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Review

The Application of eDNA for Monitoring Aquatic Non-Indigenous Species: Practical and Policy Considerations

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Abstract: Aquatic non-indigenous species (NIS) threaten biodiversity, ecosystem functions, and the economy worldwide. Monitoring NIS is of immediate concern to identify newly arriving species, assess the efficacy of mitigation measures, and report long-term indicators of introduction, spread, and impacts. The challenges associated with conventional methods of specimen collection and morphological identification have led to the development of alternative methods, such as DNA-based methods, which could offer rapid and cost-effective detection of NIS. Depending on whether a few (targeted monitoring) or many species (passive monitoring) are being monitored, environmental DNA (eDNA) can infer presence-absence and relative abundances, enabling informed decisions and actions to be made based on patterns of detection. Compared to more conventional methods, eDNA tools can increase the levels of detection and sensitivity for rare and elusive species, which is even more noticeable for some taxa when using targeted monitoring. The use of DNA-based tools not only minimizes the onus on taxonomic expertise and reduces resource demands but can also be more sensitive and cost-efficient in detecting NIS, thus proving its value as an early warning tool. As nucleic acid (DNA/RNA) methods advance rapidly for NIS detection, there must be a balance between method sensitivity, logistical requirements, and associated costs, which must be factored into future management decisions. While there are many complementary reviews available, our aim is to emphasize the importance of incorporating eDNA tools into NIS surveys and to highlight the available opportunities in this field.

Keywords: non-native species; NIS; eDNA; targeted-passive monitoring; eRNA; HTS; PCR

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1. Introduction

The introduction of non-indigenous species (NIS) into marine and freshwater ecosystems is facilitated by human activities, either deliberately or accidentally (e.g., aquaculture, fishing, international trade, ballast water, and marine litter) [1,2]. NIS are termed 'invasive' when they cause a damaging impact to local biodiversity, human health, and the economy [3]. These invasions or introductions may drastically affect not only native communities but also resource availability, ecosystem services, and functioning [4–6]. Since efforts to reduce the impact of established species are often problematic, management strategies are strongly focused on preventing introductions or spreading. One key example is that of many marine coastal invertebrates that have a benthopelagic life cycle, including marine benthic NIS, where pelagic larval stages play a major role in all steps of the invasion process (i.e., introduction, establishment, and spread) [7]. The chances of detecting pelagic larvae in the water during the introduction phase could allow early detection before establishment and possible spread [8,9]. Thus, early detection and rapid response are key to improving the success of eradication programs and aiding containment to prevent further establishment and dispersal [10–12]. Various tools are available for detecting NIS in aquatic environments, and they can be used in combination to improve the accuracy and effectiveness of NIS detection and monitoring. However, it is important to note that

each method has its own limitations and challenges, and careful consideration of these factors is necessary for rigorous NIS monitoring surveys. The objective of this review is to provide a concise overview of the latest developments in DNA-based methods for assessing NIS particularly focusing on eukaryotic aquatic NIS (e.g., meio- macrofauna, algae). In the interest of clarity and focus, we have chosen to limit our review to non-pathogenic invasive species, since the topic of invasive pathogens is complex and extensive and deserves its own comprehensive review [13]. In addition to highlighting gaps in current knowledge, we also aim to identify processes that can help advance the implementation of environmental DNA (eDNA) for biomonitoring purposes. We will guide the reader through various assessment capacities of traditional and molecular methods, including new tools such as targeted and passive detection techniques, as well as the use of eRNA and Oxford Nanopore for NIS detection (Figure 1). We will also discuss the crucial role of eDNA fate in the environment for NIS detection, which includes persistence and dispersal factors. Additionally, we will highlight other sources of errors that can affect decision and monitoring strategies. Our review evaluates the strengths and weaknesses of DNA-based tools and provides recommendations for future research and monitoring surveys based on the current state of knowledge.

