Oceanography and Marine Biology

An Annual Review, Volume 61

First published 2023

ISBN: 978-1-032-42696-9 (hbk)
ISBN: 978-1-032-54845-6 (pbk)
ISBN: 978-1-003-36387-3 (ebk)

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DOI: 10.1201/9781003363873-7

The funder for this chapter Temasek Foundation

CRC Press
Taylor & Francis Group
Boca Raton London New York

CRC Press is an imprint of the
Taylor & Francis Group, an **informa** business

Oceanography and Marine Biology: An Annual Review, 2023, **61**, 293-326

Taylor & Francis

ADVANCING AND INTEGRATING 'BIOMONITORING 2.0' WITH NEW MOLECULAR TOOLS FOR MARINE BIODIVERSITY AND ECOSYSTEM ASSESSMENTS

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Abstract Global declines in biodiversity have become increasingly severe. Traditional monitoring approaches for assessing marine species distributions and abundances are time consuming, costly, and manpower intensive. Fortunately, rapid progress of sequencing technologies from first-generation to high-throughput sequencing have resulted in improvements in experimental techniques. These advances have accelerated rates of species discovery and identification, enabling community-level biomonitoring – the 'Biomonitoring 2.0' framework. Simultaneous multispecies identifications in mixed-sample pools are now mainstream with DNA metabarcoding, upscaling monitoring from the individual specimen to the ecosystem scale. In this review, we examine the progress of DNA metabarcoding over the last decade in the characterisation of marine macrobiota to microbial communities. By melding molecular techniques and more traditional taxonomic tools, this integrative Biomonitoring 2.0 approach is tailored to improve the overall effectiveness of biomonitoring. As such, we here assess its accuracy, expertise requirement, general applicability, time, cost-effectiveness, and throughput for biomonitoring. We highlight various methodological challenges that must be considered during implementation, including completeness of reference databases, representativeness of sequencing read counts for quantitative estimates, and supplementation with environmental RNA for discerning live signals from legacy DNA. Finally, we conclude with an outlook of the enhanced Biomonitoring 2.0 framework for mass adoption by ecologists and managers, as well as the prospects of emerging rapid detection technologies for ecosystem surveillance.

Keywords: Barcoding, Bioinformatics; Environmental DNA Metabarcoding; Environmental RNA; High-Throughput Sequencing; Rapid Detection; Reference Database; Taxonomic Resolution

DOI: 10.1201/9781003363873-7

Introduction

Species declines and advances in DNA sequencing

Many species are predicted to go extinct before discovery and formal taxonomic description, as a result of the ongoing deterioration in ecosystem health and worsening biodiversity declines over the recent decades (Costello et al. 2013). Biodiversity losses have been at their highest in the last decade (IPBES 2019), and the planet is facing a sixth mass extinction, the Anthropocene extinction event (Barnosky et al. 2011, Waters et al. 2016, Ceballos et al. 2020). This situation underscores the urgent need to monitor environmental responses and assess ecosystem health to take stock of extant biodiversity so as to better formulate mitigation measures aimed at protecting Earth's natural heritage, resources, and continued supply of ecosystem services. Of the 2.2 million marine species estimated globally, more than 90% remain to be discovered or are pending formal description (Appeltans et al. 2012, Mora et al. 2011). This is because the assessment of species diversity with traditional methods requires direct organism observation and skilled taxonomic expertise for accurate identification and description, which is time consuming, costly (Miller 2007, deWitt & deWitt 2008, Carbayo & Marques 2011), and increasingly more difficult as traditional taxonomic skills decline (Hopkins & Freckleton 2002, Agnarsson & Kuntner 2007, Drew 2011). Moreover, traditional bioassessments sometimes employ highly variable methods for specimen examination at different taxonomic levels, and this inconsistency can produce results that are often not directly comparable across space and time (Friberg et al. 2011). Consequently, poor documentation of marine fauna, especially in biodiverse regions (Bouchet 2006), coupled with a large backlog of undescribed species has rendered most marine species unidentifiable and still unknown to science (Mora et al. 2011, 2013). Incomplete knowledge of species diversity hinders reliable biodiversity assessments and thus limits the effectiveness of management strategies to prevent further biodiversity loss (Isaac et al. 2004).

Fortunately, molecular techniques offer an efficient and cost-effective way to increase rates of species discovery, thereby facilitating species identification with accurate taxonomic and genetic information (Hudson 2008, Wang et al. 2018). A particular example is the use of DNA barcoding, a technique that has been repeatedly demonstrated in the past two decades to be remarkably effective for species identification and discovery (Hebert et al. 2003, Hajibabaei et al. 2007, Ratnasingham & Hebert 2007, Goldstein & DeSalle 2011, Wang et al. 2018, Ip et al. 2019). Conceptually, DNA barcoding targets a standard gene region (e.g., cytochrome c oxidase subunit I, or COI, for most metazoans, Hebert et al. 2003), generating a short DNA sequence (otherwise known as 'barcode') that is matched to curated reference sequence databases containing previously barcoded sequences for species identification (Ekrem et al. 2007).

Over the last half a century, rapid advances in molecular techniques and technologies have followed the discovery of DNA structure (Watson & Crick 1953, Heather & Chain 2016). The emergence of first-generation sequencing technologies (Holley et al. 1965) led to the development of the chain termination method, or Sanger sequencing (Sanger et al. 1977), followed by next-generation (short-read, high-throughput) sequencing at the beginning of the twenty-first century. The most recent third-generation (long-read, high-throughput) sequencing uses single molecule real-time (SMRT) and Oxford Nanopore Technologies (ONT) (van Dijk et al. 2014). Together, next- and third-generation sequencing, also known as high-throughput sequencing (HTS), has recently replaced Sanger sequencing methods in many applications due to its cost-effectiveness and efficiency (Castro et al. 2020). These technological developments have expanded DNA's utility, particularly for species discovery and identification, and this is evidenced in an exponential increase in the number of DNA barcoding studies published in the last two decades (~25,000%, Grant et al. 2021) (Figure 1).

Recognising that advanced molecular tools with sequencing technologies can revolutionise species monitoring in the field of ecology, Baird & Hajibabaei (2012) introduced 'Biomonitoring 2.0' in 2012 as a novel approach, employing next-generation sequencing to gather massive amounts of

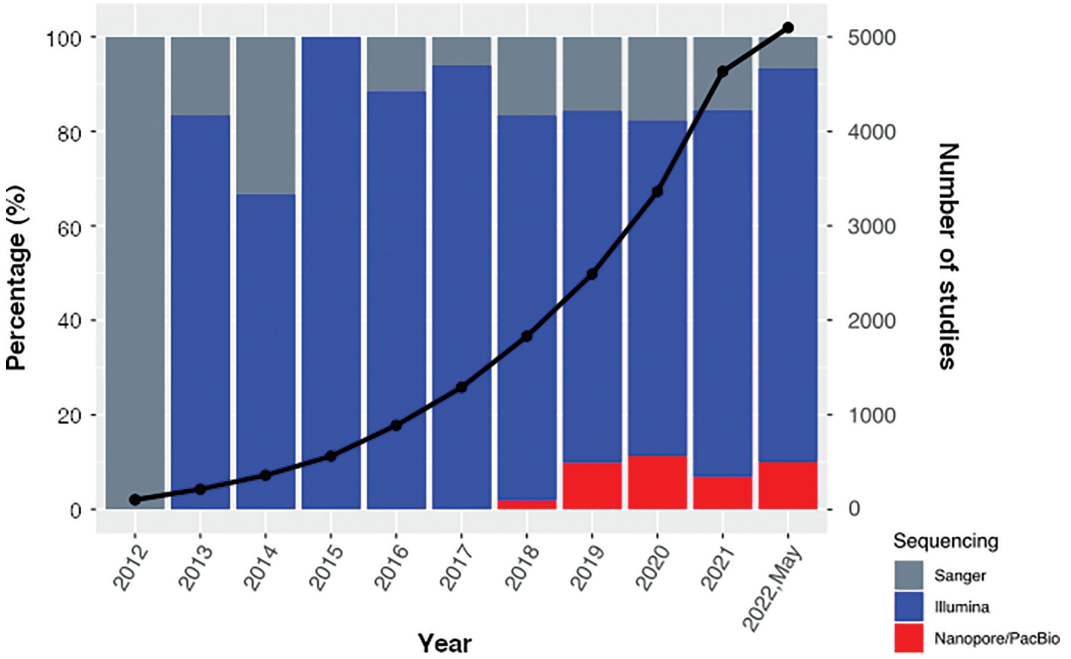

Figure 1 Line graph (right axis) showing the cumulative number of articles published in the literature compiled from Web of Science and Scopus ($n = 5079$). Stacked bar chart (left axis) showing the relative percentage of exemplar first-, second-, and third-generation sequencing strategies used in each year. Publications from years 2012 to 2022 were searched using the following keywords: marine, DNA barcoding, metabarcoding, Sanger, Illumina, iSeq, MiSeq, HiSeq, NovaSeq, Nanopore, and PacBio.

