Advantages and Limitations of Environmental DNA/RNA Tools for Marine Biosecurity: Management and Surveillance of Non-indigenous Species

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To enable successful management of marine bioinvasions, timely and robust scientific advice is required. This knowledge should inform managers and stakeholders on the magnitude of a pressure (rate of human-mediated introductions), the environmental state of an ecosystem (impacts of non-indigenous species), and the success of management response (prevention, eradication, mitigation). This advice often relies on baseline biodiversity information in the form of measureable parameters (metrics). This can be derived from conventional approaches such as visual surveys, but also by utilizing environmental DNA/RNA-based molecular techniques, which are increasingly being touted as promising tools for assessing biodiversity and detecting rare or invasive species. Depending on the stage of incursion, each approach has merits and limitations. In this review we assess the performance of biosecurity-relevant biodiversity parameters derived from eDNA/eRNA samples and discuss the results in relation to different stages of invasion and management applications. The overall performance of considered methods ranged between 42 and 90% based on defined criteria, with target-specific approaches scoring higher for respective biosecurity applications, followed by eDNA metabarcoding. Caveats are discussed along with avenues which may enhance these techniques and their successful uptake for marine biosecurity surveillance and management. To facilitate and encourage uptake of these techniques, there is a need for an international collaborative framework aimed at unifying molecular sampling and analysis methodologies. Improvement of quantitative capacity and cost-efficiency will also enhance their integration in biosecurity programmes.

Keywords: non-indigenous species, early detection, metabarcoding, eRNA, species-specific assays

INTRODUCTION

Translocation of marine organisms within and between biogeographic regions is an unavoidable consequence of modern society and has been enhanced with increasing globalization. When these organisms, referred to as non-indigenous species (NIS), arrive in a new location they can spread and become invasive (Occhipinti-Ambrogi and Galil, 2004; Katsanevakis et al., 2014), sometimes with unpredictable and undesirable effects on native communities and ecosystem functioning. This can ultimately compromise socio-economic values, and human health and wellbeing (Bax et al., 2003; Lodge and Shrader-Frechette, 2003; Molnar et al., 2008).

The importance of NIS introductions as a potential pressure on marine ecosystems is now recognized through established international organizations (e.g., International Maritime Organization [IMO], International Council for the Exploration of the Sea [ICES], Helsinki Commission [HELCOM], International Union for Conservation of Nature [IUCN]), and is addressed in a number of recent legislative initiatives worldwide (e.g., International Convention for the Control and Management of Ships' Ballast Water and Sediments [BWM], European Strategy on Invasive Alien Species and Marine Strategy Framework Directive [MSFD], New Zealand Craft Risk Management Standard [CRMS], and the United States Ballast Water Regulations). However, the range of possible responses to marine NIS introductions is limited and different response measures are applicable at different stages of the introduction and establishment process (Lodge et al., 2006; Olenin et al., 2011; **Figure 1**). Measures against NIS introductions are usually more efficient when applied at the pre-introduction stage rather than at the state change or impact stages (Lehtiniemi et al., 2015; Oesterwind et al., 2016).

It is generally accepted that for successful management of environmental issues (including NIS), timely and robust scientific advice is required (Raymond et al., 2010; Olenin et al., 2011). Ideally, this advice should inform managers and stakeholders on some or all of the following: the magnitude of the pressure/s, environmental state in relation to the pressure, appropriate, and adequate management approach/s (including effort, timeframes, and location), and the success of management response. For example, the European MSFD suggests that baseline assessments of marine ecosystems and follow-up monitoring of environmental status should be determined by assessing: NIS diversity, the number of new incursions, and their impacts on communities, habitats and ecosystem functioning (EU-COM, 2008; Lehtiniemi et al., 2015).

Baseline biodiversity information is required to support NIS related decision-making processes (Olenin et al., 2014; Lehtiniemi et al., 2015; Ojaveer et al., 2016; Darling and Frederick, 2017). This information needs to be reliably derived either pre-border (on a pathway), at the border (incursion/establishment stage), post-border (expansion/secondary spread), and sometimes from controlled experimental environments (e.g., for impact assessment or development/approval of management method). The information that is generally required can be placed into the following categories: (i) general species lists; (ii) presence/absence of target NIS species; (iii) viability of detected taxa; (iv) quantitative data on the abundance of NIS.

To derive biodiversity information by conventional morphological analysis, samples need to be collected using specific or generalist sampling devices (e.g., nets, electrofishing, filtering large water volumes, sediment cores, SCUBA diving), then sorted and individually taxonomically identified usually under the microscope. This limits how many samples and replicates can be collected and analyzed. Therefore, surveillance of extensive geographic areas is often constrained by available resources and likely to be limited to rapid assessment surveys (Awad et al., 2014; Nall et al., 2015; Minchin et al., 2016).

Molecular methods are increasingly touted as promising tools for assessing biodiversity and enhancing environmental management (Ji et al., 2013; Wood et al., 2013; Aylagas et al., 2014; Kelly et al., 2014). In recent years, rapid technological advancements have led to a range of different molecular techniques being used in biosecurity applications (Nathan et al., 2014; Comtet et al., 2015; Zaiko et al., 2015a,b; Devloo-Devla et al., 2016; Wood et al., 2017). Environmental DNA/RNA can be obtained from a range of samples, such as soil, water, and feces (Ficetola et al., 2008; Andersen et al., 2012; Epp et al., 2012; Ibáñez de Aldecoa et al., 2017). These samples contain bulk nucleic acids originating from living organisms present in the sample as well as dead cells and extracellular DNA/RNA (Taberlet et al., 2012). This overcomes the need to isolate or identify individual specimens and circumvents many of the difficulties associated with conventional morphological identification including: morphological complexities, cryptic life stages, and globally declining taxonomic expertise (Wheeler et al., 2004; Costello et al., 2013; Kelly et al., 2014).