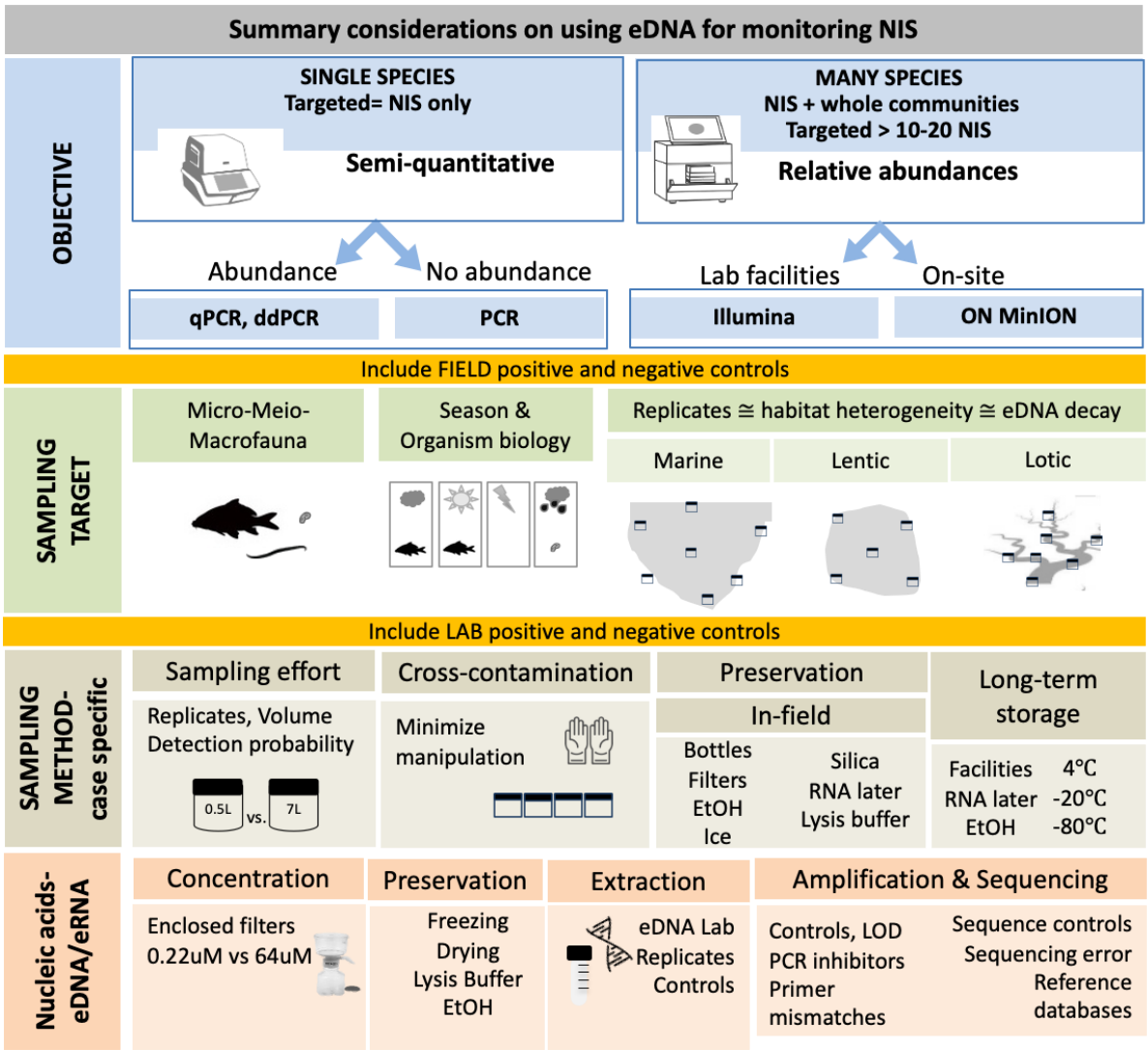

Figure 1. Summary of considerations for using eDNA to monitor NIS. Important to identify study objectives followed by biological and technical considerations (sampling target and methods).

2. Detecting Aquatic NIS: Morphology, eDNA and eRNA

Traditional detection methods, such as capture or sighting surveys, are still commonly used in monitoring programmes to determine aquatic NIS presence or absence [14]. These surveys mainly depend on morphology-based approaches that involve direct observation of the organism, which can facilitate confirmation of the identity of the target species, its size, and sometimes its abundance. Nonetheless, this can be challenging for cryptic species or early developmental stages, making it difficult to accurately identify and monitor NIS using traditional methods alone [15–17]. DNA-based methods, as a broad term covering eDNA as well as genomic DNA/RNA from animal tissue, can overcome numerous limitations associated with more traditional and morphological identification methods, such as the need for taxonomic expertise, intensive capturing effort, and invasive sampling methods [18–22]. In particular, DNA tools may offer more robust monitoring of species that are elusive, cryptic, or small, which would otherwise go unnoticed, particularly at low abundances before becoming established [22–24]. It is also a non-invasive sampling technique, which is beneficial for rare species [25]. Additionally, eDNA monitoring can be conducted rapidly, with results available within hours or days, whereas morphology-based approaches often require time-consuming and labor-intensive fieldwork and laboratory analyses [16,17]. DNA-based methods using eDNA allow the identification of organisms present in water, sediment, or air samples, where DNA is released into the environment (e.g., via mucus, skin, scales, fur, urine, and cell debris) [17]. In contrast to traditional detection methods, environmental DNA is known to be sensitive enough to monitor difficult-to-detect aquatic species at low densities [26,27], with an order of magnitude more sensitive than the former [18,28]. For example, monitoring studies of the invasive non-native quagga mussel *Dreissena bugensis* tested three DNA-based approaches, and all proved to be more sensitive than traditional kick-net sampling for its detection in flowing water [18]. Increasingly, studies are showing that eDNA surveying is the most non-invasive and cost-effective method for detecting endangered and invasive species [17,20,29,30]. Although the first studies using eDNA for NIS monitoring focused on freshwater environments [31,32], the number of studies using eDNA NIS surveillance applications in marine environments has increased over the years at a rate of ca. 6.3 papers/year [30]. Most marine studies have focused on ports, marinas, and estuaries, targeting mainly seawater, ballast water, and, to a lesser degree, sediments (reviewed by Duarte, et al. (2021)). There is an ongoing debate between morphology-based approaches and eDNA tools for NIS detection and monitoring. Some studies suggest that eDNA is a more sensitive method for species detection than traditional survey methods [22,33], whereas others advocate that, despite the higher sensitivity of eDNA, both methods offer similar ecological results [34]. Further combining morphology-based taxonomy with molecular approaches would benefit not only curating existing databases [17,35,36], as this would offer an extended taxa confirmation, but many studies also suggest complementarity when using both approaches [27,36–39]. Both methods have their own advantages and limitations, and there is no one-size-fits-all approach that can be used for all NIS detection and monitoring efforts.