information-rich biodiversity data for studying complex environmental and ecological relationships. Following a decade of developments under the Biomonitoring 2.0 framework, it is timely to review the growing literature to highlight the current status, utility, and methodological challenges influencing the trajectory of this new era of environmental genomics. Despite the numerous advantages of utilising molecular tools for biomonitoring, there is a lack of standardised guidelines ensuring accuracy, reproducibility, and scope of use, partly due to the rapid expansion of the field. Validated genomics (DNA barcoding and metabarcoding) and accompanying bioinformatics protocols have yet to be established as most techniques are undergoing optimisation (Figures 1 and 2). Additionally, one of the most desired aspects of environmental genomics – quantification of species abundance and biomass with sequence read counts from environmental DNA (eDNA) and bulk tissue samples – remains intensely debated in terms of its precision (Kelly 2016). Since most molecular protocols utilise DNA enrichment methods, they can introduce biases downstream during molecular processing, such as during gene amplification and library preparation (Figure 2). These numerous sources of bias remain unresolved and stifle DNA sequencing's potential in becoming a staple biomonitoring tool to support management frameworks (Evans et al. 2016).

In this review, we first provide a general outline of typical DNA barcoding and metabarcoding workflows used in marine studies (Figure 2). Although there are numerous ways to sample a wide range of environmental substrates for detecting, identifying, and characterising communities of marine species, we focus on a selection of widely used methods for collecting specimens of various body sizes (microbial, meiofauna, macrofauna) and trace DNA signals (eDNA) across the water column of the coral reef environment (surface seawater, pelagic, and benthic environment). Specifically, these commonly used sampling methods collect eDNA and organisms through seawater sampling, sediment grabs or coring, plankton tows, and direct capture with traps, nets

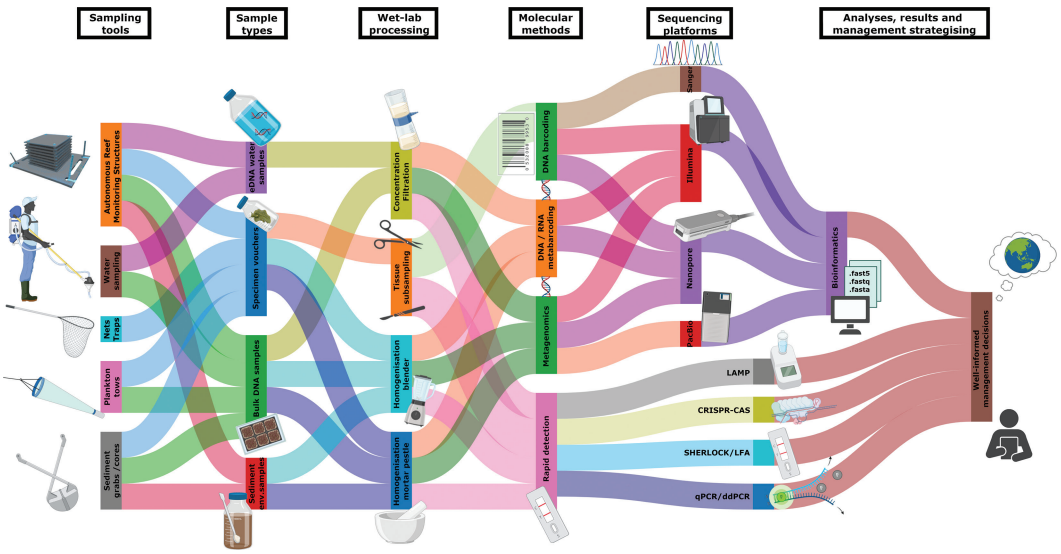

Figure 2 Overview of workflows that use common sampling tools to collect different sample types from the marine environment for molecular analyses, as well as the downstream wet-lab, molecular, analytical, and bioinformatic methods employed for species detection, identification, or community assessment of marine biodiversity. (Diagram was created with sankeymatic (https://sankeymatic.com/build/) and icons were adapted from Biorender (https://biorender.com/).)

and standardised sampling devices [e.g., Autonomous Reef Monitoring Structures (ARMS)] for molecular analyses (Figure 2). We then discuss the hurdles faced during implementation of Biomonitoring 2.0 and highlight strategies to improve accuracy and reproducibility to generate meaningful ecological results. Finally, we examine prospects for implementation of these suite of tools in an enhanced Biomonitoring 2.0 framework.

Marine biomonitoring studies and global distribution of barcoded species

All articles published from 2012 to 2022 with 'Marine' and 'DNA barcod*' or 'metabarcod*' in their title, abstract, and keywords were downloaded from Scopus and Web of Science (WoS) on 10 May 2022, and the overlapping entries between citation databases were removed (Supplementary Material 1). Subsequently, the type of sequencing technology used for each study was searched across all articles' title, abstract, and keywords, with the aim of compiling the relative proportions of sequencing technology within each of the 10 years. Next, we only selected the most popular first-, second-, and third-generation sequencing technologies by confining the search with keywords 'Sanger'; 'Illumina' or 'HiSeq', 'iSeq', 'MiSeq', 'NovaSeq'; and 'Oxford Nanopore', 'Pac* Bio*', and 'long read'. These were graphically represented by year with ggplot2 in RStudio v.1.4.1106 (R Core Team 2021).

To track the ongoing progress of marine barcoding efforts worldwide, we first obtained a complete taxonomic list of 546,847 global marine species from the World Register of Marine Species (WoRMS Editorial Board 2022). This list was filtered to retain 429,635 Animalia records that had species-level epithets. The filtered marine species list was subsequently used to search against 15 mitochondrial gene datasets downloaded from MIDORI2 web server (GenBank249 version, Longest database, downloaded 07 May 2022) (Leray et al. 2022) to compute the taxon coverage of mitochondrial barcodes for marine species available on GenBank. We then traced the metadata of the relevant accession numbers for geographic origin of the sequence, which was based on

the locality information provided by the authors who submitted sequences to GenBank. With this information, the world distribution of number of barcoded species per country and composition of barcode genes per phylum were visualised using ggplot2 in RStudio v.1.4.1106 (R Core Team 2021).

Employing DNA sequencing for species and community-level monitoring

Comparing error rates and scalabilities of common DNA sequencing platforms

Sanger sequencing produces data with the lowest error rates as compared to second- and third-generation sequencers (Table 1). It has been the gold standard for DNA barcoding (Shendure and Ji. 2008) and was the most used platform from 2012 to 2014 (Figure 1). Since 2014, HTS technologies rapidly diversified with the emergence of a variety of Illumina sequencing platforms (MiSeq, HiSeq, iSeq, NextSeq, NovaSeq) and long-read sequencers [e.g., PacBio's Sequel and ONT MinION]. The HTS data outputs are mainly classified into 1) short (≤400 bp) and accurate reads or 2) long reads (≤4 Mbp fragment size) with comparatively higher error rates (Table 1). The latter has been demonstrated to recover longer DNA barcodes with improved inter- and intraspecific resolution (Krehenwinkel et al. 2019). Most importantly, HTS platforms have the scalability to accommodate a wide range of project sizes and target taxa (Garlapati et al. 2019). As sequencing instrument sensitivities and capacities gain superiority, equally rapid advancements in computational performance and standardisation of bioinformatic analyses are needed to keep pace with the sheer amount of sequencing data produced (Langmead & Nellore 2018). These computational advances in turn prevent bottlenecks in ecological analyses and enable the timely implementation of mitigation strategies in response to anthropogenic impacts on the environment (Langmead & Nellore 2018, Mathon et al. 2021, Macé et al. 2022).