The aim of this manuscript is to review and assess the performance of eDNA/eRNA-based molecular approaches for deriving NIS-related biological information in the context of different stages of invasion and management applications (**Figure 1**). In this review we assessed a range of molecular methods currently used or considered for use in marine biosecurity applications. Hereafter, we refer to biosecurity, as protection against any risk posed to economy, health through "biological harm" caused by introducing or spreading non-indigenous species (and other harmful organisms such as diseases) in the wild (Armstrong and Ball, 2005). Our approach is based on the method-related criteria adopted from existing indicator evaluation frameworks (e.g., ICES, 2013; Krause-Jensen et al., 2015; Queirós et al., 2016). In these frameworks, criteria for selection of good indicators are suggested, in order to account for the various aspects of indicator utility, quality of underlying data, scientific robustness, and practicality. The importance of each criterion can be individually weighted, ranging from "essential" via "desirable" to "informative" and indicator compliance with criteria is assessed as "fully fitted," "not fitted," and sometimes– "partially fitted." Scoring is usually conducted based on expert knowledge and considering published information. The derived weighted scores allow shortlisting of the most effective approaches and assist managers in making informed decisions when selecting the best fit-for-purpose suite of indicators. Here,
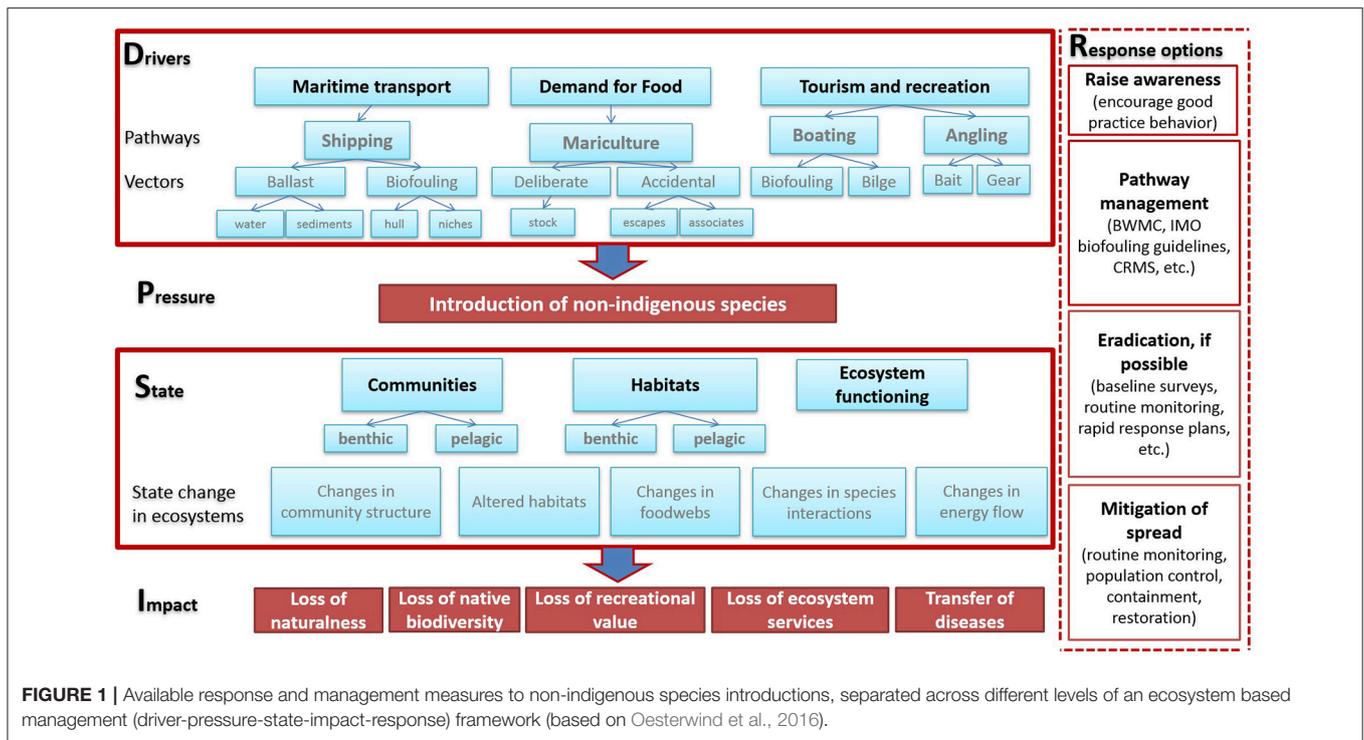
FIGURE 1 | Available response and management measures to non-indigenous species introductions, separated across different levels of an ecosystem based management (driver-pressure-state-impact-response) framework (based on Oesterwind et al., 2016).

this approach was adopted for ranking the molecular tools based on their suitability while deriving biosecurity-relevant biodiversity information. Because the review only focuses on the molecular methods and assesses their suitability for biosecurity applications, we do not directly compare nor contrast them to conventional biodiversity assessments approaches.

MOLECULAR TARGET-BASED AND INVENTORY METHODS USED IN BIOSECURITY APPLICATIONS

In this section, a brief overview of selected molecular techniques currently used in marine biosecurity studies is provided. It is not our intension to provide an in-depth account of the technical details of these methods and readers wanting more detailed explanations are referred to the multitude of internet resources which provide animations and diagrams (e.g., the DNA Learning Centre - http://www.dnalc.org). **Table 1** provides a summary of how each technique can contribute to deriving biodiversity information relevant for biosecurity applications.

Target-Based Tools: End-Point and Real-Time Polymerase Chain Reaction Amplification Methods

Despite being somewhat superseded by more sensitive methods (discussed below), traditional end-point Polymerase Chain Reaction (PCR) is still regularly used in marine biosecurity assessments. When applied to environmental samples, target-specific primers are used with the follow-up visualization

of amplicons on an agarose gel, and (if needed) Sanger sequencing of the PCR amplicons. The sequences derived from these amplicons are then compared to global databases such as Barcode Of Life Data system (BOLD) or National Center for Biotechnology Information (NCBI) database to verify the identification of target organism or undertake follow-up phylogenetic analyses.

Examples of the use of species-specific PCR in marine biosecurity include development and application of a specific end-point PCR assays to detect NIS from environmental samples include: Atlantic wedge clam *Rangia cuneata* (Ardura et al., 2015), soft-shell clam *Mya arenaria* (Ardura and Zaiko, 2018), and the Australian tubeworm *Ficopomatus enigmaticus* (Muñoz-Colmenero et al., 2017).

Quantitative Polymerase Chain Reaction (qPCR, also known as real-time PCR) is an advancement on end-point PCR and is one of the most promising molecular tools for highly specific and sensitive detection of one or a few targets. It enables rapid turnaround and simultaneous analysis of multiple samples. Quantitative PCR assays rely on primers, or primers and a probe that have been designed to be specific for the target species. The amplification of this target can then be measured in real-time either through the use of intercalating dyes (Becker et al., 1996) or probe-based detection systems (Heid et al., 1996). In recent years, assays have been designed specifically for marine pests including: dinoflagellates *Alexandrium* spp. (Galluzzi et al., 2004; Vandersea et al., 2017), sea squirts *Styela clava* (Gillum et al., 2014), and *Didemnum* spp. (Simpson et al., 2017), Amur River clam *Potamocorbula amurensis* (Smith et al., 2012), and Mediterranean fan worm *Sabella spallanzanii* (Wood et al., 2017).

TABLE 1 | Biodiversity information required for science-based biosecurity programmes, its relevance to specific management applications and available eDNA/eRNA methods for deriving this information.

