.10.035

152. Briciu-Burghina C, Heery B, Duffy G, Brabazon D, Regan F (2019) Demonstration of an optical biosensor for the detection of faecal indicator bacteria in freshwater and coastal bathing areas. Anal Bioanal Chem 411:7637–7643. https://doi.org/10.1007/s00216-019-02182-6

153. Paul J, Scholin C, van den Engh G, Perry MJ (2007) In situ instrumentation. Oceanography 20:70–78. https://doi.org/10.5670/OCEANOG.2007.50

154. Scholin C, Doucette G, Jensen S, Roman B, Pargett D, Marin R, Preston C, Jones W, Feldman J, Everlove C, Harris A, Alvarado N, Massion E, Birch J, Greenfield D, Vrijenhoek R, Mikulski C, Jones K (2009) Remote detection of marine microbes, small invertebrates, harmful algae and biotoxins using the Environmental Sample Processor (ESP). Oceanography 22:158–167

155. Pargett DM, Birch JM, Preston CM, Ryan JP, Zhang Y, Scholin CA (2016) Development of a mobile ecogenomic sensor. Ocean 2015 - MTS/IEEE Washingt. https://doi.org/10.23919/OCEANS.2015.7404361

156. Sepulveda AJ, Birch JM, Barnhart EP, Merkes CM, Yamahara KM, Marin R, Kinsey SM, Wright

PR, Schmidt C (2020) Robotic environmental DNA bio-surveillance of freshwater health. Sci Reports 2020 101 10:1–8. https://doi.org/10.1038/s41598-020-71304-3

157. Tang W, Cerdán-García E, Berthelot H, Polyviou D, Wang S, Baylay A, Whitby H, Planquette H, Mowlem M, Robidart J, Cassar N (2020) New insights into the distributions of nitrogen fixation and diazotrophs revealed by high-resolution sensing and sampling methods. ISME J 2020 1410 14:2514–2526. https://doi.org/10.1038/s41396-020-0703-6

158. Yamahara KM, Preston CM, Birch J, Walz K, Marin R, Jensen S, Pargett D, Roman B, Ussler W, Zhang Y, Ryan J, Hobson B, Kieft B, Raanan B, Goodwin KD, Chavez FP, Scholin C (2019) In situ autonomous acquisition and preservation of marine environmental dna using an autonomous underwater vehicle. Front Mar Sci 6:373. https://doi.org/10.3389/FMARS.2019.00373/BIBTEX

159. Preston CM, Harris A, Ryan JP, Roman B, Marin R, Jensen S, Everlove C, Birch J, Dzenitis JM, Pargett D, Adachi M, Turk K, Zehr JP, Scholin CA (2011) Underwater Application of Quantitative PCR on an Ocean Mooring. PLoS One 6:e22522. https://doi.org/10.1371/JOURNAL.PONE.0022522

160. Hansen BK, Jacobsen MW, Middelboe AL, Preston CM, Marin R, Bekkevold D, Knudsen SW, Møller PR, Nielsen EE (2020) Remote, autonomous real-time monitoring of environmental DNA from commercial fish. Sci Reports 2020 101 10:1–8. https://doi.org/10.1038/s41598-020-70206-8

161. Scholin C, Jensen S, Roman B, Massion E, Marin R, Preston C, Greenfield D, Jones W, Wheeler K (2006) The Environmental Sample Processor (ESP) - An autonomous robotic device for detecting microorganisms remotely using molecular probe technology. Ocean 2006. https://doi.org/10.1109/OCEANS.2006.306885

162. Roman B, Scholin C, Jensen S, Marin R, Massion E, Feldman J (2005) The 2nd generation environmental sample processor: Evolution of a robotic underwater biochemical laboratory. Proc MTS/IEEE Ocean 2005 2005:. https://doi.org/10.1109/OCEANS.2005.1639911

163. Ussler W, Preston C, Tavormina P, Pargett D, Jensen S, Roman B, Marin R, Shah SR, Girguis PR, Birch JM, Orphan V, Scholin C (2013) Autonomous application of quantitative PCR in the deep sea: In Situ surveys of aerobic methanotrophs using the deep-sea environmental sample processor. Environ Sci Technol 47:9339–9346. https://doi.org/10.1021/ES4023199/SUPPL_FILE/ES4023199_SI_001.PDF

164. Revenga C, Campbell I, Abell R, Villiers P de, Bryer M (2005) Prospects for monitoring freshwater ecosystems towards the 2010 targets. Philos Trans R Soc B Biol Sci 360:397–413. https://doi.org/10.1098/RSTB.2004.1595

165. WWF (2020) Living Planet Report 2020: Bending the Curve of Biodiversity Loss. Gland, Switzerland

166. Thomsen PF, Willerslev E (2015) Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. Biol Conserv 183:4–18. https://doi.org/10.1016/J.BIOCON.2014.11.019