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DNA Sequencing as a Tool to Monitor Marine Ecological Status

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Many ocean policies mandate integrated, ecosystem-based approaches to marine monitoring, driving a global need for efficient, low-cost bioindicators of marine ecological quality. Most traditional methods to assess biological quality rely on specialized expertise to provide visual identification of a limited set of specific taxonomic groups, a time-consuming process that can provide a narrow view of ecological status. In addition, microbial assemblages drive food webs but are not amenable to visual inspection and thus are largely excluded from detailed inventory. Molecular-based assessments of biodiversity and ecosystem function offer advantages over traditional methods and are increasingly being generated for a suite of taxa using a "microbes to mammals" or "barcodes to biomes" approach. Progress in these efforts coupled with continued improvements in high-throughput sequencing and bioinformatics pave the way for sequence data to be employed in formal integrated ecosystem evaluation, including food web assessments, as called for in the European Union Marine Strategy Framework Directive. DNA sequencing of bioindicators, both traditional (e.g., benthic macroinvertebrates, ichthyoplankton) and emerging (e.g., microbial assemblages, fish via eDNA), promises to improve assessment of marine biological quality by increasing the breadth, depth, and throughput of information and by reducing costs and reliance on specialized taxonomic expertise.

Keywords: metagenetics, metagenomics, metabarcoding, eDNA, marine biological quality element (BQE), good ecological status (GES), biodiversity and ecosystem function (BEF)

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MONITORING THE MARINE SYSTEM: SUPPORTED BY LEGAL OBLIGATION

Marine and coastal systems provide a variety of important ecosystem services, such as food, recreation, employment, medicine, and regulation of waste, disease, and climate (Liquete et al., 2013). However, marine ecosystems and transitional waters (e.g., coastal areas, estuaries, lagoons, fjords) are increasingly stressed by multiple and often interconnected factors, such as overexploitation, chemical and nutrient pollution, pathogens, harmful algae, and hypoxia. With an estimated two-thirds of the human population living in or near coastal areas

(Millennium Ecosystem Assessment, 2005), coastal population growth contributes ecosystem pressure, with virtually no marine areas unaffected by human influence (Halpern et al., 2008). In addition, sources of degradation such as elevated CO_2 and temperature threaten ecosystem integrity and the capacity for marine ecosystems to remain productive (WHCEQ, 2010; NOC, 2013; Rogers, 2013; Halpern et al., 2015).

To maintain ecosystem services, many countries develop legal and policy frameworks to guide sustainable use of marine resources (Pereira et al., 2013). Examples include the United Nations Convention on the Law of the Sea, Australia's Oceans Policy, the Canada Oceans Act and Oceans Strategy, the United States Oceans Act of 2000 and National Ocean Policy Implementation Plan, the European Union Marine Strategy Framework Directive (MSFD), and the South African National Water Act. A main objective of such policies is maintenance of good ecological status (GES) in marine waters, habitats, and resources using integrated or "holistic" approaches (Borja et al., 2008, 2016; Karsenti et al., 2011; Duffy et al., 2013; Danovaro et al., 2016). For example, an Ecosystem-Based Management (EBM) approach to marine resources considers a suite of natural physical, chemical, biological, geographic, and climatic factors in context of anthropogenic activities and impacts. The goal is to protect and maintain natural ecological function while delivering ecosystem services and societal benefits (Levin et al., 2009; Elliott, 2011).

Marine monitoring and impact assessment programs are developed to respond to sustainability requirements. Such programs may evaluate marine ecological quality to inform management actions, such as establishment of harvest guidelines, habitat and species conservation plans, and setting of requirements and practices to minimize pollutants and invasive species. In many cases, long-term monitoring programs evaluate the demographic status and trends of various marine populations (Borja et al., 2010 and references therein). Holistic approaches require integrated monitoring and assessment of both abiotic and biotic parameters, including multiple species (Arkema et al., 2006; Day et al., 2008; Curtin and Prellezo, 2010; Möllmann et al., 2014) and, ideally, multiple trophic levels to capture Biodiversity and Ecosystem Function (BEF) relationships (Strong et al., 2015).

CURRENT LIMITATIONS IN MARINE ECOLOGICAL QUALITY ASSESSMENT

Most evaluations of marine ecological status rely on biological quality element (BQE) assessments, such as monitoring of invertebrates, fishes, or phytoplankton. Assessment of BQE populations and/or ecological relationships can be translated into straightforward classifications of ecosystem status (e.g., report cards) and provided to stakeholders, management authorities, and policy makers. However, traditional BQE monitoring poses a number of drawbacks, as outlined in **Table 1**.

One drawback of traditional BQE assessments is reliance on morphological taxonomy. This causes a bottleneck in sample throughput because manual sorting and visual identification is labor-intensive and slow. Identification demands a high degree of specialized taxonomic knowledge, such expertise is required for each BQE target separately, and juvenile and cryptic species can nonetheless be misidentified (Bourlat et al., 2013; Aylagas et al., 2014, 2016; Pawlowski et al., 2014; Carugati et al., 2015; Thomsen and Willerslev, 2015; Bowers et al., 2016; Bucklin et al., 2016; Danovaro et al., 2016). Estimates suggest that between 24 and 98% of marine eukaryotic species are yet to be described (Leray and Knowlton, 2016). In addition, the quantification of early life stages for stock assessment purposes, for example ichthyoplankton eggs and larvae (Harada et al., 2015) may not accurately predict life history or therefore the future adult population, which is the target of management action (Lewis et al., 2016).