Required biodiversity information	Relevance to response and management measures	Available molecular methods
Species inventories (presence/absence)	• Pressure assessment (baseline surveys, hub monitoring) • Risk assessment • Early detection (screening for new invasions) • Status assessment (population spread/distribution) • Assessment of management success (pathway management, restoration)	eDNA metabarcoding Molecular markers for taxonomic assessment through shotgun sequencing
Presence/absence of target species	• Targeted monitoring for early detection of unwanted pests (pre-border/post-border) • Monitoring of established populations (distribution, ecological characteristics) • Assessment of management success (e.g., population containment, eradication, pathway management)	Species-specific assays, e.g., end-point PCR, qPCR, ddPCR eDNA metabarcoding
Partitioning viable biodiversity	• Risk assessment • Development of management measures (e.g., ballast water, biofouling) • Assessment of management success (e.g., population containment, eradication, pathway management)	eRNA metabarcoding eRNA based species specific assays Viability PCR using nucleic acid intercalating dyes, e.g., Propidium Monoazide
Quantitative characteristics of community/target species	• State assessment (abundance and relative abundance of NIS, as a proxy of impact) • Assessment of management success (e.g., pathway management, containment)	Species-specific assays, e.g., qPCR, ddPCR eDNA metabarcoding

From a biosecurity perspective, a potentially useful extension of the qPCR application is viability assessment for applications where determining the presence of a living organism is essential (e.g., assessing the success of applied treatment or a pest management programme; Darling and Frederick, 2017; Pochon et al., 2017). Nucleic acid intercalating dyes added to the sample before extraction (e.g., propidium monoazide [PMA]), that only penetrate damaged lipid membranes (i.e., dead cells), bind and covalently crosslink with double-stranded nucleic acids, inhibiting their extraction, and amplification (Nogva et al., 2003; Nocker et al., 2006). This approach is being used to distinguish between viable and dead cells of bacteria (Schnetzinger et al., 2013; Desneux et al., 2015). The so-called viability PCR has not yet been used for biosecurity applications and its applicability to assess viability of multicellular organisms is unknown. Substantial loss of DNA signal in viability PCR (Nocker et al., 2006) may cause false negative results in targeted surveys, e.g., failing to detect extracellular DNA of living organisms. Therefore, this method should be considered for application in combination with conventional end-point or qPCR, implying additional costs and effort. Further research is required to assess the applicability of this approach for characterizing viable eukaryotic biodiversity in the context of marine biosecurity.

Alternatively, to infer viability of the target organism(s), PCR tools can be applied to eRNA samples. Ribonucleic acid is a crucial component for protein synthesis, usually single stranded in a cell and transcribed from DNA by enzymes, i.e., produced in biologically active (living) organisms. Compared to eDNA, eRNA degrades more rapidly in the marine environment (typically hours to days, Thomsen et al., 2012b; Sassoubre et al., 2016), and is therefore considered a better proxy for detecting living biota. On the other hand, susceptibility of RNA makes it difficult to work with. Collection of RNA samples requires dedicated sampling protocols, more careful preservation, and storage. There is also additional processing time and costs associated with isolation and reverse transcription of RNA (Laroche et al., 2016), making it more expensive and challenging and thus a less attractive molecule to work with.

A recent advancement in PCR methods is droplet digital PCR (ddPCR), where target DNA is randomly allocated into discrete droplets via microfluidics and each droplet is then thermally cycled and individually screened via fluorescence measurement for the presence of target DNA. Quantitation of DNA is very accurate using ddPCR and is calculated using Poisson statistics (Hindson et al., 2011; Pinheiro et al., 2012). This negates the need for the use of standard curves and enables extremely low-level detection. A recent comparative application of qPCR and ddPCR for detecting invasive aquatic species in the Laurentian Great Lakes (Nathan et al., 2014), suggested similar sensitivities between the two methods, with higher cost-efficiency demonstrated for ddPCR (when capital expenditure was not considered). An additional advantage provided by ddPCR platforms with two fluorescence filters, is that they enable duplex or with some developments and optimization greater multiplexing capability (Dobnik et al., 2016).

Inventory-Based Tools: Metabarcoding and Polymerase Chain Reaction Free High-Throughput Methods

The advent of high-throughput sequencing (HTS) has made it possible to produce enormous volumes of sequence data rapidly. Metabarcoding has become a well-established method for characterizing the biodiversity in different types of

environmental samples (Chariton et al., 2010; Shokralla et al., 2012; Taberlet et al., 2012; Aylagas et al., 2014; de Vargas et al., 2015; Domaizon et al., 2017). It enables the identification of many taxa by matching short (typically 100–600 base pair) sequence reads obtained from HTS of PCR amplicons to reference sequences. Metabarcoding has proven to be very effective for characterizing marine communities and identifying potential pests (Pochon et al., 2013; Comtet et al., 2015; Zaiko et al., 2015c; Brown et al., 2016). However, there are some prerequisites required when applying metabarcoding for characterizing biotic assemblages and identifying potential NIS: (*i*) sufficient taxonomic resolution provided by the target gene, (*ii*) "universality" of the primers (i.e., capacity to amplify the target gene from a wide variety of taxa; see Geller et al., 2013), and (*iii*) availability of robust reference databases for reliable taxonomic assignments of obtained sequences (discussed in sections below).

Environmental RNA metabarcoding (targeting cDNA obtained through reverse-transcription PCR) is increasingly being used for characterizing viable assemblages associated with recent environmental change due to human marine activities (Pawlowski et al., 2014, 2016a; Dowle et al., 2015; Lejzerowicz et al., 2015; Pochon et al., 2015a; Visco et al., 2015; Laroche et al., 2016; Birrer et al., 2018), and is being considered for biosecurity applications (Pochon et al., 2017; Rey et al., 2018). However, there are uncertainties regarding potential biases associated with eRNA metabarcoding, resulting from e.g., overrepresentation of organisms with complex genomes and numerous copies of transcriptionally active marker genes (Gong et al., 2013), or a number of artifacts potentially occurring during RNA processing and PCR amplification (Laroche et al., 2017). Therefore, despite the potential of eRNA metabarcoding for differentiating living biodiversity, for example, in bilge (Pochon et al., 2017) or ballast water (Darling and Frederick, 2017; Rey et al., 2018), it remains challenging and requires further dedicated research and technological advancements to facilitate its uptake for biosecurity applications.

There are several emerging and rapidly advancing PCR-free methods, such as shotgun sequencing (Wang et al., 2013), mitochondrial enrichment (Zhou et al., 2013), and gene enrichment (Mertes et al., 2011; Dowle et al., 2016). These have limited application for routine biosecurity surveys to date, as they require considerable sequencing and computing effort or additional laboratory processing which increases the associated cost. These methods have an important advantage though, since they overcome PCR-inherent biases which result in the preferential amplification of certain DNA (or cDNA) templates leading to the overrepresentation of some taxa and potentially missing others.