Similarly to eDNA, environmental RNA (eRNA) is also shed by organisms and can be used for biomonitoring purposes [40–42], although it is more unstable with a higher degradation time (hours to days) than eDNA [43]. However, the consequence of its rapid degradation in the environment means that species detection using RNA may reflect signals from live and viable organisms more accurately [40]. This is in contrast to DNA, which has the potential to persist in the environment for extended periods (days to years) after the shedding organism has departed or exists elsewhere and may disperse on currents, which can result in false positives. This issue will be explored further in this review. But one advantage of including eRNA in biomonitoring studies is its capacity to function as a real-time monitoring tool [41], which is particularly useful for ecological impact surveys of contaminant exposure [19]. Recent studies further suggest that at least 25% of the taxa found in sediment and water samples are unique to either eRNA or eDNA molecules (Fonseca pers. comm.) and others showcase that eRNA surpasses eDNA levels of detection [19].

A combination of both eDNA and eRNA tools could strengthen diversity coverage and increase detection levels in monitoring studies, particularly if some NIS would be more represented by RNA molecules.

3. NIS Detection Using Targeted vs. Passive Approach: New Tools and Estimating Abundances

Environmental DNA surveys for NIS can incorporate active targeted monitoring of key species, or passive monitoring using eDNA combined with high throughput sequencing for whole-community assessments (also known as eDNA metabarcoding) (Figure 2). The targeted monitoring approach uses species-specific primers to identify the presence of a single NIS species in a given habitat, using polymerase chain reaction (PCR) methods. Conventional PCRs represent a qualitative targeted approach to determine species presence, whereas quantitative PCR (qPCR) or droplet digital PCR (ddPCR) methods have the advantage of being semi-quantitative [44]. Examples include studies exploring the ecological impacts of introduced and invasive species using species-specific markers to target NIS aquatic macrofauna [20,28,45–47]. Similar eDNA studies using active target monitoring for NIS detection make use of additional strategies to improve levels of detection using multiplex approaches [48,49] or chemical dyes in eDNA samples prior to qPCR [50,51]. For instance, Wozney and Wilson (2017) developed a multiplex quantitative PCR (qPCR) assay targeting four different species of Asian carp simultaneously. More recently, Hernandez, et al. (2020) successfully developed 60 species-specific qPCR assays (including PCR protocols, primers, and TaqMan probe sequences) for the detection of forty-five fishes, six amphibians, five reptiles, two mollusks, and two crustaceans. These assays were validated using laboratory tests and field samples, demonstrating high sensitivity and specificity for each target species. Both studies concluded that the developed qPCR multiplex assay is a reliable and efficient method for detecting the presence of multiple vertebrate and invertebrate species in environmental DNA samples. Such outputs show that multiplex qPCR greatly improves monitoring efforts and aids in the management of marine invasive and threatened species [48,49]. Other studies suggested that including a chemical compound such as propidium monoazide (PMA) in DNA samples prior to PCR amplification will greatly improve the accuracy of eDNA detection in ecological and biological surveillance studies [50]. The chemical compound PMA can penetrate damaged or dead cells and bind to their DNA, preventing PCR amplification of that DNA during subsequent analyses; due to its nature, it is also called "viability PCR" [50,51]. This can help reduce the risk of false positives in eDNA studies by differentiating between live and dead organisms, whose DNA may be present in the environment. PMA has been used for the detection of active versus non-viable, inactive bacteria [52,53] but a study conducted by Hirohara, et al. (2021) [54] found that using PMA in conjunction with different target sequence lengths of zebrafish eDNA improved the accuracy and reliability of eDNA detection in different types of water samples. The authors suggested that PMA treatment could be a valuable tool for improving the specificity of eDNA monitoring in environmental and biological surveillance applications.

A contrasting approach using eDNA metabarcoding allows the simultaneous detection of NIS as a component of the whole community [55–57]. Thus, metabarcoding can be considered "passive monitoring" due to its non-targeted nature and the fact that it can detect a wide range of species without specifically targeting any particular group. Examples of whole-community assessments for biomonitoring include both early detection and temporal/spatial monitoring of NIS in sediments and water [58–60]. This approach is particularly helpful for detecting small organisms and life stages that are hard to identify using current visual techniques [57,61]. Although it is less common and opposes the term 'passive surveillance', a metabarcoding approach using primers for predetermined taxa groups (order or family level) can also be used to reduce the number of non-NIS present in the output datasets. In fact, Westfall, et al. (2022) [62] used targeted NGS (tNGS) to improve early detection of invasive populations of the European green crab. By selectively targeting gene regions for a specific group of taxa, high sample volumes can still be processed

while potentially increasing taxonomic resolution compared to whole community-based approaches. However, it is important to note that this method can only be used to screen known taxa. Both targeted (using qPCR) and passive monitoring (using metabarcoding) can be very powerful for the early detection of NIS since they have high sensitivity levels. In addition, eDNA does not require previous isolation or visual inspection, and it is ubiquitous in any environment [63–65].