The inability to inventory microbial assemblages is another drawback of typical monitoring approaches. Microbial species dominate the ocean numerically and the majority of marine primary production is microbial in origin, produced by cyanobacteria and microalgae (Duarte and Cebrian, 1996; DeLong, 2009; Amaral-Zettler et al., 2010). Microorganisms may act as sentinels of system ecological status because they respond rapidly to natural and anthropogenic environmental pressures in terms of diversity, physiology, and function (Mock and Kirkham, 2012; Nogales et al., 2011; Mock et al., 2015). They hold promise as ecosystem indicators with regard to measures of biodiversity, toxic species, pathogens, and metabolic properties that indicate ecosystem health, such as biodegradation capacity and resistance to metals and antibiotics (Ininbergs et al., 2015; Tan et al., 2015; Caruso et al., 2016). The ocean microbiome, an assemblage of bacteria, archaea, microeukaryotes, and viruses (Stulberg et al., 2016), is the heart of marine food webs. Although the need for food web assessments in marine ecosystem status is recognized (Zampoukas et al., 2014; Caruso et al., 2016), microbes are not readily amenable to visual identification (Bowers et al., 2016; Giner et al., 2016), hindering inclusion as a BQE in monitoring frameworks. Instead, marine monitoring programs are often restricted to gross measurements such as chlorophyll to capture this critical segment of the ecosystem.

Ecosystem assessments include a suite of biotic and abiotic measurements. The call for integrated monitoring recognizes that isolated BQE measures may not capture ecological status adequately (**Table 1**) and instead should include multiple taxonomic groups (e.g., animals, protists, bacteria) and life stages (e.g., for fish: eggs/larvae, juveniles, adults) (Aylagas et al., 2016; Thompson et al., 2016; Trivedi et al., 2016). However, harmonization is challenging (Simboura et al., 2005; Borja et al., 2014). The costs and complexity of integrated assessment can limit spatio-temporal scope and hinder the ability to realize an ecosystem approach (de Jonge et al., 2006; Vince et al., 2015).

DNA SEQUENCING: A SOLUTION FOR OCEAN ASSESSMENT

From the perspective of cost and sample throughput, DNA sequencing offers a variety of advantages to marine monitoring and assessment programs compared to time-consuming visual

TABLE 1 | Summary of traditional method shortfall vs. benefits to DNA sequencing approaches.

Traditional method shortfalls	Explanation	DNA sequencing benefits
Reliance on morphotaxonomic expertise	Identification of each taxon relies on the expertise of specialized technicians in the face of declining availability to train new experts, yet diagnostics are still hampered by cryptic species, undifferentiated morphology, and damaged specimens.	Sequencing approaches do not rely on traditional taxonomic expertise, life stage, or intact specimens (although a curated reference library is needed, which requires initial collaboration with taxonomists to establish). In contrast to traditional taxonomy, workforce training in molecular and bioinformatic methods is growing.
Life stage limitations	Traditional methods rely on the species abundance of specific taxonomic groups, frequently ignoring early life stages.	Sequencing approaches can identify morphologically undifferentiated organisms (e.g., eggs, larvae, juveniles), expanding assessments.
Lack of microbial/food web assessment	Standard biotic measures do not provide information on microbial ecology, which drives the ocean productivity that supports ecosystem services.	Molecular approaches are well-developed for marine microbial assemblages, enabling improved assessment of food webs, a foundational component of an ecosystem.
Limited trophic information	Assessments may not include multiple trophic levels.	Multiple trophic levels can be assessed ("microbes to mammals").
Fail to assess biodiversity and ecosystem function	Fails to capture Biodiversity and Ecosystem Function (BEF) relationships. Data may be limited to physico-chemical measurements with biotic data indirectly inferred.	Information on taxonomy and metabolic potential, can identify key enzymes and markers of biogeochemical cycles, biodegradation, antibiotic or metal resistance, etc.
Slow throughput, high cost	Labor intensive, requires the services of multiple specialized technicians to identify each taxonomic group.	Costs for high-throughput sequencing continues to decline.
Ecosystem scale-up issues	Limited sampling in heterogeneous marine systems is performed under assumption of homogenous conditions, creating difficulties when ecological quality classification is up-scaled to the whole system.	Low per-sample costs coupled with progress in automated bioinformatic pipelines holds promise for high sample throughput, allowing higher resolution sampling and resulting in a more representative assessment.

inspection (**Table 1**). High-throughput sequencing allows faster and more accurate species identification and decreases dependence on morphological taxonomic expertise (Bourlat et al., 2013; Aylagas et al., 2014). Advantages extend to taxa traditionally monitored in marine assessment programs, such as invertebrates and fish, investigated either as individuals or assemblages (Ardura et al., 2013; Bourlat et al., 2013; Carugati et al., 2015; Harada et al., 2015; Tan et al., 2015; Zimmermann et al., 2015; Aylagas et al., 2016; Borja et al., 2016; Caruso et al., 2016; Danovaro et al., 2016; Lewis et al., 2016; Thompson et al., 2016). Information about higher trophic levels also can be gleaned from DNA extracted from filtered seawater via capture of sloughed or excreted cells, rather than direct extraction from tissue (Foote et al., 2012; Kelly et al., 2014; Kelly, 2016). Such work is buttressed by genomic-based studies which continue to advance, in part, by increased access to and application of DNA sequencing technologies (Cammen et al., 2016; Leslie and Morin, 2016). Declining costs encourage sequencing as an alternative to traditional biodiversity monitoring (Bourlat et al., 2013) and supports continued advances in conservation biology (Wallace et al., 2010; Hancock-Hanser et al., 2013).