ASSESSMENT OF BIOSECURITY-RELEVANT BIODIVERSITY INFORMATION DERIVED FROM MOLECULAR METHODS

Different criteria can be applied to select the most efficient and practical biological information or indicators for environmental management purposes (Mazik et al., 2010; Elliott, 2011; James et al., 2012; Queirós et al., 2016). Some criteria refer to intrinsic qualities of indicators, for example "scientific basis," "responsiveness to pressure," "social relevance." However, there are also criteria associated with the methodology employed for deriving underlying data (i.e., biodiversity information) and quality of that data. In **Table 1** we provide an overview of the relevance of biodiversity information derived from molecular approaches applied to environmental DNA/RNA, in the context of its utility for biosecurity management and response purposes.

Our assessment of molecular methods suitability for deriving biosecurity-relevant biodiversity information is based on the set of method-related criteria (**Table 2**) adopted from existing indicator evaluation frameworks (Elliott, 2011; ICES, 2013; Krause-Jensen et al., 2015). The relative importance of each criterion for informing adequate biosecurity response and management measures was assigned as either *essential*, *desirable* or *informative*, applying importance weighting factors 3, 2 and 1, respectively. Compliance of each considered method with selected criteria was assessed as either a criterion is *fully met* = 1.0, *partially met* = 0.5, or *not met* = 0.0. This assessment is based on evidence from our comprehensive literature review and expert knowledge. The performance score for each indicator against each criterion calculated as the product of these two values (*compliance* × *weighting factor*). For benchmarking the overall performance of the methods, the final scores were derived for each method/biosecurity application combination by summing the performance scores and then dividing the sum-product by a maximum possible score per method/application. The resulting performance value was expressed as percentage.

Compliance Scores for Technical Feasibility, Precision, and Repeatability Criterion

End-point PCR (eDNA and eRNA) = 1
qPCR/ddPCR (eDNA and eRNA) = 1
Viability PCR using nucleic acid intercalating dyes = 0.5
Metabarcoding (eDNA and eRNA) = 0.5
Shotgun sequencing and mitochondrial enrichment (eDNA) = 0.5
Shotgun sequencing and mitochondrial enrichment (eRNA) = 0
Gene enrichment (eDNA) = 0.5
Gene enrichment (eRNA) = 0

The aforementioned molecular methods (**Table 1**) are increasingly being applied in biosecurity research with multiple studies addressing species detection on pathways, in introduction hubs, and in ongoing monitoring programmes (Bott et al., 2010; Darling and Mahon, 2011; Mountfort et al., 2012; Comtet et al., 2015; Pochon et al., 2015a; Zaiko et al., 2015c, 2016; Montes et al., 2016). In general, molecular methods can be considered technically feasible for providing qualitative biodiversity information (species lists and presence/absence of target species).

In regard to documented evidence of feasibility, methods such as shotgun sequencing, gene enrichment, and viability PCR are currently less advanced comparing to more widely used metabarcoding and traditional PCR-based methods. We were

TABLE 2 | Method-related criteria (adopted from ICES, 2013; Krause-Jensen et al., 2015 and other relevant literature sources[3–10]), scoring categories and importance weighting applied in this assessment.

Assessment criterion	Compliance (scoring) categories	Importance weighting (factor) for biosecurity applications
Relevant biodiversity data should be accurately derived using **technically feasible**, **precise and repeatable methods**, with supporting evidence documented in peer-reviewed literature[1,2]	*Fully met (1)*: deriving data is technically feasible by methods that are widely adopted (with supporting evidence documented in peer-reviewed literature), signal-to-noise ratio is high; *Partially met (0.5)*: potential issues with quality assurance, or methods not widely adopted, poor signal-to-noise ratio; *Not met (0.0)*: methods are not tangible or signal-to-noise ratio is unknown.	*Essential* (**3**) for *all* biosecurity applications
Information derived by a method should be **comprehensible**-easy to communicate and understandable by policy-makers and other non-scientists.	*Fully met (1)*: information derived by a molecular method is easy to understand and communicate; *Partially met (0.5)*: information at least easy to communicate; *Not met (0.0)*: neither understandable by non-specialists, nor communicable.	*Desirable* (**2**) for *all* biosecurity applications
In biodiversity assessments (including biosecurity applications), **quantitative** measurements are generally preferred over qualitative and semi-quantitative measurements[1,2]	*Fully met (1)*: data derived by a molecular method are quantitative; *Partially met (0.5)*: data are semi-quantitative; *Not met (0.0)*: the biodiversity information derived is not quantitative.	*Essential* (**3**) for assessing the effect of NIS on environmental state of ecosystems, pathway management (e.g., monitoring compliance with legal regulations, evaluating management success); *Desirable* (**2**) for development and validating pathway management measures and risk assessments (e.g., evaluation of propagule pressure in a hub). *Informative* (**1**) for baseline surveys, early (post-border) detection, hub monitoring, assessment of spread and distribution of established populations.
It is important to derive robust biodiversity information, relying on well-established and **standardized** methodology to ensure that results are comparable within and across regions[3–5]	*Fully met (1)*: methodological protocols for entire workflow (from sampling to data analysis) are well established and standardized at the international levels, outputs are comparable across regions and ecosystems; *Partially met (0.5)*: methodological protocols are well established and standardized for some parts of the workflow and/or for certain region or ecosystems; *Not met (0)*: no unified methodologies available.	*Essential* (**3**) for development and validating pathway management measures and pathway management, since these applications require robust international validation; *Desirable* (**2**) for risk assessments, baseline surveys, early (post-border) detection, hub monitoring, assessment of spread and distribution of established populations, impact assessment. These applications are usually region-, ecosystem- or even habitat-specific, therefore require adjustments for particular ecological settings.
Sampling, measuring, processing samples should be **cost-effective**, for providing high quality biodiversity data at a relevant spatio-temporal scale[1,2,4–6].	*Fully met (1)*: relevant biodiversity information can be reliably derived at low cost independently on ecosystem type and scale required; *Partially met (0.5)*: biodiversity information can be derived at relatively low cost depending on ecosystem type and scale; *Not met (0)*: acquisition of biodiversity information is expensive/time consuming.	*Desirable* (**2**) for *all* biosecurity applications
The methods should allow **early warning** of new incursions or changes in established populations, before actual "harm" is detected-it is extremely important for empowering rapid, and therefore more efficient, responses[1,2,7–10]	*Fully met (1)*: derived data provide early warning because of method's high sensitivity and allow short response time; *Not met (0)*: long responsiveness due to low sensitivity of the method.	*Essential* (**3**) for early (post-border) detection, hub monitoring and impact assessment; *Desirable* (**2**) for risk assessments, pathway management, assessment of spread and distribution of established populations; *Informative* (**1**) development and validating pathway management measures and for baseline surveys.
In environmental studies, **low impact (non-destructive)** methods posing no or minimal harm to the ecosystem during sampling and measurements are preferable over destructive approaches[2]	*Fully met (1)*: biodiversity data obtained with no or minimal harm to ecosystem; *Partially met (0.5)*: some harm can be done to ecosystem depending on sampling approach or ecosystem type; *Not met (0)*: sampling and measurement is destructive in all cases.	*Desirable* (**2**) for early (post-border) detection, baseline surveys, hub monitoring and impact assessment, risk assessments, assessment of spread and distribution of established populations; *Informative* (**1**) for pathway management, development and validating pathway management measures