Expanding aperture of observation with Biomonitoring 2.0

Routine biodiversity assessment is necessary for managing and protecting marine ecosystems (Hampton et al. 2013, Aylagas et al. 2018). For example, long-term temporal surveillance of abundance and distribution of a wide range of taxa (micro-to-macro flora and fauna) is key to revealing the anthropogenic impacts on the environment, for instance, through tracking changes in community structure in response to rising sea temperatures, overfishing, habitat destruction, introduction of alien species, and pollutants (Aylagas et al. 2018, Hering et al. 2018, Yip et al. 2021). However, traditional approaches typically involve direct observation (e.g., benthic surveys, baited remote underwater video surveillance) and physical organism sampling (Figure 2; e.g., nets, traps, electrofishing) that are oftentimes limited in scale and scope (survey sites and target taxa). Moreover, direct observational data can make comparisons difficult as the accuracy in organismal identification varies between taxonomic practitioners and life-history stages, and may be resolved at different taxonomic levels. As such, species units could be clustered at higher taxonomic levels and the signals from species-level responses to environmental changes may be concealed, rendering biomonitoring efforts to be less effective.

Advancing genomic tools to enhance environmental monitoring can help improve understanding and management of marine ecosystems currently experiencing species decline and biodiversity loss (Dirzo et al. 2014). Deep sequencing with HTS, coupled with increased utility of bioinformatics, provides an opportunity to leverage the power of phylogenetic and population genomic approaches. Most importantly, these advancements facilitate the progression from single and multispecies traditional assessments to whole ecosystem surveillance with DNA metabarcoding (Leray

& Knowlton 2015, Miya et al. 2015, Ip et al. 2021b). Leveraging the concepts of DNA barcoding with HTS, DNA metabarcoding enables high-throughput, multispecies identifications in bulk environmental samples, with reduced reliance on taxonomic expertise for presorting (Beentjes et al. 2019, Djurhuus et al. 2018, Mauffrey et al. 2021). Limitations in scope faced by traditional sorting based on Sanger sequencing workflows can also be addressed, since organisms from all size ranges and even visually cryptic taxa can be sampled with DNA metabarcoding; this results in data that tend to be complementary and comparable with conventional surveys (Lobo et al. 2017, Cahill et al. 2018, Di Muri et al. 2020). Even as HTS has been shaping the field of molecular ecology (Baird and Hajibabaei 2012, Aylagas et al. 2016, Pawlowski et al. 2018), Sanger sequencing and morphological methods remain relevant, as taxonomic assignments of metabarcoding sequences are largely dependent on reference databases that have thus far been built primarily from Sanger-sequenced barcodes (Steyaert et al. 2020).

Nevertheless, ecosystem management approaches benefit from monitoring efforts that collect relevant ecological data consistently over extended periods of time (Compson et al. 2020). Advancements in HTS technologies are important here, as they have fuelled development of novel environmental monitoring frameworks. In particular, Biomonitoring 2.0 (Baird & Hajibabaei 2012) establishes a universal comparison scheme with DNA-based species identification for targeting a wide range of biodiversity across different ecosystems with reduced reliance on taxonomic expertise (Zhang et al. 2018, Carvalho et al. 2019, Pearman et al. 2020, Ip et al. 2022a). Since its introduction in 2012, there has been a sharp increase in marine studies published using either DNA barcoding or metabarcoding techniques for biodiversity assessment and biomonitoring (Figure 1). At least 5,079 articles were published in the last decade, of which 1,734 were from the last 1.5 years (Figure 1). This increase can be attributed to the increased user accessibility and utility of HTS, following the inverse trends of technological advances and lowering costs over time (Grant et al. 2021). Moreover, the rapid proliferation of commercial sequencing companies in the last 5 years has also contributed to the diversification of sequencing applications (Slatko et al. 2018, Singer et al. 2019).

(Meta)barcoding for community-level biomonitoring in marine ecosystems

Current studies focus on a few prominent taxonomic groups, such as indicator, keystone, foundation, megafauna, and abundant species that are easily observable or already well-studied for overall community assessments (Hermosillo-Núñez et al. 2018, Mustika et al. 2021, Seymour et al. 2020, Mendez et al. 2021). Recognising that most studies conducted so far show varying levels of taxonomic biases, the next step forward would be to ascertain which taxa are ecologically significant before expanding the monitoring scope to track these informative taxa that are often overlooked by observers (Carvalho et al. 2019, Seymour et al. 2020, Ip et al. 2022a). With the recent progress in ability of DNA metabarcoding tools for simultaneous detection of multiple species from a range of body sizes (Porter & Hajibabaei 2018c), there is great promise in quantifying compositions of microbial, meiofauna, and macrofauna communities and for comparing species relative abundances within or between environmental bulk samples (Figure 2) (Pearman et al. 2019, Gaither et al. 2022, Klunder et al. 2022, Pawlowski et al. 2022). The enhanced throughput of organism detection has triggered wide-ranging applications in a broad range of habitats that include many not amenable to traditional survey techniques, demonstrating the broad utility of DNA metabarcoding across sample types (e.g., Antarctic sediment, Fonseca et al. 2022; sedimented seawater, Ip et al. 2021b; biofilms, Rivera et al. 2022). Most field-collected samples types are suitable for metabarcoding analyses after laboratory processing, which include bulk tissue samples that have to be homogenised by blending or pestle grinding of tissue, and environmental samples with the concentration and recovery of trace DNA from water and sediment samples (Figure 2). With bulk

sample processing, DNA metabarcoding can circumvent the time-consuming specimen sorting component of traditional workflows and also detect rare or visually cryptic taxa that are typically missed by observers (Carvalho et al. 2019, Pearman et al. 2019, Ip et al. 2022a). As for environmental samples, genetic materials are being recovered from shed cells, excretions, and mucus suspended in water or sediment – also known as environmental DNA (eDNA) (Goldberg et al. 2016, Jo et al. 2022a). As such, non-invasive biomonitoring approaches utilising eDNA tools are becoming one of the most popular metabarcoding applications today as it reduces field-experimental challenges by eliminating the need for direct observation or organism capture. Hence, this allows for the indirect detection of rare, endangered, or elusive organisms that are typically challenging to survey or capture (Boussarie et al. 2018, Ip et al. 2021a, Mathon et al. 2022, Richards et al. 2022, Zainal Abidin et al. 2022). Notably, the same DNA metabarcoding techniques have also been applied in environmental RNA metabarcoding, which can further elucidate the viability of signals from active communities and quantify organismal responses to environmental changes (Marshall et al. 2021, Ankley et al. 2022, Zaiko et al. 2022).

Enhancing Biomonitoring 2.0 with new molecular tools

Integrating molecular techniques and taxonomic tools

Typical traditional monitoring methods such as direct sampling, organism capture, camera trapping, and visual census (Figure 2, Wong et al. 2018, Lim et al. 2020, Taira et al. 2020) are susceptible to various sampling limitations, including destructive sampling, site inaccessibility, observer biases, and overlooking neglected taxa. They also tend to focus on conspicuous groups (Pearman et al. 2018, Ip et al. 2022a), leading to high levels of false negatives, and overall, inaccurate community assessments and biodiversity estimates.

An integrative approach with molecular techniques complementing traditional methods enhances the efficiency of monitoring marine biodiversity (Chang et al. 2022a, Czachur et al. 2022, Wang et al. 2018, Ip et al. 2019, Richards et al. 2022). Instead of starting with conventional morphological identification, a reverse workflow approach would mean molecular methods are first used to identify specimens by matching them to databases of previously identified and barcoded species. This can accelerate the process of species discovery and identification, since it eliminates the prerequisite of involving trained taxonomists in species monitoring efforts (Wang et al. 2018). Even for undescribed species with no reference barcodes, the reverse workflow can help rapidly sort specimens into molecular operational taxonomic units (MOTUs) based on sequence similarity, and subsequently, direct follow-up morphology-based identification work.

Notwithstanding these advantages, appropriate experimental designs are crucial for effective species detection with molecular techniques. It must be emphasised that pilot experiments are needed to assess feasibility of study designs and to optimise methods (Furlan et al. 2016, Goldberg et al. 2016) since there is no single template procedure suitable for processing every sample type while targeting all species (Barnes & Turner 2016, de Souza et al. 2016). Furthermore, methodological biases can potentially be introduced at every experimental stage, from sample collection, gene amplification, sequencing to data analysis, which could result in erroneous ecological inferences (Gold et al. 2022, van der Loos & Nijland 2021, Zinger et al. 2019). Prevailing considerations in study designs include minimalisation of potential biases across all steps of the workflow, optimisation of the bioinformatics pipeline, selection of appropriate gene region(s) of interest, taxon coverage of barcoded genes in reference databases, as well as managing contamination and misidentification (Gold et al. 2022, Martins et al. 2021, Richards et al. 2022, van der Loos & Njiland 2021, Zaiko et al. 2022).