Although information derived from DNA sequencing is yet to be formally included in current marine status assessment programs (Bourlat et al., 2013), there is widespread recognition of the importance of this approach, and an increasing number of projects (**Table 2**) generate sequence-based biodiversity assessments. For example, molecular approaches were found to be more time-efficient compared to visual census (Yamamoto et al., 2017) and were successfully used to identify nonindigenous species in marine waters and to retrieve taxa from benthic samples that were not identified by morphological analysis (Zaiko et al., 2015; Aylagas et al., 2016). Progress moving molecular assessments into formal monitoring is being made as evidenced by adaptation of the AZTI Marine Biotic Index (AMBI), determined by traditional manual sorting and visual identification of benthic macroinvertebrates, to an index based on genetics (gAMBI) (Aylagas et al., 2014, 2016). Success with this approach was extended to bacterial assemblages (Aylagas et al., 2017).

DNA sequencing addresses the challenges of microbial assemblage inventory. Limitations of visual identification and culture methods drove microbial ecology to become an early adopter of molecular methods (Giovannoni et al., 1990; Handelsman, 2004; Venter et al., 2004; Rusch et al., 2007). Sequencing technology and bioinformatics revolutionized the study of marine biology, providing new insights into how the "hidden majority" (Rappé and Giovannoni, 2003) mediates cycles of carbon, nutrients, oxygen, metals, and toxins and responds to ecosystem change. The field is now sufficiently mature to be considered in routine marine monitoring efforts. For example, DNA sequencing could be used to monitor marine food webs, as explicitly called out in the EU Marine

TABLE 2 | Examples of projects promoting molecular-based biodiversity assessment.

Project	Methods employed	Aims
BioMArks biomarks.eu	Metabarcoding	Develop taxonomically curated DNA barcode reference database for unicellular eukaryotes (protists) and bioinformatics and statistical tools to "provide a complete toolbox for modern, cheap, and accurate biomonitoring of marine eukaryotic biodiversity."
Consortium for the Barcode of Life (CBOL) www.barcodeoflife.org	Barcoding	Construct a richly parameterized barcode reference library as a global standard for identification of biological species.
DEVelopment Of innovative Tools for understanding marine biodiversity and assessing good Environmental Status (DEVOTES) www.devotes-project.eu	Metagenetic vs. traditional methods	Development of analysis pipelines complementary to traditional tools that translate genomic data into indicator metrics to be used by stakeholders.
Earth Microbiome Project (EMP) earthmicrobiome.org	Metagenetic (16S V4 region)	Massively collaborative effort to characterize microbial life across the globe, including marine biomes.
FishPopTrace https://fishpoptrace.jrc.ec.europa.eu	Sequencing/gene expression/proteomic	Develop a wide range of traceability tools for assigning fish and fish products back to their origin population.
Global Genome Initiative (GGI); Global Genome Biodiversity Network (GGBN) ggi.si.edu; ggbn.org	Barcoding/metabarcoding/metatranscriptomic	Endeavors to capture and understand the Earth's genomic biodiversity with emphasis on sample archival.
International Census of Marine Microbes https://icomm.mbl.edu/	Metagenetic (V9 region)	Inventory of marine microbial diversity inclusive of bacteria, archaea, protists and associated viruses.
Marine Biodiversity Observation Network (MBON) www.marinebon.org/	Metagenetic/metagenomic/eDNA	Work includes development and application of standardized molecular methods to elucidate the role of marine microbes to connect biodiversity and ecosystem function.
Bioplatforms Australia - Marine Microbes bioplatforms.com/marine-microbes	Metagenetic/metagenomic	Investigation of changes in the diversity of Australian marine microbes from multiple environments including seawater, sediment, sponges and seagrass.
Marine Microbial Eukaryotic Transcriptome Sequencing Project (MMETSP) marinemicroeukaryotes.org	Metatranscriptomic	Provide a significant base for integrating microbial eukaryotes into marine ecology generating functionally annotated, and publicly available transcriptomes.
Moorea Biocode; mooreabiocode.org	Barcoding	Catalogue specimens, photographs, and DNA sequences for species in marine, freshwater, and terrestrial habitats on the island of Moorea, French Polynesia.
Ocean Sampling Day (OSD), Marine Microbial Biodiversity, Bioinformatics, Biotechnology (MicroB3) www.microb3.eu	Metagenetic (16S V4, 18S V4 and V9)/metagenomic	A snapshot of 16S, 18S, and marine metagenomic sequences taken on the same day across the globe to promote standardized methods in sample and metadata collection.
Tara Oceans http://oceans.taraexpeditions.org	Prokaryotic and eukaryotic plankton V9 rDNA metabarcoding/metagenomic	Probe morphological and molecular diversity, evolution and ecology of marine plankton to explore how they are impacted by changes in the Earth's climate.

Strategy Framework Directive (MSFD) (Zampoukas et al., 2014). Examples of projects (**Table 2**) focused on microbial assemblages include Ocean Sampling Day (Kopf et al., 2015), the TARA Oceans project (Karsenti et al., 2011; Bork et al., 2015), the Earth Microbiome Project (Gilbert et al., 2014), the Marine Biodiversity Observing Network (Duffy et al., 2013; Muller-Karger et al., 2014), and Bioplatforms Australia—Marine Microbes (NCRIS, 2016).