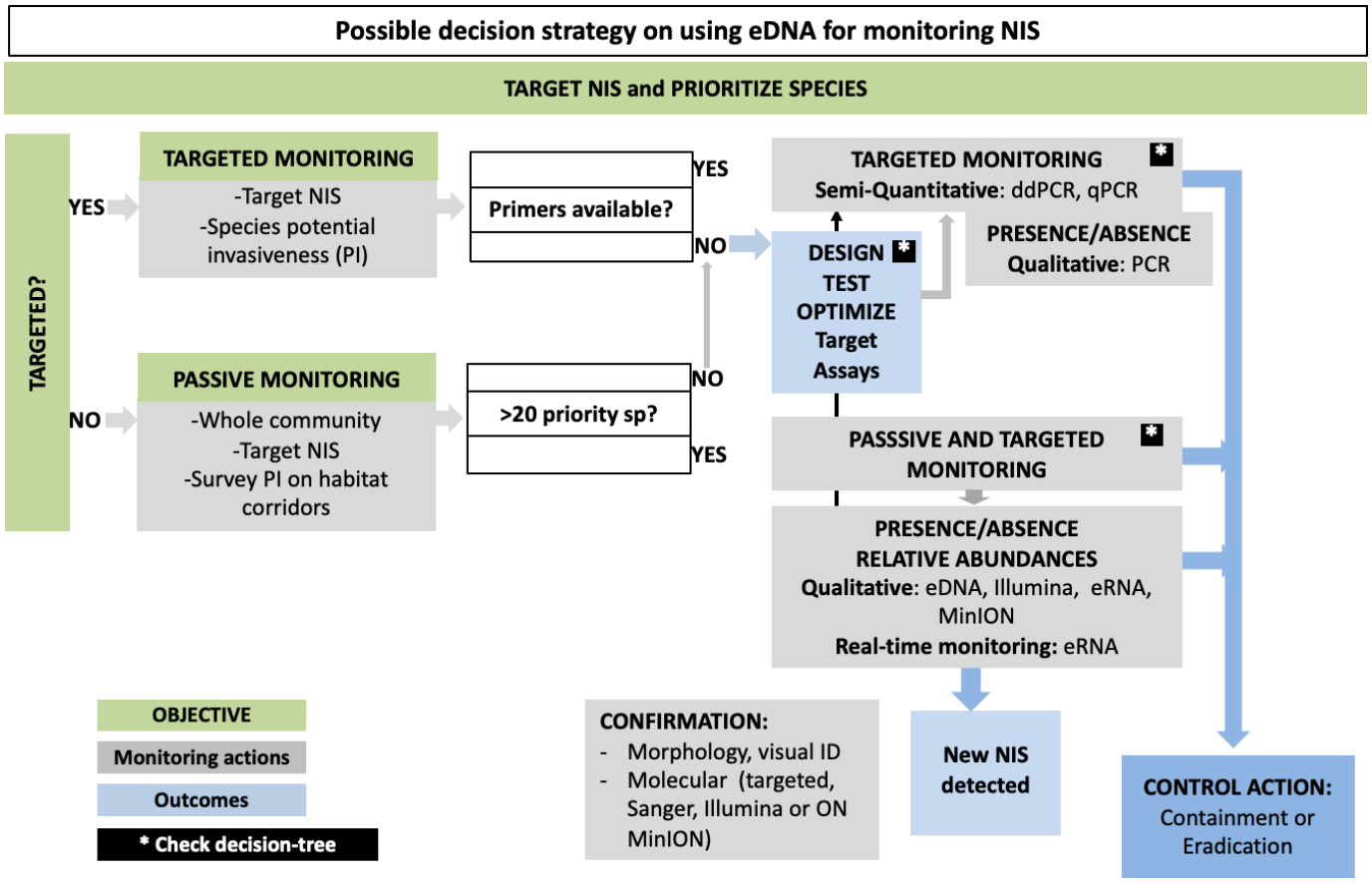

Figure 2. Possible decision strategy for using eDNA to monitor NIS. Decision steps are highlighted and include objectives, monitoring actions, and outcomes. Decision-tree to be consulted prior to PCR-based approach.

Decisions on whether to monitor NIS using passive or active monitoring, or a combination, will depend on several factors. eDNA tools can be more sensitive when using a targeted approach (e.g., qPCR) [18,65]. For example, freshwater monitoring studies on redfin perch, *Perca fluviatilis*, using eDNA metabarcoding and qPCR, found that the latter was more sensitive [66]. Passive monitoring through eDNA metabarcoding can be more costly for monitoring purposes if the number of target NIS is small [67], but conversely, it will provide additional information on a wider set of species, some of which might even be of unknown existence in that specific habitat [27,59]. Another advantage of using eDNA metabarcoding is the opportunity to apply nanopore-based sequencing technologies (Oxford Nanopore Technologies, ONT), which can sequence longer reads and thus offer a higher species resolution power [68]. This technology has been used in biodiversity and biomonitoring surveys [69], and it is known for its portability, which allows the processing of small batches of samples with less turnaround time, and real-time monitoring with on-site sequencing capacity [70]. This approach was recently tested in eDNA water samples outperforming the Illumina platform for the detection of invasive mussel species in Italy and Portugal. The study concluded that Nanopore technology was able to detect all invasive bivalve species with much longer reads and at reduced turnaround

times [69]. Nanopore technology is often the optimal choice for applications requiring (a) in-field testing, (b) the ability to perform surveillance for multiple NIS simultaneously, and (c) sequence data to determine the identity of the NIS [68,69].