Sample collection, fixation, and processing

Although there are many different techniques for sample collection, we focus on five commonly used sampling tools as depicted in Figure 2. The type of specimens collected with these sampling tools range from water, sediment, bulk to specimen vouchers, each of which are typically processed distinctly (Figure 2). Downstream molecular methods are relatively consistent following the sample processing stage, but the type of final data output for ecological analyses is largely contingent on the specific research question (Figure 2). This may either involve sequencing on different platforms or employment of rapid detection technologies without sequencing involvement (Figure 2).

DNA barcoding studies focus primarily on biodiversity assessments using DNA-based identification of collected specimens. They typically engage in tissue subsampling for molecular analyses and preserve whole specimen vouchers for morphological identification. Hard-bodied organisms, such as invertebrates with shells (molluscs) or exoskeletons (arthropods), are preserved as vouchers in 70% ethanol; while soft-bodied organisms like polychaetes, fish, and flatworms are often fixed in formalin (Vivien et al. 2018). It is noteworthy that formalin-fixed tissues are suboptimal for DNA sequencing (Hahn et al. 2022, Raxworthy et al. 2021). Nevertheless, formalin fixation ensures minimal loss of key features for morphology-based taxonomic work, while subsamples from expendable body parts are used to generate high-quality DNA barcodes tagged to properly identified vouchers.

Furthermore, DNA metabarcoding is employed by studies with the aim of simultaneous identification of multiple taxa or characterisation of community composition from mixed-sample pools and bulk samples. Environmental bulk samples are collected by hand (e.g., surface water sampling with sterile plastic bottles), with a horizontal (e.g., Van Dorn) or vertical (e.g., Niskin) water sampler for seawater sampling, and a grab or corer is used for sediment sampling (Figure 2). Since environmental DNA signals in seawater can be distinct between sampling depths (DiBattista et al. 2019, Jeunen et al. 2020, Monuki et al. 2021, but see Ip et al. 2021b), working understanding of the target species' biology is key for determining the appropriate seawater sampling depth. For instance, detecting pelagic species requires sampling of the mid-water column while collecting seawater near the seabed can improve the detection of benthic species (Antich et al. 2021a). Vacuum or peristaltic pumps are typically used to concentrate genetic material from the seawater on to a porous membrane (via ultrafiltration) for downstream molecular processing. For sample preservation, filter membranes can be dry-frozen, stored at -20°C in ethanol or cell-lysis buffers (e.g., Longmire's solution, Sarkosyl buffer), desiccated using silica beads or with self-preserving filter membrane housing units until further work is conducted (Thomas et al. 2019, Williams et al. 2016, Mauvisseau et al. 2021).

As for sediment subsampling, it is done by collecting sediment with sterile spatulas or syringes from the surface and the centre of the grab or core sample, so as to ensure sample integrity while avoiding vertical admixture (Lins et al. 2021, Pawlowski et al. 2022). The subsamples are stored in 100% molecular grade ethanol or dimethyl sulfoxide (DMSO) solution and frozen till further processing (Ransome et al. 2017). Notably, preprocessing of samples may be required before seawater filtration or sediment sampling. Depending on the nature of the sample, prefiltering of turbid seawater samples with larger pore-sized membranes prevents clogging of filters and can help reduce amplification inhibition in downstream molecular processes, but also risks decreasing DNA yield (Stoeckle et al. 2017, Hunter et al. 2019). Similarly, sieving sediment samples that may contain the larger body-sized macrofauna helps to isolate these larger animals present within the sediment (Aylagas et al. 2018, Gielings et al. 2021), which could otherwise contribute disproportionately more DNA to the mixed-sample pools and mask signals from other rare taxa during metabarcoding.

Lastly, studies that utilise standardised sampling devices, e.g., Autonomous Reef Monitoring Structures (ARMS, Leray & Knowlton 2015), are focused on the sampling of the reef matrix, which can generate many different sample types, such as meio- and macrofauna specimen vouchers, bulk samples of encrusted material, and sediment and seawater environmental samples. As such, ARMS require a combination of wet-lab processing techniques to collect and preserve the samples

(Leray & Knowlton 2015), which include preprocessing with serial sieving (<2 mm mesh size) of meio- and macrofauna for tissue subsampling and fixation (Ransome et al. 2017), collection of sediment from encrusted plates for homogenisation (Aylagas et al. 2016), and filtering of containment seawater for the concentration of genetic material (Figure 2, Aylagas et al. 2016, Ip et al. 2021b, Nichols et al. 2022).

Choice of gene markers and taxonomic resolution

The same primers used for DNA barcoding of single species specimens can be applied for DNA metabarcoding assays that target the same gene loci for species delimitation. Critically, slight modifications must be made to the 5′ terminus of the primer sequence to render it suitable for DNA metabarcoding. The addition of a short (8–9 bp) and unique oligonucleotide sequence distinguishes between sets of primers used on different samples in the same assay, thereby permitting sample multiplexing during sequencing (Meier et al. 2016). These uniquely tagged primers are assigned to individual or mixed samples and must not overlap with one another so that downstream demultiplexing or bioinformatically sorting and assigning of sequences back to their respective sample 'bins' can occur.

Nevertheless, the selection of gene regions for (meta)barcoding is closely associated with the availability of published molecular markers and the robustness of reference barcode databases for providing sufficient resolution in species identification. It is imperative to target the most suitable or well-represented gene locus for barcoding or metabarcoding assays since the downstream analyses hinge on the species delimitation resolution that the gene marker provides. To maximise the barcoding gap that separates intra- and interspecific variation (Meyer & Paulay 2005), in silico experiments can identify gene regions with adequate variation for unambiguous species delimitation, while ideally being flanked by conserved regions to allow primer binding for polymerase chain reaction (PCR). Therefore, mitochondrial gene fragments like COI (26.6% of marine metazoan GenBank sequences), 12S rRNA (11.8%), and 16S rRNA (19.3%) are common target loci since these are relatively well represented in reference databases (Figure 3) and sufficiently variable for species delimitation in many taxa (Thomsen et al. 2012, Kelly et al. 2014, Valentini et al. 2016, Sigsgaard et al. 2020, but see Anthozoa (Huang et al. 2008, McFadden et al. 2011), Annelida (Sun et al. 2012), and Platyhelminthes (Vanhove et al. 2013)). To ensure meaningful taxonomic assignments of sequence data, emphasis must be placed on having comprehensively curated and updated sequence databases, which can also provide feedback on the specificity of the assay design. Ideally, a local database specific to the study area should be established to improve the precision of sequence matches (Bucklin et al. 2021, Dugal et al. 2022, Gold et al. 2021, Ip et al. 2019, Keck & Altermatt 2022).

Even when a gene marker offers species delimitation resolution, incomplete reference sequence databases can result in the barcodes not matching with species-level epithets, while primer mismatches and poor binding affinities can also lead to false negative detections. Intuitively, sequences without species names may seem meaningless, for instance, in conservation or invasive species monitoring programmes (Locatelli et al. 2020). However, for studies focused on overall diversity estimation and community-level responses to environmental changes, they remain relevant as the putative species units from taxonomy-free approach becomes the common denominator for comparisons of poorly studied, rare, and morphologically cryptic organisms across various habitats (He et al. 2019, Rodrigues et al. 2021). Furthermore, poor primer binding affinities due to mismatches of priming sequences between different species could lead to false negative detections, especially when the same universal primers designed for single-specimen DNA barcoding are used for bulk or environmental metabarcoding (Deagle et al. 2014, Ip et al. 2021b). Primer mismatches also inevitably occur due to varied binding affinities of primers to different species' template DNA in mixed-sample pools (Piñol et al. 2015, Piper et al. 2019, Ip et al. 2021b), potentially resulting in