Overall, DNA sequencing is a promising approach for integrated ecosystem assessments. Sequencing data can provide information about individuals, populations, and communities, and data can be used to understand stress responses and adaptation capacity. Such information can be distilled into ecosystem indicators and potentially integrated in ecosystem models aimed to inform people dependent on marine ecosystem services.

DNA SEQUENCING APPLIED TO MARINE MONITORING

Technical Context

DNA sequencing and analysis approaches vary widely, with opportunities to serve a variety of marine monitoring applications. Terminology usage shifts as methods change (e.g., the evolution of long-read Sanger to short-read Illumina sequencing) and varies among applications, particularly between microbial/macrobial and aquatic/benthic specialties. For example, the term "metagenomics" is defined simply and broadly as the analysis of genetic materials obtained directly from environmental samples, including analysis of sequenced amplicon (Xu, 2015). Alternatively, this broad definition is rejected in favor of keeping terminology for amplicon sequencing separate (e.g., "metagenetics" or "metaprofiling") rather than as a subset of metagenomics (Esposito and Kirschberg, 2014; Escobar-Zepeda et al., 2015; Mendoza et al., 2015; Creer et al., 2016). A similar situation exists for "environmental DNA" (eDNA) which is broadly defined in some cases (Pedersen et al., 2015). However, the term eDNA is increasingly used to connote animal DNA obtained indirectly from environmental samples rather than tissues *per se* (Bohmann et al., 2014; Kelly et al., 2014; Kelly, 2016), and that connotation is generally used here. Additional ambiguities arise from advances in sequencing technologies. For example, both Sanger (first generation) and later platforms such as Roche 454, SOLiD, and Illumina (second generation) may be referred to as next generation sequencing (NGS) in the literature, and both second and third-generation (PacBio, Ion Torrent, Oxford Nanopore) may be referred to as massively parallel or high-throughput sequencing (HTS). Other examples of terms with ambiguous usage include: conservation genomics (Garner et al., 2016), genetic fingerprinting, tag sequencing, targeted metagenomics, metabarcoding (Mendoza et al., 2015), and community analysis. With continued modifications to technology, expanding applications, and development of bioinformatic tools, terminology is expected to remain a challenge.

In generic terms, it is anticipated that DNA sequencing as a tool in routine marine monitoring will employ high-throughput sequencing coupled with a bioinformatic analysis pipeline. Sequencing may target whole genomes or specific regions that may be amplified by PCR prior to sequencing. DNA may be obtained from an individual, an assemblage of organisms ("meta" approach), or an assemblage of partial organisms (eDNA). For the purposes here, the focus is on high throughput, short-read sequencing of amplicons from macroeukaryotic assemblages (metabarcoding) or individuals (barcoding); amplicons from assemblages of maroeukaryotes obtained indirectly from the environment rather than from whole organisms or tissues (eDNA); amplicons from prokaryotic/microeukaryotic assemblages (metagenetics); and shotgun sequencing of prokaryotic/microeukaryotic assemblages (metagenomics). A variety of references regarding extraction (Cox and Goodwin, 2013; Hazen et al., 2013), amplification, and sequencing strategies for water, sediments, and tissues are available herein, and eDNA is also reviewed extensively elsewhere (Bohmann et al., 2014; Rees et al., 2014; Pedersen et al., 2015; Thomsen and Willerslev, 2015; Shelton et al., 2016); therefore, only a brief technical overview is provided here.

Sequencing of PCR-amplified marker genes is often employed to identify organisms (Patwardhan et al., 2014). This targeted sequencing approach can provide detection of species in relatively low abundance (DeSantis et al., 2006; Sogin et al., 2006; Hamady and Knight, 2009; Quast et al., 2013) and better depth of coverage compared to shotgun whole-genome approaches (Zhou et al., 2015). For example, amplification of a nuclear 16S rRNA gene segment is typical for taxonomic classification of marine prokaryotic assemblages (Klindworth et al., 2013). Amplification of 18S rRNA regions is used for a variety of communities, including phytoplankton and other microeukaryotes (Dunthorn et al., 2012; Hugerth et al., 2014; Johnson and Martiny, 2015; del Campo et al., 2016; Giner et al., 2016), and amplification of the 23S rRNA gene is used to classify organisms such as zooplankton (Hirai et al., 2015a; Bucklin et al., 2016). Amplification of mitochondrial DNA is used to identify an assortment of organisms, with cytochrome oxidase I (COI), cytochrome b, and mitochondrial 16S as examples of popularly employed target regions (Dauble et al., 2012; Pawlowski et al., 2014; Cowart et al., 2015; Guo et al., 2015; Harada et al., 2015; Johnson and Martiny, 2015; Aylagas et al., 2016; Bucklin et al., 2016; Creer et al., 2016; Leray and Knowlton, 2016; Thompson et al., 2016; Trivedi et al., 2016). Newer approaches are being used to identify fish (Miya et al., 2015) and marine mammals (Foote et al., 2012; Ma et al., 2016) from seawater samples.