Currently, one of the most pressing questions in the field of eDNA research is whether these methods can be used to estimate abundance or biomass, going beyond species presence or absence. For the targeted approach, the use of qPCR and ddPCR, which is by definition a quantitative technique, has shown promise for estimating organism density or biomass based on eDNA concentration [10]. Quantitative and digital PCR will provide information on the number of copies of a specific DNA fragment or gene present in the sample [71]. The number of DNA copies can then be used as a proxy for the number of individuals, but this relationship can vary depending on factors such as the number of gene copies per individual and the eDNA shedding rate in the environment [72]. For the 'passive approach', several studies have also found a correlation between biomass and read counts derived from HTS of eDNA samples [62,73]. Interestingly, Westfall, et al. (2022) [62] found that the number of sequencing reads from targeted NGS (tNGS) was significantly correlated with the invasive green crab catch per unit effort (CPUE), whereas Ct values from qPCR were not. The authors suggested that tNGS may offer more power for detecting spatial variation in eDNA availability particularly for early detection, making it suitable for species of known low abundances where a positive detection could have high economic or environmental consequences.

The combination of eDNA metabarcoding and qPCR has also been successfully applied to correlate eDNA concentration or number of reads with fish biomass [74,75]. It is widely recognized that the relationship between eDNA concentration and organism abundance is complex, non-linear, and dependent on a multitude of variables. These include variations in eDNA shedding rates and degradation rates, environmental conditions such as differences in water flow and sedimentation, and the use of different eDNA extraction methods or PCR primers [23,76]. Additionally, the type of DNA marker used, multicellular individuals, sampling method, and species biology can all introduce challenges in accurately estimating absolute abundances from eDNA data [23,60,77]. Firstly, multicellular organisms have multiple copies of genes within their genomes [77,78]; therefore, the amount of eDNA detected may not directly correlate with the number of individuals present, making it difficult to obtain accurate estimates of absolute abundance [77]. Secondly, some genes, such as ribosomal and mitochondrial DNA, can be present in multiple copies per cell, making it difficult to accurately estimate the number of individuals based on read counts [77]. This is because a higher copy number of a particular gene may lead to an overestimation of the number of individuals, whereas a lower copy number may lead to an underestimation. Additionally, different loci can have different read depths and amplification efficiencies [79], and the number of reads generated per species can be influenced by factors such as the length of the amplified DNA fragment, the sequencing platform used, and the bioinformatic pipelines used for data analysis [78,80]. Thirdly, species biology can also influence the accuracy of absolute abundance estimates [10,81]. For example, some species may shed more eDNA into the environment than others, namely their metabolic activity, the rate of tissue turnover, and the rate of shedding of various bodily fluids or eDNA may also degrade at different rates depending on the season [9,10,81,82]. This means that even if the number of individuals is known, the rate of eDNA shedding can vary, leading to uncertainty in the absolute abundance estimates [81]. Additionally, some species may have low detection rates even if they are present in high numbers, which can lead to an underestimation of their abundance [83]. These issues might be minimized when dealing with unicellular species such as bacteria, diatoms, and other unicellular organisms, whereby the number of reads can be used as a proxy for abundance when the gene copy numbers per taxa are known [72]. Nonetheless, it is important to validate and optimize the eDNA HTS protocols to obtain accurate and reliable estimates of abundance, particularly for NIS detection. The estimation of the number of individuals in a sample based on eDNA metabarcoding data is an area of active research, and methods are being

developed to improve the accuracy of these estimates. Recent studies have found that the relationship between eDNA particle concentration and organism abundance in nature can be more reliably measured using allometric scaling [76,84]. This method describes how the relationship between an organism's body size and various biological processes can improve the accuracy of estimating organism abundance using eDNA predictive models [76,84]. The authors demonstrated how estimates of allometrically scaled mass derived from eDNA samples in 'unknown' systems can be converted to biomass or density estimates with additional size structure data [76]. Others have also shown how controlling allometric relationships can enhance the accuracy of estimating bycatch (the unintentional capture of non-target species) using eDNA as a tool to improve the accuracy of estimating the biomass of different aquatic species and to better understand the impacts of fishing practices on marine ecosystems [84]. Although it has not yet been applied to detect NIS, allometric scaling shows promise as a modeling approach to enhance the reliability of eDNA concentration as an indicator of abundance.

4. eDNA Fate and Impact on NIS Detection

Understanding how DNA persists, travels, and spatially distributes within different habitats is crucial for data interpretation, and although many knowledge gaps remain, some studies enabled a general assessment of eDNA behaviour. Independent of the approach used, the fate of DNA in the environment (e.g., freshwater, marine, and sediments) plays a key role in moderating the efficacy of species detection [85]. Stratified and dynamic environments, such as aquatic systems, can easily disperse eDNA from its original source [86], and DNA can be transported through both active and passive mechanisms in the environment [20,87,88]. Active dispersal consists of the intentional or unintentional movement of eDNA by the organism itself. For example, organisms actively release eDNA into the water through excretion, sloughing off skin cells, scales, and extracellular DNA, which could float or adhere to sediment particles and other microscopic fractions [17,20]. In some cases, eDNA may also be transported by other organisms through predation or scavenging, whereas passive dispersal involves the movement of eDNA by environmental factors, such as water currents, wind, or wave action [17]. This can result in eDNA being carried long distances from the source organism, and it can potentially lead to false positives if eDNA is found in areas where the species is not actually present [87]. Pilliod, et al. (2014) [89] found that eDNA can be detected in flowing freshwater systems (lotic) within 5 m of its source, but in aquatic ecosystems, eDNA can be detected hundreds of kilometers away from its source. [70,74,90–92].