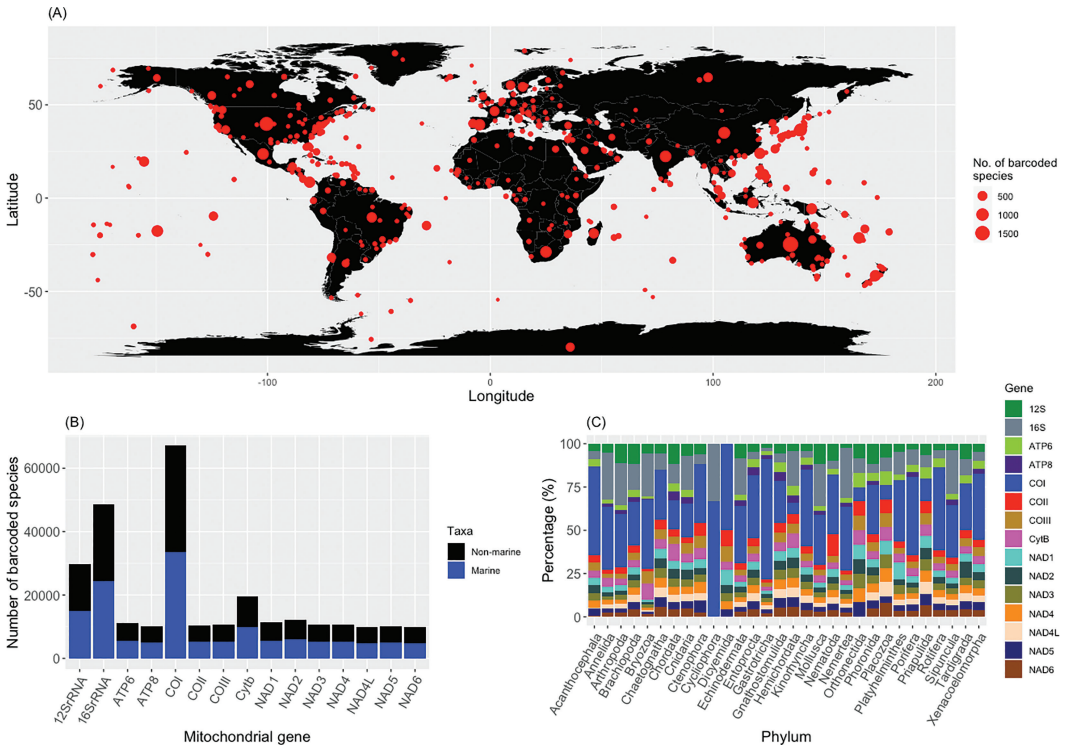

Figure 3 (A) Global distribution of the 37,223 marine animal species with mitochondrial DNA barcodes, based on author-submitted locality metadata from GenBank Release 249, consolidated by MIDORI2. (B) Stacked bar chart showing numbers of mitochondrial barcoded marine and non-marine species for each gene. (C) Stacked bar chart showing the relative frequencies of the 13 mitochondrial protein-coding and 2 ribosomal RNA barcodes available for all marine taxa.

amplification biases. A multimarker approach would thus help circumvent these issues, as it could recover species diversity more inclusively or precisely, and reduce false negative detections from primer binding incompatibilities or barcode underrepresentation in reference databases. Notably, the multilocus approach is also contingent on barcode representation on global databases. For instance, there exist 37,223 marine animal species with mitochondrial DNA barcodes, according to MIDORI2 (GenBank Release 249) (Figure 3). Among the 13 mitochondrial protein-coding and two ribosomal RNA loci from across 31 marine animal phyla, the top three most well-represented loci are COI (13.3–69.7%), 16S (11.2–33.3%), and 12S (11.3–11.9%). Since the representation is much poorer for the remaining mitochondrial genes (0.05–12.6%), studies designing multilocus experiments should target at least COI, 16S, or 12S (Figure 3).

Target amplicon size in relation to sequencing platform

Different sequencing platforms return different read lengths (Table 1), which in turn can impact taxonomic resolution (Krehenwinkel et al. 2019, Latz et al. 2022, Martijn et al. 2019, Yeo et al. 2020). For example, with Illumina platforms, the maximum possible barcode length that can be generated with minimal error rates is up to 500 bp, which means that primers must be designed to target gene regions of less than 400 bp (Table 1). Since the performance of most HTS platforms is limited to sequencing shorter fragments, universal DNA barcoding markers like the 658

Table 1 Comparison of performance, yield, error rates, and costs between Sanger and five representatives of high-throughput sequencing platforms (Chang et al. 2020a, Dohm et al. 2020, Quail et al. 2012, Shendure & Ji 2008, Stoler & Nekrutenko 2021)

		Short-read platform			Long-read platform	
	Sanger	Illumina Miseq V2/V3	Illumina HiSeq 4000/2500	Illumina Novaseq 6000 S2	ONT MinION	PacBio Sequel II
Sequencing cost per sample (USD)	~$18	~$30	~$7/~$20	~$4	~$6	~$10
Manpower for preparation	Low	Medium	Medium	Medium	Low	High
Maximum read length	1–2-kbp	151 bp/251 bp	151 bp/251 bp	151 bp	~2 Mbp	~100 Kbp
Throughput	Single consensus barcode per sample	8.5 Gbp/15 Gbp	1500 Gbp/300 Gbp	1250 Gbp	20 Gbp	160 Gbp
Error rate	~0.001%	0.473%	0.112%	0.109%	R9.4.1: ~6% R10.3: ~4%	13%
Run time	~4 hours	56 hours	84 hours	56 hours	0.5–72 hours	15 hours

bp region introduced by Folmer et al. (1994) are incompatible for paired-end sequencing. Instead, only mini-barcoding markers like the 313 bp fragment by Leray et al. (2013) are feasible with HTS. Contrastingly, Sanger-based DNA barcoding can target gene regions of up to 1–2 kbp. While shorter fragments can be amplified and sequenced more easily (Cruaud et al. 2017), the longer amplicons typically have higher discriminatory power to resolve closely related species (Krehenwinkel et al. 2019, Latz et al. 2022, Martijn et al. 2019).

The third-generation ONT's MinION sequencer's long-read capabilities can bypass some of the utility shortcomings of both Sanger and Illumina technologies for DNA barcoding and metabarcoding (Pomerantz et al. 2018, Krehenwinkel et al. 2019). However, the MinION's single flow cell capacity has a relatively low throughput of ~50 Gbp. Only ONT's GridION or PromethION platforms can comfortably replicate Illumina's sequencing yield (Table 1), which have higher theoretical maximum outputs of 250 Gbp – 14 Tbp from being able to accommodate multiple flow cells (5–48) simultaneously for sequencing (Pervez et al. 2022). Indeed, such applications will be less restricted by the amplicon length output with longer-read technology, which shifts the focus to planning considerations concerning sequencing depth and the nature and state of samples. On the one hand, degraded samples are expected to have more fragmented DNA and targeting shorter fragments (still >150 bp) likely increase detection successes (Wainwright et al. 2018, Choo et al. 2021). On the other hand, fresh tissue samples can be targeted for longer barcodes (or full-length genes even, Günther et al. 2022) to attain a more unambiguous delimitation of species (Quek et al. 2019, 2021, Chang et al. 2022a). As such, a suite of sequencing technologies can be assembled to suit each study's needs through the review of sample DNA quality (fresh or degraded) or funding available.

Decontamination and replication

Contamination is a major challenge for molecular approaches as it can potentially skew results and generate erroneous conclusions (Goldberg et al. 2016, Hansen et al. 2020). These are not limited to laboratory settings and can also arise during fieldwork and equipment assembly. Clean, aseptic

practices must be implemented at all stages of wet-lab work to reduce contamination risks (Schweiss et al. 2020). Disinfection protocols range from UV irradiation, autoclaving, rinsing with distilled water, soap water, enzymatic treatment with DNases, and wiping down with bleach to inactivate any residual biological materials. Overall, 10% household bleach and the use of single-use, sterilised and DNase-treated consumables are strongly encouraged for ensuring decontamination (Goldberg et al. 2016, Ip et al. 2021a). Besides rigorous disinfection protocols, it is critical to include negative controls at every stage of the experiment to facilitate identification of contamination sources and prompt rectification procedures (Goldberg et al. 2016, Williams et al. 2019), such as taking additional steps in lab cleaning or bioinformatic filtering (Barba et al. 2014). Most importantly, these negative controls must be processed the same way as samples (Goldberg et al. 2016).

Biological replicates are repeated collections of samples from the same sampling site. They are critical for estimating diversity and determining the frequency of species occurrences, especially for rare taxa (Bessey et al. 2020, West et al. 2020). Technical replicates are subsamples generated from within the same biological sample (Fonseca 2018). Increasing the number of technical replicates can help improve detection consistency and confidence as well as enhance likelihood of detecting rare taxa (Ficetola et al. 2015). On one hand, a cumulative approach of combining signals across replicates reduces false negative detections of rare taxa; while on the other hand, a minimum threshold approach increases statistical confidence of species detection, e.g., deemed a true signal if detected in >50% of the replicates (Van den Bulcke et al. 2021). Nevertheless, considerations must be made regarding the rise in costs and labour intensity with larger numbers of replicates. As such, it is recommended that future studies conducting DNA metabarcoding minimally use three technical replicates, and species present in at least two replicates can be more confidently interpreted as a true biological signal (van der Loos and Nijland 2021).