In comparison to amplifying and sequencing a specific genomic region, shotgun metagenomic methods sequence broadly across entire genomes (Thomas et al., 2012; Escobar-Zepeda et al., 2015; Sharpton, 2014; Guo et al., 2016). In general, DNA extracted from a sample is fragmented, a DNA library is prepared (Head et al., 2014) using a limited number of PCR cycles or via PCR-free methods (Mendoza et al., 2015), and the assortment of random DNA fragments is sequenced. The resulting sequences can be compared directly against genomes of known taxa or can be assembled into longer segments (contigs) which can be further annotated for gene content, function, and taxonomic assignment (Segata et al., 2012; Teeling and Glöckner, 2012; Wood and Salzberg, 2014; Bengtsson-Palme et al., 2015). This approach provides a broad view because a variety of genes are detected, including those that encode metabolic enzymes and pathways. If the goal is to characterize the functional potential of an ecosystem, shotgun sequencing is desirable because whole-genome data provides information on potential metabolic function, in addition to taxonomic information (Handelsman, 2004; Thomas et al., 2012). Furthermore, shotgun sequencing is less affected by primer bias and chimeras compared to amplicon sequencing (Guo et al., 2016). However, obtaining adequate depth of coverage can be problematic (Zhou et al., 2015), motivating some studies to utilize both amplicon and whole-genome approaches (e.g., Ocean Sampling Day, **Table 2**).

Regardless of the DNA sequencing approach used, bioinformatics is critical to understand the information delivered by DNA sequencing efforts. A plethora of bioinformatic tools are available, with a complete review beyond the scope of

this article. Many bioinformatic tools and platforms are now tailored to gene sequences derived from environmental samples, particularly for prokaryotic assemblages (Kanehisa et al., 2008; Meyer et al., 2008; Caporaso et al., 2010; Abubucker et al., 2012; Thomas et al., 2012; Kozich et al., 2013; Hunter et al., 2014). Furthermore, de-novo assembly is increasingly being used to derive nearly complete genomes of previously unknown ecologically important organisms from metagenomes (Garza and Dutilh, 2015; Hugerth et al., 2015). A growing number of assemblers (Boisvert et al., 2012; Peng et al., 2012) and binning tools (Alneberg et al., 2014; Imelfort et al., 2014; Nurk et al., 2017) are available that assemble short sequencing reads into larger contigs and subsequently combine them into bins based on, for example, sequence similarity (Teeling and Glöckner, 2012). In addition to standard biodiversity metrics such as alpha, beta, and gamma diversity (Escobar-Zepeda et al., 2015; Zhou et al., 2015), network analysis tools aid visualization of complex ecological relationships (Mitra et al., 2010; Hurwitz et al., 2014).

Applications of DNA Sequencing to Marine Assessment Programs

Marine microbes were among the first communities to be explored by DNA sequencing (Giovannoni et al., 1990; Venter et al., 2004). Sequencing was initially applied to prokaryotic and microeukaryotic communities in seawater with techniques developed for these whole organisms because traditional culture methods were inadequate to describe microbial biodiversity in water and soil (Rappé and Giovannoni, 2003; Handelsman, 2004). The Global Ocean Sampling expedition helped establish DNA sequencing as a fundamental tool in marine ecosystem research (Rusch et al., 2007; Dupont et al., 2015). Potential benefits of DNA sequencing of microbial communities to marine assessment programs include characterization of food webs, assessing responses to disruption and stress, and detection of sensitive, rare, threatened, toxic, or invasive taxa (Hulme, 2006; Kudela et al., 2010; Mock and Kirkham, 2012; Lindeque et al., 2013; Muller-Karger et al., 2014; Pawlowski et al., 2014; Chown et al., 2015; Mock et al., 2015; Tan et al., 2015; Zaiko et al., 2015; Bowers et al., 2016; Bucklin et al., 2016).

Sequencing of environmental samples such as seawater is now applied in a macrobial sense across a range of trophic levels ("microbes to mammals") (Trivedi et al., 2016; Valentini et al., 2016), and approaches used to inventory individuals (Ardura et al., 2013; Harada et al., 2015; Thompson et al., 2016) are being applied to assemblages (Carugati et al., 2015; Zimmermann et al., 2015; Aylagas et al., 2016). Studies demonstrate DNA metabarcoding to be a reliable method for biodiversity assessment with potential for inferring biotic indices for marine ecosystem quality assessment (Aylagas et al., 2014; Pawlowski et al., 2014; Cowart et al., 2015; Elbrecht and Leese, 2015; Visco et al., 2015; Ferrera et al., 2016). Comparable results between molecular and traditional approaches are reported in a number of studies (Hirai et al., 2015a; Lejzerowicz et al., 2015; Aylagas et al., 2016, 2017; Valentini et al., 2016), suggesting that assessment programs that require manually intensive sorting and visual inspection will be among the first to formally integrate molecular techniques. For example, larval rockfish time-series are currently used to quantify spawning stock biomass off California, USA and are integral components of rockfish stock assessment (He et al., 2015). However, the utility of the traditional approach for counting rockfish larvae based on morphology is limited because only a few species can be visually identified to species. Recent research to improve stock assessment has genetically identified rockfish larvae collected off southern California and increased from 6 to 36 the number of species with time-series (Chen, 2017). The genetically-derived data is now being considered for integration into formal stock assessments.