Environmental DNA transport in lotic freshwater systems can be modelled effectively [93], but in marine environments, such predictions are more challenging due to much more complex hydrodynamics [94]. Limited dispersal levels were found in nearshore environments for both benthic and planktonic taxa [95], but moderate dispersal up to several kilometres from its source has also been identified [96]. The rate of passive dispersal can be affected by factors such as water flow, temperature, and the presence of physical barriers, whereas active dispersal can have a more limited range compared to passive dispersal, but it can provide more accurate information about the actual presence of the species [97]. In less mixed marine conditions (low flow), sharp gradients of eDNA concentration are more likely to occur closer to its source [98]. For example, Jeunen, et al. (2020) identified specific eDNA signals from several taxa, including fish, crustaceans, and echinoderms, between samples taken 4 m apart across a strong halocline. Similarly, different species assemblages in different kelp forest habitats separated by as little as 60 m can also be discriminated [99]. The existence of eDNA gradients from its source can be such that sometimes eDNA is used as an alternative method for the identification of NIS living in the direct vicinity of the sampled site [58], assuming that current dynamics are known. In lentic environments, eDNA dispersion is even more complex and difficult to predict and detect, [100] but clearly such variations in water flow dynamics add additional complexity to eDNA detection and its interpretation. It is important to keep in mind that the presence of DNA does not

necessarily indicate biological activity [88]. Thus, both passive and active eDNA dispersal models can influence eDNA detection and quantification and should be considered when designing and interpreting eDNA studies [87,88].

We are just starting to understand the role that biotic and abiotic factors play in DNA dispersal and degradation in the environment, and a recent metanalysis by Lamb, et al. (2022) [82] identified that higher temperatures and marine environments (as opposed to freshwater) speed up eDNA decay. Other factors contributing to eDNA decay include microbial load [101], pH, enzyme activity, and fragment length [82,102,103]. Environmental DNA can persist in the water from a couple of days up to several weeks [85,104,105], especially so if it is offshore [106]. In sediments, DNA can remain detectable for longer periods of time, from up to one year [107] to several years [108], and even millions of years [109]. So much so that marine sediment ancient DNA (*seda*DNA) has been used to identify past diversity patterns of dormant and extinct species that lived thousands or millions of years ago, namely, the occurrence of invasive species that might have led to extinctions [110,111]. Fragments of *seda*DNA are typically more fragmented and degraded than recent eDNA, but they remain preserved in the sediments due to factors such as very low temperatures and oxygen concentrations and an absence of UV radiation [111]. Because most suspended particles sink and accumulate in the superficial sediment layer [17,45,58,61], sediments contain DNA from pelagic organisms at a higher concentration than eDNA present in the water column [108]. Such differences have been observed in benthic and pelagic aquatic samples, for the detection of NIS [9,58,112] and for eDNA studies in general [58,108,113,114].

5. NIS Detection Using PCR-Based Approaches: False Positives, False Negatives, and Sources of Error

False positives (species that are not currently present at the study site but have been detected via eDNA) are another concern in eDNA studies [23,70]. Several hypotheses have been proposed to explain the existence of false positives, such as the release of eDNA through revolving sediments [115], long-distance transport of eDNA, or even detection from the faeces of predatory animals, including migratory birds [70,116]. Consequently, understanding eDNA behaviour and habitat ecology is crucial for eDNA data interpretation, particularly in the case of false positives. These uncertainties may hinder assessments at the species distribution level, but their effects on community diversity estimates are still unknown [23]. False positives may be the consequence of sampling processing, in particular, sample manipulation, but they are also derived from non-target amplification [117] (Figures 1 and S1). Nonetheless, laboratory cross-contaminations or unspecific PCR assays can be minimized through standards and laboratory controls, including optimized storage conditions, minimized handling, and increased replication [17] (Figure S1). False positives can also occur when the reference library contains errors or inaccuracies [36]. Using multiple reference libraries, including local or in-house databases, and cross-checking results can help reduce the likelihood of errors and improve the reliability of the analysis [36]. In addition, validation of eDNA results through taxonomic ID using voucher specimens could further improve taxonomic assessments.

The presence of false negatives (species is present, but not detected through eDNA) could derive from inadequate field sampling, low-sensitivity assays, or inhibition. This has led to the development of refined approaches to overcome detection limitations, such as increasing biological (sample number) and technical replicates (e.g., DNA and PCR replicates) [17,26], including internal positive and negative controls [118], using positive field controls and inhibitor removal with clean-up kits [17], or incorporating multigene approaches [61,119]. Performing a PCR inhibition test by adding an artificial sequence to the samples can also be a useful method for identifying and quantifying the effects of PCR inhibition on eDNA detection and concentration estimations, which can lead to false negatives [120]. By adding an artificial sequence of known concentration and detecting it alongside the target sequence, it is possible to determine the degree of PCR inhibition and adjust the analysis accordingly (e.g., dilution, PCR additives such as BSA or DMSO,

purification, and optimizing PCR conditions) [120]. This will also help determine the limit of detection (LOD) in the PCR, which is the minimum amount of DNA that can be reliably detected in a given sample [44,120]. Lower detection limits indicate higher sensitivity and the ability to detect smaller amounts of NIS DNA. Klymus, et al. (2020) [44] further highlights that the limit of quantification (LOQ) can also be a problematic aspect of eDNA studies because it can vary depending on PCR efficiency, DNA extraction method, and sequencing technology, which is the same for LOD. The authors suggest that NIS detection using eDNA should focus on developing standardized methods for determining the LOQ and LOD to avoid false negative and positive detections, respectively. This will further help to ensure that the results obtained are comparable across studies and that the data obtained are reliable and useful for managing NIS.