Bioinformatics

Today, a myriad of open-source, command-line programs (Table 2), such as OBITools (Boyer et al. 2016) and DADA2 (Callahan et al. 2016), can capably handle the analyses of large biodiversity datasets containing billions of sequence reads in a short time. The bioinformatic processing of sequences is the last step of any barcoding or metabarcoding workflow (Figure 2) in which the different ways that the sequencing data are processed and analysed with varied filtering thresholds can influence the final detection outcomes of target species (Mathon et al. 2021). It is noteworthy that strict quality filtering thresholds are intuitively useful for removing erroneous sequences for downstream analyses, but caution must be exercised regarding the over-elimination of reads, which can lead to false negatives of rare taxa.

Most HTS data are generated via paired-end sequencing, and they have forward and reverse sequence reads. The first bioinformatics step is to begin with the merging of forward and reverse reads using a minimum Phred quality score and base overlap threshold (Ewing et al. 1998),

Table 2 List of open-source bioinformatic program examples for DNA (meta)barcoding analyses

Name	Web resource	Type	Reference
OBITools	https://pythonhosted.org/OBITools/welcome.html	Command line	Boyer et al. (2016)
DADA2	https://benjjneb.github.io/dada2/index.html	Command line	Callahan et al. (2016)
eDNAFlow	https://github.com/mahsa-mousavi/eDNAFlow	Command line	Mousavi-Derazmahalleh et al. (2021)
FROGS	http://frogs.toulouse.inra.fr/	GUI	Escudié et al. (2018)
QIIME2studio	https://docs.qiime2.org/2019.4/interfaces/q2studio/	GUI	Bolyen et al. (2019)
SLIM	https://trtcrd.github.io/SLIM/	GUI	Dufresne et al. (2019)
TaxonTableTools	https://github.com/TillMacher/TaxonTableTools	GUI	Macher et al. (2021)

which are determined by the user examining the sequencing run profile on the FASTQC program (Andrews 2019). Since barcoding and metabarcoding libraries are multiplexed with large numbers of samples for cost-effectiveness on HTS platforms, the next step involves sorting or assigning sequences back to their respective samples by using the unique oligonucleotide tags (8–9 bp, Bohmann et al. 2022, Ip et al. 2021a,b, see also Section on Choice of gene markers and taxonomic resolution) at each read's 5′ terminal ends for demultiplexing. The adapters, unique multiplexing tags, and primer sequences are also removed during the demultiplexing step. Subsequently, quality filtering removes sequencing errors, retains sequences with the correct fragment size and a minimum number of read counts for statistical confidence (Mathon et al. 2021). Next, clustering with arbitrary thresholds [e.g., maximum 3% difference in bases for COI sequences (Hebert et al. 2003); 2% difference for 12S V5 (Riaz et al. 2011)] groups highly similar sequences together into putative species units or MOTUs. This application of 'universal' clustering thresholds assumes a distinct gap between inter- and intraspecific variation but, in actuality, can vary considerably among taxa (Collins & Cruickshank 2013). Alternatively, users can avoid the use of arbitrarily employed thresholds and retain unique sequences, such as in the form of amplicon sequence variants (ASVs) (Callahan et al. 2016) or zero-radius operational taxonomic units (zOTUs) (Edgar 2016b). The generation of ASVs is a denoising technique as erroneous sequences are removed and retained sequences are distinguishable by a single nucleotide difference (Eren et al. 2013). Compared to clustering of sequences into MOTUs using arbitrary thresholds, the utility of ASVs confers advantages in allowing finer distinction of sequences, experimental reproducibility, and comparison across different studies. It is noteworthy that the generation of MOTUs and ASVs involves different sequence processing approaches that may influence inferences in biodiversity assessments. Nevertheless, both methods have produced largely consistent biological conclusions (Glassman & Martiny 2018), with a few studies recommending that ASVs are more suitable for recovering microbial diversity (Chiarello et al. 2022). Moreover, emerging studies have suggested that both sequence processing methods are more complementary than equivalent (Antich et al. 2021b, Cholet et al. 2022, Schloss 2021), with the incorporation of both approaches into COI metabarcoding bioinformatic pipelines highly recommended (Antich et al. 2021b). Lastly, sequence alignment and similarity searches with BLAST allow the taxonomic assignment of ASVs or MOTUs through the matching of the query sequences to the most similar references on the sequence database (Altschul 1990).

Despite being the staple for ecological analyses of HTS data, bioinformatics can be overwhelming for new users, as the required skillsets are distinct from the technical expertise of conventional molecular ecologists. In such instances, the bioinformatics work can either be commercially outsourced or unfamiliar users can consider employing Graphical User Interface (GUI) programs (Table 2), which will have broad user applicability since coding proficiency is not required. This breaks down the complexity of command-line scripting and paves the way for new users to analyse their own DNA metabarcoding data.

Managing misidentification

Misidentification during taxonomic work is inevitable, since the accuracy in species identification is contingent on a multitude of factors like the taxonomist's proficiency, quality of taxonomic keys (details can be lost in translation rendering them incomplete), and the preservation state of the specimen (Bush et al. 2019). DNA-based methods can potentially be advantageous in scenarios where they can reduce taxonomic misidentifications and complement conventional morphological tools (DeSalle 2006). In particular, DNA barcoding allows more consistent species identification of taxa that have been formally described and previously barcoded with markers possessing adequate species delimitation resolution (Wang et al. 2018). However, DNA-based methods are not without flaws and remain largely dependent on reliable morphological identifications, suitable marker choice,

and robustness of the reference barcode databases (Hleap et al. 2021). Critically, a specimen that is taxonomically misidentified will have an incorrect species name tagged to the DNA barcode in global reference databases, and such instances have been routinely uncovered (Liu et al. 2017, Porter & Hajibabaei 2018c). This will cause chain misidentifications when following studies search and BLAST query sequences against erroneous reference sequences (Leray et al. 2019, Locatelli et al. 2020).

We recommend, where available, to also match query sequences to alternative reference databases that may be smaller but better curated. For instance, the Barcode of Life (BOLD) can be used in conjunction with GenBank for more accurate matching of COI sequences (Ratnasingham & Hebert 2007). Moreover, BOLD has advanced its ability to automate and organise batch sequence searches with BOLDigger (Porter & Hajibabaei 2018a, Buchner & Leese 2020), and the recent emergence of curated reference databases like MIDORI2 (Leray et al. 2022) and PR2 (Guillou et al. 2012) can collectively address the misidentification challenges, thereby increasing the accuracy of DNA-based species identification. Curated reference databases are not limited to COI sequences – marine eukaryotes barcoded at other gene loci like the nuclear 18S have the curated PR2 database for sequence matching. Lastly, curated databases like MIDORI2 and PR2 have results output formats that are compatible with Bayesian or taxonomic classifier programs like CONSTAX2 (Liber et al. 2021), RDP classifier (Wang et al. 2007), and SINTAX (Edgar 2016a). Classifier programs provide statistical support for every level of taxonomic classification of the query sequences, raising identification confidence and reducing the likelihood of barcode misidentification.

Challenges and prospects of implementation

Reference databases

The effectiveness and efficiency of DNA barcoding and metabarcoding are dependent on reliable taxonomic assignment by matching to curated databases with robust representation of species sequences. However, the problem of incomplete databases that have insufficient taxonomic resolution for a large portion of published DNA sequence data remains unaddressed (West et al. 2021, Rourke et al. 2022). Improvements in reference sequence matching and primer resolution are urgently needed. The former requires enhancing the completeness of reference databases, since most sequences from metabarcoding studies remain unassigned at the species level. For instance, COI barcodes with matches at <97% to reference sequences or >97% to database records without species epithets are considered 'unidentified' and only used as unnamed assemblages or putative species for coarse community assessments (Ip et al. 2021b). While single-gene Sanger barcoding has helped build sequence databases and should continue to be so, high-throughput technologies for the rapid barcoding of specimens (Srivathsan et al. 2019, Chang et al. 2020a, 2022a) or assembly of mitogenomes (Quek et al. 2019, 2021, Chang et al. 2022b) may in certain cases exponentially expand reference databases (Porter & Hajibabaei 2018b), although DNA barcoding and species identification of marine organisms in many geographic areas are currently still lacking (Figure 3A). More importantly, complementary morphology-based taxonomic work should be a requirement for barcoded specimens to be assigned species names before submission of DNA barcodes to reference databases. This measure would reduce the proportion of database entries with inaccurate or imprecise names, and saves the time and effort needed to correct erroneously tagged specimen vouchers and barcodes.