Newer eDNA methods allow detection of higher trophic levels, including fish and mammals used to evaluate ecological status, from DNA extracted from sediment or filtered seawater. The eDNA approach promises information about bioindicators and commercially important or protected species without the need to collect tissue or trawl through sensitive habitats (Foote et al., 2012; Bohmann et al., 2014; Thomsen and Willerslev, 2015; Evans et al., 2016; Lacoursière-Roussel et al., 2016; Shelton et al., 2016). Some studies show promising results with regard to abundance estimates (Hänfling et al., 2016; Port et al., 2016), but others show significant differences between eDNA results and traditional tows for epibenthic macroinvertebrates (Kelly et al., 2017) and zooplankton (Hirai et al., 2015b), suggesting that these methods require further research.

Indications of Ecosystem Stress

DNA sequencing offers insight into ecosystem stress via changes in relative abundance or completeness of enzymatic pathways associated with chemical biodegradation or resistance. For example, a number of microorganisms carry genes that confer resistance to antibiotics or heavy metals. Increased exposure to anthropogenic chemicals promotes production and dissemination of these genes. Increases in the abundance of resistance genes and of integrons, genetic elements involved in horizontal gene transfer, are documented in polluted sediments (Thureborn et al., 2013). Resistance to multiple antibiotics is more frequent at polluted sites, and resistance to antibiotics and heavy metals can co-occur (Henriques et al., 2016). A number of studies demonstrate environmental gradients showing co-occurrence of anthropogenic inputs and gene occurrence in marine environments (Tacão et al., 2012; Chen et al., 2013; Thureborn et al., 2013), providing an opportunity to use individual gene markers (e.g., blaCTX-M, merA) or a full metagenomic profile to indicate pollution in ecological status assessments.

Disrupted ecosystems can also present shifts in particular taxa or overall biodiversity (Nogales et al., 2011; Mason et al., 2014; Mukherjee et al., 2017), opening the possibility to evaluate ecological status according to such metrics. Toward this goal, Aylagas et al. (2017) developed two composite indicators: (1) a sediment quality index that included organic matter content, redox potential, and metal, PAH and PCB concentrations; and (2) a bacterial index of environmental quality adapted from the AMBI. The indices were correlated, a promising result with regard to inclusion of prokaryotic assemblages into future assessment programs (Caruso et al., 2016). Furthermore, DNA

sequencing approaches are used for public health applications related to human pathogens derived from fecal contamination (Tan et al., 2015; Staley and Sadowsky, 2016) and to investigate the impacts of anthropogenic stressors on the coral microbiome (Ziegler et al., 2016; Staley et al., 2017). Kelly et al. (2016) demonstrate that eDNA in seawater can be used not only for biodiversity measurement but also for assessing anthropogenic disturbance in coastal environments. In that study, sequencing of metazoan mitochondrial 16S DNA show greater diversity of sessile taxa in more urbanized sites. Higher diversity in a disturbed site also is reported for estuarine benthic eukaryotes using 18S rRNA metabarcoding (Chariton et al., 2015). In contrast, lower diversity of benthic foraminifera is reported for sites impacted by fish farming (Pawlowski et al., 2014). Such a combination of results suggests that a sole biodiversity index may be inadequate to evaluate ecosystem status and indicates that an AMBI-like approach, which weights taxa abundance by pollution tolerance, may be needed to convert taxonomic measures into indices of ecosystem function.

HURDLES AND CHALLENGES

Standardization

While DNA sequencing holds promise for marine assessment programs (**Table 1**), improvements are required to adapt these powerful tools to formal monitoring frameworks. One of the greatest needs, cutting across all aspects of the endeavor, is the need for standardization. Standardization is particularly critical for marine monitoring applications because long-term data set generation is a hallmark of such efforts. Standard methods and practices can be applied at every stage: study design; metadata collection and curation; sample collection and preservation; DNA extraction; inclusion of control samples; approaches to amplification, library preparation and sequencing; sequence quality control and other downstream processing; bioinformatic method documentation; and depositing of sequence results with appropriate metadata.

Standardization of metadata, a minimum common set of measurements to be recorded at the time of sample collection, is critical. Adequate metadata is required to integrate DNA sequencing information into marine ecosystem assessment programs, provide environmental context to sequence data, simplify annotation, facilitate data mining, and to allow comparisons between studies. Progress is being made to create standardized protocols (Field et al., 2008; Chain et al., 2009; Gilbert, 2015; Droege et al., 2016). Efforts that promote community collaboration and participation to produce standard practices include the MIxS standard from the Genomic Standards Consortium (GSC) (Yilmaz et al., 2011; ten Hoopen et al., 2015), the Environmental Ontology (Buttigieg et al., 2013, 2016), and data standards of the Global Genome Biodiversity Network (GGBN) (Droege et al., 2016). Despite widespread recognition of its importance and the realization that subsequent data curation is costly and time-consuming (ten Hoopen et al., 2016), compliance with metadata collection and curation standards is lacking. Gold standards with regard to data analysis, applied algorithms, and reference data sets comparable to those sought for the human medical space will improve the reliability and usability of results gained from marine sequencing studies. Recent sampling efforts such as the Ocean Sampling Day (Kopf et al., 2015), TARA Oceans project (Karsenti et al., 2011; Bork et al., 2015) and the Australian Marine Microbes Project (NCRIS, 2016) mark first steps to standardize sample preparation and sampling procedures for marine microbial ecology.