(Continued)

TABLE 2 | Continued

Assessment criterion	Compliance (scoring) categories	Importance weighting (factor) for biosecurity applications
The **cross-applicability** of derived metrics/information (e.g., for assessing different pressures in an ecosystem or calculating different environmental state indices), is considered a desirable advantage[1,2]	*Fully met (1)*: derived data are applicable for other ecosystem surveillance/research purposes; *Not met (0)*: derived data are biosecurity-specific and not cross-applicable.	*Desirable* (**2**) for *all* biosecurity applications

[1](ICES, 2013); [2](Krause-Jensen et al., 2015); [3](Lehtiniemi et al., 2015); [4](Ojaveer et al., 2016); [5](Olenin et al., 2011); [6](Staehr et al., 2016); [7](Forrest and Hopkins, 2013); [8](Magaletti et al., 2017); [9](Piola et al., 2009); [10](Williams and Schroeder, 2004).

unable to find any documented examples of eRNA-based PCR-free methods been applied for marine biosecurity, therefore the criterion was considered not met for these methods. There are also some uncertainties regarding the reproducibility and signal-to-noise ratio in data derived from metabarcoding (both eDNA and eRNA), as sensitivity and accuracy for individual species detection can be impeded by incompleteness of reference databases, marker resolution, and amplification biases, especially in complex samples from diverse communities (Briski et al., 2016; Hatzenbuhler et al., 2017; Leray and Knowlton, 2017). Extrinsic factors, that may affect precision and repeatability of these methods include heterogeneous distribution of eDNA/eRNA in the water, susceptibility to both field and laboratory cross-contamination, variability of nucleic acid degradation due to microbial activity and environmental conditions, and variation in eDNA/eRNA shedding rates (Goldberg et al., 2016).

Compliance Scores for Comprehensibility Criterion

End-point PCR (eDNA) = 1
End-point PCR (eRNA) = 0.5
qPCR/ddPCR (eDNA) = 1
qPCR/ddPCR (eRNA) = 0.5
Viability PCR using nucleic acid intercalating dyes = 0.5
Metabarcoding (eDNA and eRNA) = 0.5
Shotgun sequencing and mitochondrial enrichment (eDNA and eRNA) = 0.5
Gene enrichment = 0.5

Generally, biodiversity information derived by target-based tools is more straight-forward, as positive eDNA signals are easy to interpret and communicate to stakeholders. This is much trickier for inventories, as there is a number of factors that should be considered for robust interpretation of eDNA-based biodiversity estimates, including resolution of the marker used, quality and completeness of reference databases, bioinformatics pipelines, etc. (Cristescu, 2014). There are also some difficulties interpreting detection or non-detection of rare taxa due to sequencing errors, preferential sequencing and aforementioned caveats (Darling and Frederick, 2017). The complex analyses applied to the molecular inventory data also makes communication of results to stakeholders more difficult than simple presence-absence data from targeted detections. Interpretation of viability assessment results from molecular analyses can also be complex and difficult

to understand, given all potential uncertainties (see the above sections).

Compliance Scores for Quantitative Capacity Criterion

End-point PCR (eDNA and eRNA) = 0
qPCR/ddPCR (eDNA and eRNA) = 0.5 (Note: can be 1 for prokaryotes, provided an appropriate empirical calibration is undertaken)
Viability PCR using nucleic acid intercalating dyes = 0.5
Metabarcoding (eDNA and eRNA) = 0.5
Shotgun sequencing and mitochondrial enrichment (eDNA and eRNA) = 0.5
Gene enrichment = 0.5

The ability to quantify the impacts of NIS using standardized methods has been identified as a priority in biosecurity research (Olenin et al., 2007, 2011; Lehtiniemi et al., 2015; Ojaveer et al., 2016). Although no universal framework has been agreed upon, most impact assessments utilize quantitative information on NIS populations: abundance, biomass, proportional contribution to relevant communities, distribution range (Kotta et al., 2001; Olenina et al., 2010; Zaiko et al., 2011; Ojaveer and Kotta, 2014). One of the key limitation of most molecular methods is that they usually cannot provide exact measures of abundance and/or biomass information (Yu et al., 2012), although some methods have higher quantitative capacity (e.g., qPCR, ddPCR, PCR-free approaches) than others (e.g., end-point PCR, HTS metabarcoding; Dowle et al., 2016).

Semi-quantitative biological information can be derived from molecular studies, for example, relative quantities of species in the community can be inferred from the percentage of sequence reads obtained through metabarcoding (Hajibabaei et al., 2011; Zhan et al., 2013; Evans et al., 2016; Pawlowski et al., 2016b). However, a number of studies have highlighted clear discrepancies between microscopically-derived absolute abundance data and metabarcoding-based abundance data (Medinger et al., 2010; Stoeck et al., 2014; Sun et al., 2015).

Although qPCR, ddPCR, and PCR-free methods enable quantification of targeted genes (e.g., Hindson et al., 2011), there are still a plethora of possible factors influencing biological biases when estimating abundances. These include intra- and inter-species variation due to the presence of different number of

nuclei, gene copies, genome sizes, and/or biovolume (Martin-Laurent et al., 2001; Weber and Pawlowski, 2013), PCR and sequencing errors, primer biases, and differential amplification efficiencies (Farrelly et al., 1995; Suzuki and Giovannoni, 1996; Polz and Cavanaugh, 1998; Acinas et al., 2005). It is generally accepted that it is extremely challenging to determine absolute abundance, and some authors advocate for the exclusive use of presence-absence data when using molecular information generated from HTS or quantitative PCR (Chariton et al., 2015; Zaiko et al., 2016). Many conventional biotic indices require only relative abundance data (e.g., AZTI Marine Biotic Index [AMBI], Borja et al., 2000; Benthic Quality Index [BQI], Rosenberg et al., 2004; Infaunal Quality Index [IQI], Kennedy et al., 2011). Recent studies have used or developed metabarcoding-based indices and demonstrated the potential of using semi-quantitative HTS data for measuring diversity change of marine benthic assemblages along pollution gradients (Lejzerowicz et al., 2015; Pochon et al., 2015a; Pawlowski et al., 2016a; Keeley et al., 2018).