Species identification based on mitochondrial DNA barcoding is feasible for most marine animals as several gene loci (12S, 16S, COI) are adequately variable and possess a distinct barcoding gap for species delimitation (Meyer & Paulay 2005). However, there are a few marine taxa that are either difficult to barcode due to unspecific binding of universal primers or there is a lack of a barcoding gap in the commonly targeted barcode regions for identification. For instance, serpulid

calcareous tubeworms (Annelida) are challenging to amplify for COI due to poor primer binding affinities (Sun et al. 2012), while species delimitation is ineffective with the COI gene for Platyhelminthes (Vanhove et al. 2013) and Anthozoa (Huang et al. 2008, McFadden et al. 2011). Nevertheless, taxon-specific primers or the use of multimarker approaches targeting different gene regions can help distinguish closely related taxa and collectively increase the overall resolution of taxon delimitation (Pearman et al. 2018, Ip et al. 2022b), addressing both DNA barcoding and metabarcoding issues associated with the lack of barcoding gap, amplification biases, and insufficient primer delimitation resolution. Fortunately, improved markers are continually designed for barcoding anthozoans in the COI region (McFadden et al. 2011, Nichols & Marko 2019, Shinzato et al. 2021), while barcoding alternate mitochondrial loci such as 12S, 16S, mitochondrial protein-coding gene (msh1), COI intergenic region (igr1), or nuclear gene regions, such as 18S, 28S, and internal transcribed spacers (ITS), have led to improved resolution for delimiting flatworm and coral species (Afiq-Rosli et al. 2019, Ip et al. 2022b, Vanhove et al. 2013). However, some of these alternative mitochondrial gene loci for most marine phyla remain poorly represented (Figure 3B and C), highlighting an urgent need to ramp up efforts for more barcoding work or mitogenomic skimming and assembly. Furthermore, non-specific amplification can be circumvented with blocking primers or bioinformatic filtering to remove non-target DNA and sequencing reads in downstream analyses, which would enhance species detection success (Piñol et al. 2015, Huggins et al. 2020, Rabbani et al. 2021).

Read counts for quantitative estimates

An intensely debated topic involves the utilisation of metabarcoding sequence data for quantitative estimates of species abundance (Kelly 2016), which is highly informative for mapping home ranges and distribution patterns (Barnes & Turner 2016), among others. Critically, raw read numbers for each species may not be directly proportional to its biomass or abundance (Evans et al. 2016, Kelly 2016). Studies have been divided in their findings, for instance, Lamb et al. (2019) found a weak relationship between eDNA read counts and organism biomass, but Li et al. (2021) reported otherwise for large-sized organisms' eDNA. While some groups have shown the incongruence between the amount of sequence reads and absolute abundance of morphological data (Evans et al. 2016, Kelly 2016), others have reported congruent patterns between molecular (relative abundance) and morphological derived abundance data for marine plankton, diatoms, fish, and invertebrates (Abad et al. 2016, Aylagas & Rodríguez-Ezpeleta 2016, Kimmerling et al. 2018, Vasselon et al. 2018, Hoshino et al. 2021). Strong positive correlations between read counts and relative species abundances have also been demonstrated while tracking ecologically significant events like mass spawning (Bista et al. 2018, Tillotson et al. 2018, Rourke et al. 2022, Ip et al. 2022b) and characterising spatiotemporal dispersal patterns of fish larvae (Kimmerling et al. 2018). In particular, Nichols and Marko (2019) highlighted the potential for inferring eDNA sequence abundances for reef coral cover estimates, although West et al. (2022) warned against applying read count data for this purpose, especially at more diverse sites. Moreover, Yates et al. (2019) found stronger positive correlations in *ex situ* experiments, but failed to replicate the same trends with *in situ* experiments. These inconsistencies in read counts for abundance inferences can be attributed to the use of PCR for metabarcoding, which generate biases by impairing the proportional relationship between the quantity of DNA pre- and post-amplification (van der Loos & Nijland 2021). Additionally, different metabarcoding primers may perform differently with the template DNA in mixed-sample pools (Hajibabaei et al. 2019), and primers with fewer mismatches with the target loci of template DNA generally yield more reliable quantitative results (Piñol et al. 2019).

These limitations have motivated the normalisation of read count data to be used as a rough index for abundance measures (Lawson Handley et al. 2019, Laporte et al. 2021), allowing for inferences of relative, rather than absolute, abundances. Accordingly, a multimarker approach using

species-specific primers (Beng and Corlett 2020, van der Loos & Nijland 2021) and read count normalisation (Laporte et al. 2021) is necessary for reducing potential biases from PCR and primer choice. PCR-free and shotgun sequencing can also eliminate PCR biases, and these have also successfully demonstrated positive correlations between read counts and abundance estimation (Bista et al. 2018, Ji et al. 2020). These precautionary alternatives appear effective, as more than 90% of DNA metabarcoding studies in the past 2 years have demonstrated positive correlations between normalised read counts and relative taxon abundances (Rourke et al. 2022). Future studies can employ Hellinger transformation of read count data (Laporte et al. 2021) or include internal standard DNA for copy number correction (Ushio et al. 2018) to improve the reliability of species abundance inferences.

Environmental RNA for differentiating between living and dead organisms

Environmental DNA have been shown to mostly exist in the environment as 1–10 μm sized particles (Barnes et al. 2021, Jo et al. 2022a), suggesting that eDNA from macroorganisms are likely subcellular tissue fragments and intracellular DNA from shed cells (Moushomi et al. 2019). As such, eDNA is frequently used as a proxy for detecting organism presence, although shedding and decay models have shown high variability in eDNA degradation and persistence profiles between different species and environment types (Harrison et al. 2019, Zhao et al. 2021). Despite few studies reporting short persistence times of less than 8 hours with dead eDNA (Ely et al. 2021), the majority have highlighted that one of DNA metabarcoding's key limitations is its inability to differentiate between the living and the dead in environmental samples, resulting in false positive results as the detected organisms could have been dead or inactive at the point of sample collection (Jo et al. 2022b, Pochon et al. 2017, Marshall et al. 2021). Environmental RNA (eRNA) has been proposed as an alternative to complement eDNA-based monitoring programmes (Giroux et al. 2022, Greco et al. 2022, Yates et al. 2021), and recent studies have reported eRNA's ability to distinguish between living and dead (legacy) signals (Giroux et al. 2022, Greco et al. 2022, Pochon et al. 2017, Marshall et al. 2021). Conceptually, because RNA is physiochemically more unstable and easily degradable compared to DNA, it does not remain for long in the environment (Jo et al. 2022b, Kagzi et al. 2022). Therefore, eRNA is expected to provide more a reliable 'time stamp' for eDNA-detected signals (Jo et al. 2022b, Pochon et al. 2017, Kagzi et al. 2022). However, this may not always be the case as Wood et al. (2020) found that eDNA and eRNA shared similar half-life profiles. Depending on the target organism's physiological state, there could also be prolonged eRNA detection due to upregulation of selected genes in response to environmental stimuli (Cristescu 2019, Jo et al. 2022b, Ikert et al. 2021). Nevertheless, the majority of the studies that have compared eRNA and eDNA performances have presented evidence that eRNA results are more precise in recognising only signals from living organisms (Giroux et al. 2022, Greco et al. 2022, López-Escardó et al. 2018, Miyata et al. 2021).

Despite being a viable alternative to eDNA metabarcoding, the laborious nature and logistical challenges of eRNA experiments (extra steps in preservation and wet-lab processing) obstruct its widespread applicability in research and routine monitoring programmes. Due to the unstable nature of RNA, proper preservation of bulk and environmental samples for RNA work is more demanding, including field requirements, such as flash freezing with liquid nitrogen, or specialised and costly preservatives like RNAlater (Invitrogen) to maintain RNA integrity between sample collection and storage (Passow et al. 2019). If these logistical challenges can be addressed, the concurrent use of eRNA and eDNA tools will surely advance the feasibility of genomic tools for environmental monitoring (Von Ammon et al. 2019, Greco et al. 2022, Marshall et al. 2021). Most importantly, eRNA is highly relevant for studies that need to determine the viability of detected signals, such as for invasive species management and endangered species monitoring (Sepulveda et al. 2020, Farrell et al. 2021, Veilleux et al. 2021).