Controls and Replication

An adequate set of negative and positive control samples (e.g., mock communities, both DNA and whole-cell) are needed to track DNA preservation, extraction efficiency, contamination, and sequence errors, which can significantly affect results (Zhou et al., 2015; Allen et al., 2016; Mulcahy et al., 2016; Siegwald et al., 2017). Work initiated in the area of standardizing laboratory procedures includes much-needed development of reference materials and improved DNA extraction methods, as exemplified by establishment of the International Metagenomics and Microbiome Standards Alliance (IMMSA) (NIST, 2016). Controls used to assess artifacts and bias introduced by sample preparation, PCR, and sequencing are reviewed elsewhere (Pinto and Raskin, 2012; Elbrecht and Leese, 2015; Pedersen et al., 2015; Tan et al., 2015; Zhou et al., 2015; Aylagas et al., 2016; Danovaro et al., 2016) and should be used in conjunction with standard quality control pipelines for sequence quality, including chimeric removal (Smyth et al., 2010; Teeling and Glöckner, 2012; Zhou et al., 2014; Escobar-Zepeda et al., 2015; Jeon et al., 2015). Including mock communities in DNA sequencing efforts can assess technical issues such as incomplete DNA extraction or library preparation, PCR, and sequencing errors (Schirmer et al., 2015) and provide correction factors (Tan et al., 2015; Aylagas et al., 2016). Furthermore, simulated datasets can test the performance of both sequencing technology and analysis pipelines (Bonilla-Rosso, 2015), and bioinformatic tools can be used to mask technical variability (Leek et al., 2012).

The technical variation in sequence data is compounded by the considerable temporal and spatial biological variability in marine systems. Environmental heterogeneity is both natural (e.g., salinity gradients in transitional waters) and driven by proximity to anthropogenic influences. Study design should account for expected variability and include appropriate types and amounts of biological and technical replicates to ensure that meaningful comparisons and statistical analysis, suited to the question at hand, can be provided rather than *ad-hoc* sequencing based on available budget (Prosser, 2010; Knight et al., 2012; Pinto and Raskin, 2012; Thomas et al., 2012; Zhou et al., 2015). Greater sequencing coverage can aid the ability to differentiate environmental variability, with the balance of biological and technical replicates dependent on the study questions and community complexity (Teeling and Glöckner, 2012; Zhou et al., 2015).

Taxonomic Classification and Quantification

A challenge to DNA sequencing of environmental samples is obtaining accurate taxonomic classification, particularly from short DNA sequence reads. The issue is exacerbated when

reference genomes are lacking, such as for marine ecosystems and particularly for eukaryotic and viral components (Leray and Knowlton, 2016; Roux et al., 2016). Taxonomic mis-classification of reads impacts the accuracy of abundance estimates (Aylagas et al., 2016). Lack of intercalibration between morphological and molecular methods presents an additional issue, and transferability across taxa is a concern, particularly if the initial assessment used to establish the database is limited to one or two taxonomic groups (Kelly, 2016). Although comparable molecular and traditional approaches are reported (Lejzerowicz et al., 2015; Aylagas et al., 2016, 2017; Hänfling et al., 2016; Valentini et al., 2016), other studies show discrepancies (Cowart et al., 2015; Hirai et al., 2015b; Mohrbeck et al., 2015; Giner et al., 2016; Kelly et al., 2017); therefore, it has been advised to use both approaches in a complementary fashion rather than outright substitution of morphotaxonomic approaches (Borja et al., 2008; Pedersen et al., 2015; Thomsen and Willerslev, 2015). Although such an approach may deliver a holistic perspective of marine ecological status, it would not produce the desired effect of streamlining monitoring efforts, which is needed to transition research into practice (de Jonge et al., 2006).

Bioinformatic tools to aid microbial classification include similarity based approaches (Liu et al., 2011; Menzel et al., 2016), phylogenetic placement of sequences to known reference trees (Matsen et al., 2010), and using a combination of methods (Darling et al., 2014; Dröge et al., 2015). The reliance of current methods on known sequences, however, is a major drawback, as only a fraction of marine genomes (whole genomes or marker gene regions) are sequenced to date. It is worth noting that sequences obtained from metabarcoding efforts may provide value to ecosystem assessments even if reliable taxonomy cannot be assigned to them. A biodiversity index that does not require taxonomic assignment—a reference-free approach (Mendoza et al., 2015)—could in principle substitute for existing indices targeting specific species. It is noted that diversity in terms of operational taxonomic units (OTUs) vs. strict taxonomic assignment of sequences could satisfy requirements of the EU MSFD (Danovaro et al., 2016). Several bioinformatic tools are available for microbial sequences that could aid this goal (Eren et al., 2013; Callahan et al., 2016; Amir et al., 2017). For example, the Earth Microbiome Project (earthmicrobiome.org) employed Deblur (Amir et al., 2017) to deliver a set of unique sequences with single-nucleotide resolution, rather than representative sequences delivered by OTU clustering. This strategy enabled sequences to be tracked across a variety of studies and habitats and, coupled with environmental metadata, allowed global inferences regarding microbial community structure. Patterns were not dependent on taxonomic assignments, but because exact sequences were provided, taxonomy can later be assigned as reference databases improve. Such approaches can be extended from metagenetics to metagenomics and should continue to improve as longer sequence reads delivered from high-throughput platforms become more routine (Teeling and Glöckner, 2012).