The intrinsic nature of eDNA may differ between multicellular eukaryotes and prokaryotes or micro-eukaryotes. For example, there is a higher probability of detecting intracellular DNA from microbes, as intact individuals are more readily isolated from small volumes of material. For larger eukaryotes, there is increased probability of detecting free-floating or extracellular DNA and DNA from non-living cells or unicellular/small propagules (gametes, eggs; Ibáñez de Aldecoa et al., 2017). Consequently, any quantitative biodiversity information derived from eDNA samples may need to be treated differentially for micro- and macro-communities in biosecurity applications, especially if only intact or viable organisms are of interest (e.g., for confirming compliance with the BWMC regulations; IMO, 2004).

Compliance Scores for Standardization Criterion

End-point PCR(eDNA and eRNA) = 0.5
qPCR/ddPCR (eDNA and eRNA) = 0.5
Viability PCR = 0
Metabarcoding (eDNA and eRNA) = 0
Shotgun sequencing and mitochondrial enrichment (eDNA and eRNA) = 0
Gene enrichment = 0

While this criterion is being met at least to some extent for developed species-specific assays, further work is needed for international cross-validation to account for regional genetic variation (Bohmann et al., 2014; Ardura et al., 2016).

Despite recent attempts to develop and optimize standardized protocols for eDNA metabarcoding (Taberlet et al., 2012; Deiner et al., 2015; Elbrecht and Leese, 2015; Aylagas et al., 2016; Porter and Hajibabaei, 2018), they are often restricted to a portion of the biotic community (e.g., macrofauna), or only focus on a segment of the protocol (e.g., sampling, DNA extraction, library preparation, bioinformatics). A unified, internationally calibrated protocol for marine biosecurity applications is clearly needed. Currently, such a document does not exist and research laboratories employing metabarcoding for marine biosecurity applications often develop their own analytical workflows

and in-house reference sequence databases, which impedes the transferability/comparability of results at international and regional scales.

Two widely used genes for characterizing entire eukaryotic communities are the 18S ribosomal RNA (18S rRNA) and the mitochondrial *Cytochrome C Oxidase subunit I* (COI). While it is relatively straightforward to design universal primers for the highly conserved 18S rRNA gene, it does not provide sufficient resolution to identify organisms at the species (or even genera) level within many groups (Pochon et al., 2013; Fletcher et al., 2017). Use of the COI gene enables finer resolution in taxonomic assignments, and primers which cover a short region of this gene are now widely used (Hajibabaei and McKenna, 2012; Leray and Knowlton, 2015; Wangensteen et al., 2017). The common drawback of applying these two markers for biosecurity assessment, however, is incompleteness of open-access reference databases (Ratnasingham and Hebert, 2013; Zaiko et al., 2016). Despite efforts to build standardized barcode reference libraries for many major animal and plant phyla (Ratnasingham and Hebert, 2010; Guillou et al., 2013; Quast et al., 2013) including recent pest-focused initiatives (Diaz et al., 2004; Dias et al., 2017), numerous gaps remain. Even among groups with well-developed reference databases greater than 20% of entries may be incorrectly identified or lack important data e.g., geographic location (Nilsson et al., 2006). Other marker genes suggested or considered for metabarcoding applications, e.g., 16S ribosomal RNA (Zhan et al., 2014) or taxon-specific markers proposed for meiofauna, protists and plants, e.g., chloroplast DNA or the ribosomal Internal Transcribed Spacer ITS region (Medinger et al., 2010; Yao et al., 2010; Freeland, 2016; Hume et al., 2018) are less frequently applied for determining marine biodiversity, and therefore are less likely to have substantial representation of relevant taxa in publicly available databases. The deficiency of reference sequence databases for international marine pests (Briski et al., 2016) limits effective uptake and standardized application of metabarcoding in marine biosecurity programmes (Bohmann et al., 2014; Zaiko et al., 2016).

Comparability of metabarcoding results can be further impeded by a broad array of bioinformatics pipelines currently applied and with continuously emerging novel analytical tools coupled with ever-developing computing and sequencing technologies (Coissac et al., 2012; Cristescu, 2014; Callahan et al., 2016; Anslan et al., 2017). Biodiversity information produced by different sequencing platforms (Zaiko et al., 2015a; Speranskaya et al., 2018) and/or algorithms used for filtering, de-noising, clustering, and taxonomic assignments are generally comparable at high-level overview of community composition, but can be divergent at genus or species levels (Plummer et al., 2015). When data is not easily reconcilable at lower taxonomic levels this can create challenges for biosecurity applications over extended spatio-temporal scales (e.g., long-term national hub monitoring or implementing international policies).

The need for uniformity and standardization (from protocols to databases and bioinformatics pipelines) has been already recognized by large consortia working in the field of molecular research, for example, 1000 Genomes (The 1000 Genomes Project Consortium, 2015) and CBOL Protist Working Group (Pawlowski et al., 2012). Similar initiatives should be

also encouraged for biosecurity applications, through inter-calibration sequencing experiments and developing consistent analyses approaches (Muir et al., 2016).

Compliance Scores for Cost-Efficiency Criterion

End-point PCR (eDNA) = 1
End-point PCR (eRNA) = 0.5
qPCR/ddPCR (eDNA) = 1
qPCR/ddPCR (eRNA) = 0.5
Viability PCR = 1
Metabarcoding (eDNA and eRNA) = 0.5
Shotgun sequencing and mitochondrial enrichment (eDNA and eRNA) = 0
Gene enrichment (eDNA and eRNA) = 0

Molecular methods can rapidly detect target species or be used to characterize comprehensive biodiversity from small sample sizes, and can be effectively applied to a wide range of environmental samples and spatio-temporal scales: from localized mesocosm experiments to temporally replicated site or nation-wide surveys (Shaw et al., 2016; Minamoto et al., 2017; Stat et al., 2017). Only small volumes of water or sediments are needed to obtain molecular traces of an organism (Thomsen et al., 2012a; Pochon et al., 2015a; Shaw et al., 2016). Thus, when compared to the traditional morphology-based biodiversity assessment, the estimated cost per sample when applying molecular methods is significantly less due to the constantly increasing high-throughput and multiplexing capacities (Ji et al., 2013; Shokralla et al., 2015). The actual cost however can vary significantly depending on the methodology (e.g., end-point PCR is generally cheaper than quantitative PCR, which is less expensive than HTS metabarcoding), the aim of the study, and the scale of the survey.