Advancing field-based molecular applications

Despite the performance advantages of HTS, the molecular processes are nonetheless restricted to lab-based settings, while data generation and bioinformatic analyses remain limited by sequencing run times and computational power (Table 1). Recently, portable- and non-sequencing-related innovations have enabled *in situ* field molecular experiments, accelerating the rate of biodiversity assessments (Figure 2) (Baerwald et al. 2020, But et al. 2020, Chang et al. 2020b, Doi et al. 2021). Field-based applications eliminate the need for storage and transport to the laboratory as processing work can be done *in situ*, thereby increasing efficiency and output through time savings (Chang et al. 2020b, Doi et al. 2021) and extending the range (distance and time) of sampling expeditions (Baerwald et al. 2020). Clearly, field-ready equipment and reagents are needed, such as BentoLab, miniPCR™, and Genie® for molecular processes like DNA extraction and PCR. MinION sequencer, two3™, and Genie® portable qPCR devices, commercial colorimetric and lateral flow kits are required for field sequencing analyses and visualising data output (Baerwald et al. 2020, But et al. 2020, Doi et al. 2021). Unless using equipment that includes a mini-centrifuge, e.g., BentoLab, reagents should preferably require minimal centrifugation in the field, such as the QuickExtract™ (Lucigen) for DNA extractions, Recombinase Polymerase Amplification (RPA, TwistDx) reagents for isothermal DNA extraction/amplification, and AMPure XP (Beckman Coulter) magnetic beads for clean-up procedures (Chang et al. 2020b). While the MinION sequencer, Genie®, and two3™ qPCR thermocyclers allow real-time analyses of high-throughput sequence and species detection data without reliance on a laboratory (Doi et al. 2021, Pomerantz et al. 2022), we note that these are sophisticated equipment. It may also be challenging to visualise data or explore bioinformatics in depth in the field.

User-friendly applications without the need for electrical power, portable thermocyclers, or specialised equipment are increasingly available and will be highly advantageous for research in developing countries or remote survey sites where lab-based experiments are not cost-effective or logistically feasible. In particular, loop-mediated isothermal amplification (LAMP) assays with colorimetric kits (But et al. 2020, Porco et al. 2022) and clustered regularly interspaced short palindromic repeats technology's (CRISPR) specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) method with lateral flow devices (LFA) (Baerwald et al. 2020) are available for easy data visualisation, although these are currently appropriate only for species-specific detection rather than whole-community analyses. SHERLOCK-LFA is a remarkably sensitive detection tool that has been highly successful as a non-invasive molecular tool for field-based species identification (Baerwald et al. 2020), but its potential for targeted species detection in bulk or environmental DNA metabarcoding studies has not yet been explored. Nevertheless, given the recent successful optimisations of eDNA detection of important food fish species with CRISPR from water samples (Williams et al. 2019, 2021), we would also expect SHERLOCK-LFA to replicate such application successes in environmental monitoring in the near future. These ongoing developments are exciting prospects for field molecular techniques to further enhance biomonitoring efficiency, biodiversity assessments, species discovery, documentation, and diversity estimation (Creer et al. 2016, Deiner et al. 2017, Hering et al. 2018, West et al. 2021).

Research gaps and future priorities

The immediate priorities for enhancing the ecological applications of Biomonitoring 2.0 are to extend the utility of the molecular tools for robust species monitoring, continue testing the feasibility and innovating more reliable solutions for using sequencing reads to estimate abundances of target organisms. Recent developments in the field have seen expansions in the scope and range of species monitoring. This is evident with the increasing number of studies administering the Biomonitoring 2.0 framework and conducting molecular work outside of lab-based settings in the

field (Chang et al. 2020b, Pomerantz et al. 2022). Molecular processes have also been streamlined to be more user-friendly, so that amateur and citizen scientists can also contribute to species monitoring efforts (Larson et al. 2020, Miya et al. 2022).

Although species abundance is crucial for numerous ecological applications, the inference of relative abundances from sequence read counts remains a challenge (Pawlowski et al. 2018). Currently, there is no straightforward remedy for this sequencing quantification and abundance estimate issue. We acknowledge these limitations and recognise that it is technically impractical to infer absolute abundance, and only relative abundance estimates may be credible from standardised and quality-controlled sequencing read count data. As such, the most reliable utility of sequencing data for ecological applications remains at determining species presence or absence, characterising community composition, determining multispecies site occupancy, inferring rough relative abundances, as well as evaluating food webs and species co-occurrences (Kang et al. 2022, Lawson Handley et al. 2019, Valdivia-Carrillo et al. 2021). Future work interested in using sequencing read counts for relative abundance measures has to consider correction factors in their experimental designs for both the wet- and dry-lab components. To improve the accuracy of relative abundance estimates, internal positive controls (Ushio et al. 2018), application of quantitative sequencing techniques (Hoshino et al. 2021), and normalisation or transformation of read count data during bioinformatic analyses need to be considered and tested (Lawson Handley et al. 2019, Laporte et al. 2021, Rourke et al. 2022).

Conclusions and outlook

Recent advancements in molecular laboratory processes, DNA sequencing technologies, and bioinformatics have all engendered integrative genomic approaches that can be leveraged for species discovery to keep pace with and even outpace the rate of defaunation and extinction (Dirzo et al. 2014). DNA barcoding and metabarcoding have been increasingly applied in the last decade to establish biodiversity baselines (Figure 1), which can be used to flag environmental changes and allow prompt implementation of management measures (Figure 2). Consequently, these approaches have proved highly effective in expediting species discovery and identification, demonstrating capabilities in the rediscovery of locally missing taxa, flagging potential new records, and drawing focus on neglected marine species diversity. Besides updating biodiversity estimates, optimised genomic approaches have immense potential for investigating food web interactions and studying organism responses to climate change and other anthropogenic impacts.

The growing number of threatened species worldwide (Davidson & Dulvy 2017) calls for cost-effective and reliable methods for mapping species distributions and diversity to inform management decisions. Fortunately, a decade's worth of progress with Biomonitoring 2.0 has demonstrated numerous innovative applications for effective species monitoring of marine habitats. The number of DNA barcoding and metabarcoding applications continues to rise (Figure 1), and with the limitations continually being addressed, we foresee that genomic tools for large-scale environmental monitoring will continue to be optimised, streamlined, and eventually mainstreamed. The foundational role of database assembly with DNA barcodes of properly identified, vouchered specimens for biodiversity studies cannot be neglected (Chang et al. 2020a). Therefore, more work is needed to expand and upgrade reference databases for robust sequence matching (Porter & Hajibabaei 2018b, Figure 3), which in turn will help improve interpretation of molecular data and draw more meaningful ecological conclusions.

The apparent trends in reduced sequencing costs and rise in sequence data yield will drive expansion and diversification of ecological applications (Grant et al. 2021), requiring novel study approaches. As such, the experimental challenges are seeing a shift from sequence throughput to bioinformatics analyses and drawing biological inferences from the increasingly complex

molecular data output. Biomonitoring 2.0 has created a novel form of 'vouchers', where raw sequence data and nucleic acid extracts from environmental samples can be considered as 'community-level vouchers' that should be viewed as analogous to the museum-archived specimen vouchers. These materials are frozen in time as they store a historical snapshot of the community, which can be revisited for establishing baselines and time series in future studies. While the sequencing technological improvements have motivated advances in computational biology for effective data analyses, other molecular techniques have also been developed to simplify analytical processes while accomplishing similar research or surveillance goals, including quantitative and digital droplet PCR (qPCR and ddPCR, respectively) (Schweiss et al. 2020, Kwong et al. 2021, Yip et al. 2021), LAMP (But et al. 2020, Porco et al. 2022), and SHERLOCK-LFA (Baerwald et al. 2020).

Biomonitoring 2.0 has demonstrated broad applicability for a wide range of environmental research. Importantly, it has enhanced the scale and scope of genomic research for numerous ecological applications. Capitalising on the current technological trajectory, which is centred on upscaling data throughput and automation, and in lockstep with the rapid progress in genomic approaches, reference database expansion, and ecological modelling, Biomonitoring 2.0 will continue to push the field towards new frontiers (Cordier et al. 2019, Compson et al. 2020, Havermans et al. 2022).

Acknowledgements

We would like to thank the World Register of Marine Species for making their data available to us. This research is supported by the Temasek Foundation under its Singapore Millennium Foundation Research Grant Programme. Lastly, we thank the reviewers and editors for comments and suggestions that have helped improve this manuscript.

Author contributions

Y.C.A.I. and D.H. conceived the idea for the manuscript. Y.C.A.I. wrote the draft with contributions from J.J.M.C. and D.H. Data were collected by J.J.M.C., while Y.C.A.I. conducted the analyses and constructed figures with inputs from D.H. and J.J.M.C. All authors read and approved the final version of the manuscript and declared no conflicts of interest.

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Supplementary Materials are provided online at: https://www.routledge.com/ 9781032426969