Primer-based amplification steps are still widely considered to be the primary source of bias in eDNA studies [17,23]. This is due to the potential for selective amplification of certain species, whereas others may be underrepresented or completely missed during PCR amplification. While the so-called 'universal primers' cover a broad range of metazoan phyla [121], some taxa groups will always fail to amplify, leading to false negatives. It is unlikely that this can ever be completely resolved since all primers have inherent biases. Thus, to mitigate such sources of error, it is common to use various bioinformatic tools to optimize probe/primer design and test their specificity. The design and validation of species-specific oligonucleotides for NIS detection are crucial steps in ensuring the reliability and accuracy of eDNA-based monitoring and surveillance programmes [36,67]. Firstly, it is required to identify specific genetic markers that are unique to a target species or group of species. This typically involves searching public DNA databases, such as GenBank or BOLD, for relevant sequences and aligning them to identify conserved regions [36]. To ensure specificity across a range of taxa, a more conserved region of the genome is usually selected [30,67]. However, targeting more variable regions of the genome can increase the specificity of the probe for a specific target species. Secondly, once potential target regions have been identified, in silico optimization is required to design primers or probes that specifically amplify or bind to the target DNA sequences [30]. In silico optimization involves various steps, such as designing primers or probes with appropriate lengths and melting temperatures, avoiding potential cross-reactivity with non-target sequences, and testing for potential secondary structures or hairpins. Once the primers or probes have been designed, they need to be validated experimentally using appropriate controls and reference samples. This typically involves testing their specificity, sensitivity, and accuracy using PCR, qPCR, or other molecular technique [122]. Despite the careful design of probes and primers, there are several potential sources of error, including (1) cross-reactivity due to unspecific primers leading to false positives; (2) DNA degradation leading to false negatives which can be mitigated by using short DNA fragments or better preservation methods [122]; (3) PCR bias leading to over- or under-representation of certain taxa [30,122]; (4) design errors such as choosing the wrong target DNA region or designing probes with suboptimal specificity, which can also lead to false positive or false negative results; (5) incomplete reference databases can limit the ability to design specific probes [30,36]; and (6) intra-specific variability can make it difficult to design probes that are specific to a single species.

In addition to the aforementioned approaches to overcome detection limitations, it is crucial that tests of assays are conducted on mock communities, systems, or species to understand the nature of such biases before implementation to make management decisions [23]. Standardization of protocols and procedures for DNA-based NIS monitoring is important to ensure consistent and comparable results across different laboratories and studies [30,37,39]. This can help to minimize variability and errors in data interpretation and improve the overall reliability of the monitoring system [17]. When stakeholders are informed about the methods, the potential benefits, and the standardization of procedures, they are more likely to support the implementation of DNA-based monitoring for a specific group of organisms [16]. By addressing issues such as false positives and negatives, including errors in reference libraries and establishing clear regulations and

guidelines, DNA-based monitoring can become a powerful tool for biodiversity research and monitoring, with the potential to inform conservation decisions and management actions [16,30,123,124]. Thalinger, et al. (2021) [123] and Evans, et al. (2017) [124] encouraged implementing validation scales in order to aid eDNA monitoring standardization. These standards range from in silico analysis and in vitro tests on the target species, DNA extraction, and PCR protocols to extensive testing using eDNA and specificity testing [123]. This validation scale helps to interpret eDNA results in cases where the target is detected or not detected, promoting standardization and facilitating decision making (possible outcomes in Figure S1).

6. eDNA as a Biomonitoring Tool: Challenges and Looking Forward

Environmental DNA has shown great advantages over traditional biomonitoring methods, where sampling is far less invasive, allows a more targeted approach, easier sampling in more remote areas, and is more cost-effective overall. This is particularly the case for targeted and passive monitoring, where eDNA tools have proven to be highly efficient and sensitive to infer occurrences of rare organisms in the environment [125], including NIS, early detection of unwanted organisms, surveillance of pathogens, and identifying paths of invasion [126]. Despite being a cutting-edge method, eDNA tools pose certain challenges that need to be overcome. One such challenge is the potential for limitations in signal detection, which can arise from various factors, including sample collection and processing, as well as bioinformatics analysis. Additionally, the properties of DNA itself can also affect the accuracy and reliability of eDNA analysis [17,127]. The detection of eDNA over long distances and time is likely to depend on many factors, including the flow rate of the water, the persistence of DNA in the water, the sensitivity of the detection method used, and the time between DNA shedding and sampling [82]. To circumvent some of these challenges, the experimental and sampling design must acknowledge such spatial representativeness and sampling efforts, which are crucial for augmenting the probability of detection, but the target taxa (micro to macrofauna) and habitat type (e.g., marine environment eDNA is more diluted and lentic environment is more patchy) (Figure 1) should also be considered [17]. Thus, depending on the type of environmental sample (e.g., shallow lake water, coastal surface water, and deep mesopelagic water) and the specific biotic and abiotic processes, sampling strategies must be optimized [23], and the results should be interpreted carefully regarding eDNA fate and transport [94]. The early detection of aquatic NIS will be facilitated by regular passive metabarcoding (using Illumina or ON sequencing) of high-risk locations, such as estuaries, ports, marinas, and recreational water [23]. In the event of NIS identification through passive monitoring, a targeted approach would be necessary to monitor their spread (qPCR and ddPCR), but a quick response time hinges on the availability of species-specific PCR primers [23]. Therefore, it is crucial to have a range of molecular assays available to support NIS monitoring surveys. In the absence of such primers, a viable alternative could be the utilization of ON-MinION metabarcoding until specific primers become available. Despite technological advances and concerted efforts to use DNA-based tools for NIS biomonitoring, the use of eDNA as a biomonitoring tool for policy actions or managers to detect NIS is still quite scarce (e.g., Canada's Marine Protected Areas Program since 2020) [128]. Given the potential financial and ecological consequences of a false negative or false positive result, it is imperative that eDNA analysis maintain a high level of accuracy. However, it is important to note that, as with many conventional detection methods, imperfect detection rates are common, particularly in the case of false negatives [6]. Nevertheless, minimizing the likelihood of false results through careful experimental design and rigorous quality control measures is essential for ensuring the reliability of eDNA analysis. Some suggest the use of decision support trees to help balance eDNA surveillance, management actions, and risk tolerance upon action or no action measures [129,130]. A consensus view exists that eDNA results should be considered in policy and management actions when assays are validated and results provide a clear workflow to managers, and eDNA should be used as another tool for the detection and