Sequencing services are becoming increasingly affordable for routine application, enabling larger numbers of samples to be processed and enormous volumes of sequencing data to be generated (Muir et al., 2016). Analysis of these data requires increased computational resources and analytical efforts. This affects the overall cost structure of biodiversity research projects requiring a larger budget allocated to the analysis component, compared to traditional research where most of the cost is spent on experimental work and data generation (Sboner et al., 2011). Such shift in focus should be accounted for when planning molecular-based studies, and allocating funding resources for biosecurity surveillance.

Compliance Scores for Early Warning Criterion

End-point PCR (eDNA and eRNA) = 1
qPCR/ddPCR (eDNA and eRNA) = 1
Viability PCR = 1
Metabarcoding (eDNA and eRNA) = 1
Shotgun sequencing and mitochondrial enrichment (eDNA and eRNA) = 1
Gene enrichment (eDNA and eRNA) = 1

Surveillance programmes using eDNA-based tools may enable the detection of NIS arrival or population spread at an earlier stage compared to programmes relying exclusively on traditional survey methods (Pochon et al., 2015b; Brown et al., 2016; Xiong et al., 2016). A further benefit of molecular methods is the ability to detect cryptic marine species at early life stage, when visual identification is difficult or impossible.

Molecular surveillance can generally ensure higher sensitivity of detection due to legacy DNA (extracellular or non-living material). This may in some cases be an advantage as it provides information on potentially present, but not observable taxa (Zaiko et al., 2015c, 2016; Ardura et al., 2016). The down-side of legacy DNA is that it may lead to the detection of false positive signals, e.g., from non-viable organisms, untargeted sources, incidental contamination, or (in HTS metabarcoding) as a result of marker- or reference-related biases (Bohmann et al., 2014; Ficetola et al., 2015).

Eliminating false positives from biodiversity assessment remains a major challenge in eDNA-based studies (Bohmann et al., 2014). There are several pathways for minimizing the probability of false positives through e.g., stringent control of contamination, including the use of multiple blank controls at different steps of sample collection and processing (Jerde et al., 2011); and/or confirming species detection by HTS using complementary analyses such as multi-loci metabarcoding, and phylogenetic analyses or species-specific assays (Kelly et al., 2017; Wood et al., 2017). A range of statistical methods are also available for optimizing the quality and strategy of a survey and account for both imperfect detection and false positive signals (Ferguson et al., 2015; Ficetola et al., 2015; Lahoz-Monfort et al., 2015). It should be noted though, that appropriate fit-for-purpose sampling design and monitoring strategy is important for efficient implementation of molecular surveillance. Similarly as recommended for conventional approaches (Lehtiniemi et al., 2015), thorough consideration should be given to sampling time, locations, spatial coverage required, which will vary depending on the objectives of each survey or monitoring programme.

Compliance Scores for Low Impact (Non-Destructive) Criterion

End-point PCR (eDNA and eRNA) = 1
qPCR/ddPCR (eDNA and eRNA) = 1
Viability PCR = 1
Metabarcoding (eDNA and eRNA) = 1
Shotgun sequencing and mitochondrial enrichment (eDNA and eRNA) = 1
Gene enrichment (eDNA and eRNA) = 1

Conventional biological surveillance methods vary in the magnitude of disturbance involved. High-impact methods such as electrofishing (Nielsen, 1998), may inflict significant injuries and mortality on non-target taxa. Medium-impact methods such as net sampling may interfere with a range of biota and induce some physiological stress and casual mortalities (e.g., Eleftheriou, 2013). Methods using underwater cameras and/or acoustic methodologies (Matsua et al., 2009; Doehring et al.,

TABLE 3 | Comparative performance of environmental DNA/RNA tools for deriving biodiversity information relevant for advising biosecurity response and management measures.

Biosecurity application	Biodiversity information required	Relevant eDNA/eRNA based methods	Feasible, precise, repeatable	Comprehensible	Quantitative	Standardized	Cost-efficient	Early warning	Low impact	Cross-applicable	Overall performance
Risk assessment	Species inventories (pres/abs)	eDNA metabarcoding	1.5	1	1	0	1	2	2	2	70.0%
		eDNA shotgun sequencing	1.5	1	1	0	0	2	2	2	63.3%
	Target species (pres/abs)	end-point PCR (eDNA)	3	2	0	1	2	2	2	0	80.0%
		qPCR/ddPCR (eDNA)	3	2	1	1	2	2	2	0	86.7%
		Gene enrichment (eDNA)	1.5	1	1	0	0	2	2	2	63.3%
	Viable biodiversity/ target species	eRNA metabarcoding	1.5	1	1	0	1	2	2	2	70.0%
		end-point PCR (eRNA)	3	1	0	1	1	2	2	0	66.7%
		qPCR/ddPCR (eRNA)	3	1	1	1	1	2	2	0	73.3%
		eRNA shotgun sequencing	0	1	1	0	0	2	2	2	53.3%
		Gene enrichment (eRNA)	0	1	1	0	0	2	2	2	53.3%
		Viability PCR	1.5	1	1	0	2	2	2	0	63.3%
Development and validating pathway management measures	Viable biodiversity/target species	eRNA metabarcoding	1.5	1	1	0	1	1	1	2	60.7%
		end-point PCR (eRNA)	3	1	0	1.5	1	1	1	0	60.7%
		qPCR/ddPCR (eRNA)	3	1	1	1.5	1	1	1	0	67.9%
		eRNA shotgun sequencing	0	1	1	0	0	1	1	2	42.9%
		Gene enrichment (eRNA)	0	1	1	0	0	1	1	2	42.9%
		Viability PCR	1.5	1	1	0	2	1	1	0	53.6%
Pathway management (e.g., compliance control)	Species inventories (pres/abs)	eDNA metabarcoding	1.5	1	1.5	0	1	2	1	2	62.5%
		eDNA shotgun sequencing	1.5	1	1.5	0	0	2	1	2	56.3%
	Target species (pres/abs)	End-point PCR (eDNA)	3	2	0	1.5	2	2	1	0	71.9%
		qPCR/ddPCR (eDNA)	3	2	1.5	1.5	2	2	1	0	81.3%
		Gene enrichment (eDNA)	1.5	1	1.5	0	0	2	1	2	56.3%
	Quantitative characteristics of community/ target species	eDNA metabarcoding	1.5	1	1.5	0	1	2	1	2	62.5%
		qPCR/ddPCR (eDNA)	3	2	1.5	1.5	2	2	1	0	81.3%
		eDNA shotgun sequencing	1.5	1	1.5	0	0	2	1	2	56.3%
		Gene enrichment (eDNA)	1.5	1	1.5	0	0	2	1	2	56.3%
	Viable biodiversity	eRNA metabarcoding	1.5	1	1.5	0	1	2	1	2	62.5%
		Viability PCR	1.5	1	1.5	0	2	2	1	0	56.3%
		eRNA shotgun sequencing	0	1	1.5	0	0	2	1	2	46.9%