Presence/absence detection appears sufficient for certain ecological quality assessment applications (Aylagas et al., 2016), although improvements in quantification would surely extend the utility of DNA sequencing for marine monitoring purposes. Some studies report adequate estimates of relative abundance (Aylagas et al., 2014, 2016; Elbrecht and Leese, 2015; Tan et al., 2015; Thomsen and Willerslev, 2015) even though read abundance does not necessarily provide a direct correlation to organism abundance because of issues that include variations in copy number, genome size, and growth condition. Bioinformatic solutions to the issue of quantification include estimation of organism abundance based on normalized read counts of clade-specific marker genes (Segata et al., 2012; Sohn et al., 2014), polymorphisms in universal markers (Luo et al., 2015), or accounting for incorrect taxonomic classification through probabilistic models (Lu et al., 2017). Differences in sequencing depth, i.e., the number of reads obtained from an environmental sample, significantly affects results (Rodriguez and Konstantinides, 2014) and can potentially render a study unable to detect low-abundance strains. In cases in which detection or abundance estimation is critical, amplicon sequencing with deep sequence coverage coupled with quantitative PCR (qPCR) may be a workable strategy.

The challenge that residual or ancient DNA poses to quantitation, particularly in benthic environments where DNA turnover is relatively slow, is reviewed extensively elsewhere (Bohmann et al., 2014; Pedersen et al., 2015; Thomsen and Willerslev, 2015). Recent studies in aquatic systems focus on DNA decay rates to better understand the potential of, and limits to, applying eDNA to marine monitoring and stock assessment (Sassoubre et al., 2016). In some cases, qPCR of eDNA is used to address the need for quantification (Laramie et al., 2015). In other cases, qPCR alone is suggested for abundance measurements of harmful algae in marine monitoring programs (Zamor et al., 2012). Overall, overcoming and/or circumventing issues that compromise abundance estimates is a research need that requires attention on all fronts—from the first steps of sample collection and DNA extraction, to routine generation of longer sequence reads, and development of better bioinformatic tools.

Bioinformatics Infrastructure and Expertise

DNA sequences can be obtained cheaply and quickly; a decade ago a standard sequencing run returned a few thousand DNA base pairs. Now for the same cost, sequencers routinely generate hundreds of billions of base pairs in a single run. Despite the enormous opportunities posed by this technological revolution, the vast amount of data generated requires new solutions for data handling, storage, processing, documentation, visualization, and dissemination (Desai et al., 2012; Muir et al., 2016). This, in turn, drives a critical need (and current gap) for bioinformatic infrastructure and expertise. Adequate investment must be paid to obtain and maintain the tailored IT infrastructure needed to handle the increasing data volume and sophistication of processing. New developments such as cloud computing provide cost efficient solutions for decentralized storage and analysis of Big Data (Stein, 2010) while facilitating research collaborations. However, a common problem for sequencing projects is a dearth of experienced and skilled bioinformaticians. This is partly due to the rapid expansion of the field; the need has expanded faster than the ability to train scientists (Hughey and

Karplus, 2003). In addition, the skill sets needed are diverse and project-dependent. The result is that many bioinformatic tools are executed by researchers without sufficient training and knowledge of the underlying algorithms. Choosing the correct pipeline is important (Siegwald et al., 2017), and competence in bioinformatics is required to choose the appropriate analysis software from the vast number of available tools. Despite the critical importance of bioinformatics expertise to sequencing studies, bioinformaticians can lack appropriate career and development opportunities (Chang, 2015). There is a need for institutions and funding bodies to address this gap and to create attractive career paths and salary models for current and future bioinformaticians. Meanwhile, education and training opportunities are being created to close the bioinformatic workforce gap (Edwards et al., 2013; Atwood et al., 2015), creating a fundamental difference between the expertise gap in bioinformatics and the one for traditional taxonomic identity (**Table 1**).

FUTURE OF BIODIVERSITY ASSESSMENTS USING SEQUENCING APPROACHES

Although there is progress to be made, environmental sample DNA can now be routinely analyzed by high-throughput sequencing methods. Furthermore, there is growing ability to apply other 'omic approaches to environmental samples, such as metatranscriptomic, proteomic, and epigenetic analyses. Sequencing approaches empower mechanistic understanding of ecosystem dynamics and integration into marine monitoring and assessment programs will allow "environmental intelligence" (who is there, what they do, and how they are impacted by changing conditions) to be gathered with scope and detail never before available. In addition to improving routine assessment methods that rely on sorting & visual inspection (e.g., benthic invertebrates, ichthyoplankton), sequencing can provide a detailed inventory of the microbial portion of the food web. Inclusion of this fundamental component should add value to marine monitoring programs, allowing holistic ecological assessment from both a taxonomic and functional perspective. Simultaneously, eDNA approaches offer the ability to inventory higher organisms from seawater samples. Moreover, molecular approaches are amenable to integration into automated *in-situ* or remotely operated platforms (Yamahara et al., 2015; Bowers et al., 2016), further opening the possibility to increase temporal or spatial sample coverage while realizing economies compared to ship or satellite sensing. Environmental managers seek to take advantage of DNA sequencing for environmental assessment and management given potential savings in labor costs, faster sample throughput, and the relative ease of integration across trophic levels. As research science makes progress in developing techniques, standardizing practices, and demonstrating efficacy for monitoring missions, increased integration of DNA sequencing into formal monitoring programs is expected.

AUTHOR CONTRIBUTIONS

KDG was responsible for overview of DNA sequencing as a tool applied to marine ecological status monitoring. TK and LRT contributed to bioinformatics portions of the review. BD, IC, and JCM contributed to comparisons between traditional and metagenomic biodiversity assessments and application to ecological indexes. ART contributed to eDNA and fisheries perspectives.

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