monitoring of species of interest [17,123,129,130] (overview Figure 2). There is an urgency in establishing standardized methods for rapid and accurate detection, not only for aquatic NIS detection but also for other eDNA applications (e.g., endangered species detection and pathogen surveillance). To some extent, method standardization might be limited due to the different provenance of samples, target taxa, the rapid development of methods, and concomitant use of different bioinformatic analyses. In the medium term, however, the use of standardized methods throughout would be a necessity for quality control, proficiency testing, and data analysis in order to obtain consistent and comparable results [23]. However, among factors such as habitat type, the targeted taxa might be a limiting factor for a universal standardized method, so standardization should be applied in different use cases [17]. For example, for some macrofauna species (e.g., fish) the sampling effort in terms of water volume required may be substantial for detection (e.g., more than 15 L per site), despite sample representativeness being more important than volume in some cases, especially when DNA is patchily distributed [17]. Case-specific studies can provide an initial basis for better defining the focus of intensive traditional sampling efforts [131]. Similarly, the existing methods using eDNA assays are now well recognised and provide baselines to reduce and quantify errors (e.g., the use of field and internal technical controls, the removal of inhibitors to avoid false negatives, and primer specificity to target taxa) [17,129]. Environmental DNA methods have advanced substantially since 2010 [129], but how can we transition from the well-recognized eDNA assays for NIS detection to its application? In theory, it should be simple. According to Thalinger, et al. (2021), if a set of guidelines is followed for targeted eDNA assays (e.g., validation scales and reporting standards), communication between academia, policy, and the government will be facilitated and transparent. Following such guidelines for specific NIS, case studies should then aid its implementation [129], as occurs in other applications where eDNA analyses are used for decision making, such as the detection of *Enterococcus* spp. bacteria [132] or determining the presence of the great crested newt *Triturus cristatus* for legal planning decisions in the UK [133]. As a general recommendation, eDNA assays should report as much information as possible on the validation steps taken [123]. Existing studies that provide guidelines for best practices and validation workflows in the field and laboratory should be taken into consideration [23,44,123,134,135]. Thalinger and colleagues (2021) [123] also have a website (https://edna-validation.com (accessed on 20 March 2023)) that summarizes key validation processes to help decision making and best practices.

7. Concluding Remarks

Conventional methods for identifying non-indigenous species (NIS) in high-risk locations, such as morphological identification through dive surveys, may currently be perceived as a reliable option by policy makers due to their ability to detect tangible, physical evidence of the organisms. Despite some initial reservations, it is becoming increasingly clear that DNA-based monitoring has a crucial role to play in environmental monitoring; in fact, the transition is already underway and gaining momentum. This approach is becoming increasingly popular due to its accuracy, sensitivity, and ability to detect species even in small quantities of environmental samples. The infrastructure and expertise for DNA-based analyses are expanding, resulting in a reduction in associated costs, while taxonomic expertise is becoming scarce. The adoption of eDNA monitoring aligns with the "take once, use many" sampling approach, which allows for multiple environmental indicators, including NIS, to be detected from a single environmental sample. Now is the opportunity to utilize both molecular and conventional monitoring methods simultaneously to validate any remaining uncertainties in eDNA monitoring. This combined approach can offer a more comprehensive understanding of environmental conditions, which is valuable for natural resource management and conservation. While the scientific community increasingly recognizes the value of DNA-based tools for monitoring, this acceptance may not necessarily extend to policy workers and stakeholders. Therefore, it is the responsibility of the scientific community to promote an understanding of DNA-based

technologies and facilitate transparent discussions on their limitations, while emphasizing the importance of exercising caution in their application and interpretation. By doing so, the scientific community can help bridge the gap between scientific research and policy making and promote the responsible use of DNA-based monitoring tools.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15050631/s1, Figure S1: Decision tree to aid in NIS eDNA PCR-based approaches. The decision steps highlighted include external sources of variability (problem sources) that lead to false negatives and false positives (Problem) followed by measures to mitigate PCR assay problems (solution). The possible outcomes and decision steps of eDNA result from the NIS surveys are also exemplified. LOD-Limit of detection. Sample handling: use of gloves and minimized sample handling to mitigate sample cross-contaminations.

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