(Continued)

TABLE 3 | Continued

Biosecurity application	Biodiversity information required	Relevant eDNA/eRNA based methods	Feasible, precise, repeatable	Comprehensible	Quantitative	Standardized	Cost-efficient	Early warning	Low impact	Cross-applicable	Overall performance
Baseline surveys	Species inventories (pres/abs)	eDNA metabarcoding	1.5	1	0.5	0	1	1	2	2	69.2%
		eDNA shotgun sequencing	1.5	1	0.5	0	0	1	2	2	61.5%
Early (post- border) detection	Species inventories (pres/abs)	eDNA metabarcoding	1.5	1	0.5	0	1	3	2	2	73.3%
		eDNA shotgun sequencing	1.5	1	0.5	0	0	3	2	2	66.7%
	Target species (pres/abs)	End-point PCR (eDNA)	3	2	0	1	2	3	2	0	86.7%
		qPCR/ddPCR (eDNA)	3	2	0.5	1	2	3	2	0	90.0%
Hub monitoring	Species inventories (pres/abs)	eDNA metabarcoding	1.5	1	0.5	0	1	3	2	2	73.3%
		eDNA shotgun sequencing	1.5	1	0.5	0	0	3	2	2	66.7%
	Target species (pres/abs)	End-point PCR (eDNA)	3	2	0	1	2	3	2	0	86.7%
		qPCR/ddPCR (eDNA)	3	2	0.5	1	2	3	2	0	90.0%
		Gene enrichment (eDNA)	1.5	1	0.5	0	0	3	2	2	66.7%
Assessment of population status	Species inventories (pres/abs)	eDNA metabarcoding	1.5	1	0.5	0	1	2	2	2	71.4%
		eDNA shotgun sequencing	1.5	1	0.5	0	0	2	2	2	64.3%
	Target species (pres/abs)	End-point PCR (eDNA)	3	2	0	1	2	2	2	0	85.7%
		qPCR/ddPCR (eDNA)	3	2	0.5	1	2	2	2	0	89.3%
		Gene enrichment (eDNA)	1.5	1	0.5	0	0	2	2	2	64.3%
Impact assessment	Abundance and relative abundance of NIS/ target species	eDNA metabarcoding	1.5	1	1.5	0	1	3	2	2	70.6%
		qPCR/ddPCR (eDNA)	3	0	1.5	1	2	3	2	0	73.5%
		eDNA shotgun sequencing	1.5	1	1.5	0	0	3	2	2	64.7%
		Gene enrichment (eDNA)	1.5	1	1.5	0	0	3	2	2	64.7%

Weighted scores from performed assessment are given for each method/application combination and each considered criterion. The highest performance scores for each criterion are emphasized by gradient cell shading (green color), the blue data bars visualize the overall benchmarked performance.

2011; Donaldson et al., 2014; Zhang et al., 2016) are considered least-to-non-destructive. The latter, however, have limited use for biosecurity applications as they cannot accurately identify most taxa, and are therefore not applicable for characterizing biodiversity and identifying the dispersive stages of potential pests.

The non-destructive nature of molecular surveillance methods has been highlighted in studies of endangered or protected species (Foote et al., 2012; Rees et al., 2014). Although NIS are not protected species, it is important to note that their efficient detection using traditional surveillance methods might require extracting substantial portions of accompanying native biota. This can be a particularly big issue in fragile ecosystems such as marine reserves or protected areas, like coral reefs and polar sanctuaries (Gutt, 2001; Leray and Knowlton, 2015).

Compliance Scores for Cross-Applicability Criterion

End-point PCR (eDNA and eRNA) = 0
qPCR/ddPCR (eDNA and eRNA) = 0
Viability PCR = 0
Metabarcoding (eDNA and eRNA) = 1
Shotgun sequencing and mitochondrial enrichment (eDNA and eRNA) = 1
Gene enrichment (eDNA and eRNA) = 1

Biodiversity information derived from molecular methods, even though intended for biosecurity applications, can be used to address many other questions in marine environmental research, from general biodiversity assessment and detection of spatio-temporal patterns to deducing environmental quality status for management purposes (Aylagas et al., 2014, 2016; Pochon et al., 2015a; Darling and Frederick, 2017; von Ammon et al., 2018). Target-specific methods like end-point PCR, qPCR, or viability PCR might not be widely applicable for other research and surveillance purposes. However, being more affordable for in-house implementation, they may be applicable in citizen science or local educational programmes (Ardura et al., 2015; Biggs et al., 2015).

OVERALL RESULTS AND CONCLUSIONS

As evidenced from the assessment above and resulting weighted scores (**Table 3**), the overall performance of the considered molecular methods for deriving biosecurity-relevant biodiversity information ranged from 42 to 90%. In general, target-specific tools like end-point PCR and qPCR/ddPCR scored higher for marine biosecurity applications, followed by eDNA metabarcoding which was especially applicable for deriving inventory information.

To facilitate and encourage effective uptake of molecular approaches, there is a need for an international collaborative framework aimed at unifying molecular sample processing and analysis methods for marine biosecurity applications. Given the complex range of potential biosecurity-related management questions, creating a single standardized protocol will be a challenging task. However, a universal/flexible protocol to ensure detection on different pathways, from varying habitats, and for a range of purposes could be developed through consultation with scientists and stakeholders working in this field globally.

Despite the current limitations of eDNA-based techniques, they have a great potential for deriving biodiversity information and complementing marine biosecurity programmes worldwide. For example, even in the lack of quantitativeness, biodiversity information derived from molecular analyses (e.g., community metabarcoding or target species detection with qPCR) often surpasses that from conventional approaches (e.g., microscopy and morphological assessment) in terms of taxonomic resolution, precision, and sensitivity (Zaiko et al., 2016; Fletcher et al., 2017). This information can be effectively used for identifying new arrivals at different temporal and geographic scales. Data on new arrivals can help assess introduction rates in relation to pathways and vectors of introduction, which is crucial for biosecurity management and, eventually, may be used to measure the effectiveness of legal and administrative instruments aimed at the prevention of new incursions.

Molecular techniques are advancing rapidly and it is likely that extensive scientific effort in this field will overcome many of the current caveats resulting in more robust and cost-efficient methods for use in a wide range of biosecurity applications. However, even with these advancements it is unlikely that all marine biosecurity questions will be answered by molecular methods alone. Comprehensive marine biosecurity programme should integrate complementary scientific approaches including traditional surveys, mathematical modeling, risk assessment frameworks, and molecular techniques. Effective collaboration and communication between experts working in different fields of marine biosecurity will be essential to successfully protect native marine biodiversity, marine ecosystems and associated environmental, economic, social, and cultural values.

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