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Review

Microbial, holobiont, and Tree of Life eDNA/eRNA for enhanced ecological assessment

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Microbial environmental DNA and RNA (collectively 'eNA') originate from a diverse and abundant array of microbes present in environmental samples. These eNA signals, largely representing whole organisms, serve as a powerful complement to signals derived from fragments or remnants of larger organisms. Integrating microbial data into the toolbox of ecosystem assessments and biotic indices therefore has the potential to transform how we use eNA data to understand biodiversity dynamics and ecosystem functions, and to inform the next generation of environmental monitoring. Incorporating holobiont and Tree of Life approaches into eNA analyses offers further holistic insight into the range of ecological interactions between microbes and other organisms, paving the way for advancing our understanding of, and ultimately manipulating ecosystem properties pertinent to environmental management, conservation, wildlife health, and food production.

Highlights

Microbes (bacteria, Archaea, microeukaryotes, and viruses) are highly diverse taxonomically and functionally in most environments, and as symbionts of other organisms.

Microbes are represented in eDNA/eRNA samples largely by viable cells, reflecting close association between function and taxonomy at high spatial and temporal resolution, and offering a wealth of information for inferring biotic indices and bioindicators that remains largely untapped.

Microbial environmental DNA and RNA (eNA) from signals from across the Tree of Life are complementary, capturing responses to stressors and ecological change at different spatial and temporal scales, and integrating the effects of change across the whole food web.

Holobionts and microbial consortia represent ecological units which may be more sensitive indicators of ecosystem function and health than eNA signals solely based on individual species or environmental biodiversity.

Environmental bioindicators: morphology to molecules

Bioindicators are organisms or biological processes that represent and respond to changes in environmental conditions and can serve as early warning signs of environmental disturbances, such as pollution, habitat degradation, or climate change. Biotic indices commonly applied for assessing ecological quality mostly still rely on morphological taxonomic identification of benthic macrofauna and are often limited to a narrow taxonomic range and level of resolution. A few microbial groups, for example Cyanobacteria, ammonia-oxidising Archaea, diatoms, ciliates, and foraminifera [1–3] are used in a comparable way. Many other prokaryotic and eukaryotic taxa and assemblages could be used, but with the exception of diatoms [4], have yet to be adopted into standardised/accredited protocols [5–7]. For most of these microbial groups traditional (i.e., morphology-based) assays are incapable of providing the taxonomic or spatiotemporal resolution necessary to exploit their full potential as bioindicators, and this is where the new generation of molecular approaches and **eDNA/eRNA** (= **eNA**) (see Glossary) tools offer particular promise for the future.

To provide some context for the potential transformative power that could be added to biomonitoring schemes by adopting eNA approaches, microbes constitute 70% of the world's marine biomass, and a similar proportion of living terrestrial biomass, and more than 99% of the planet's biodiversity [8]. They play key roles in cycling essential nutrients through the food web, bioremediating polluted environments, modulating environmental suitability for larger organisms, generating oxygen, and fixing carbon from atmospheric CO_2, and, by extension, even regulating the global climate [9]. Both the taxonomic and functional diversity of microbes is strongly associated with salinity, pH, temperature, and nutrient availability, providing a means to connect phylogeny, traits, and the major environmental gradients and drivers that shape the wider ecosystem [10,11]. Microbes respond rapidly to environmental change, making them ideal barometers of

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both organismal and ecosystem health [12,13] and for measuring anthropogenic impacts that are difficult or impossible to gauge using traditional techniques [14,15]. In addition to these sensitive response traits, microbes can also have powerful effect traits and can modify their environment via a range of biochemical and biophysical mechanisms [16]. This combination of strong response and effect traits makes many microbes ideal indicators of both the presence of a given stressor and also of its wider ecological impacts, and their huge biodiversity provides orders of magnitude more bandwidth than is possible for the far more limited diversity within macroorganisms for disentangling the combined effects of multiple stressors. The molecular ecology and functional diversity of bacteria in environmental samples have been extensively studied since the 1990s, principally based on 16S rRNA gene regions, and more recently using metagenomic (shotgun) and long-read sequencing techniques, and multi-omic analyses [17,18]. The functional diversity of micro-eukaryotes is less well documented [19] but represents a rich source of phylogenetic and functional information to exploit at the lower end of the eukaryotic size spectrum.

Integrating microbes into holistic eNA studies

Advances in both technical scope and scalability of eNA techniques have expanded our capabilities to survey a much wider range of organisms of all sizes, across trophic levels and the entire phylogenetic **Tree of Life** (prokaryotes, micro- and macro-eukaryotes, and, increasingly, viruses), whilst minimising detection biases due to conspicuousness or size [20]. Molecular data also enable taxonomic discrimination between very small organisms, which is often difficult or impossible using traditional methods due to their lack of easily measurable distinguishing characteristics [21]. Microbial eNA studies range from targeting individual microbial taxa to conducting kingdom-wide **metabarcoding** and **shotgun sequencing** analyses of entire microbial assemblages, resulting in comprehensive community profiles that encompass both microorganisms and macroorganisms (Table 1). The ability to view both the visible and invisible biodiversity simultaneously, and using common currencies, has only been available in the past decade or so, and easily affordable for sampling at scale within only the last few years.

eNA studies can be deployed in three main ways, targeting: (i) single-species targets (e.g., [22]), (ii) taxon-specific assemblages (e.g., [1,23,24]) and phylogenetically broader groups (e.g., [10,14,19,25,26]), or (iii) holobiont hosts and associated microbes (e.g., gut microbiomes, coral holobionts [27], and pathobiomes [28]). These types of investigation can be visualised on a triangle (Figure 1), illustrating the potential for combining their elements in holistic studies incorporating the whole Tree of Life and multiple trophic levels, spanning microbes and macroorganisms [29].

Using microbial and viral eNA for ecosystem assessment

Most ecological assessments based on microbial eNA still use PCR-based methods – targeting specific species/genera or wider phylogenetic diversity via a range of metabarcoding methods (Table 1). Bacterial metabarcoding (most commonly using regions of the 16S rRNA gene) can be used as bioindicators and early warning systems, based on differing sensitivities and taxonomic shifts under environmental change, such as climate change, pollution, and other human activities [5,6,10,12–14]. For almost every potential pollutant on Earth there are bacteria that can utilise it as a substrate, and these very specific consumer–resource relationships can provide a gateway for detecting not just the presence of a huge array of pollutants, but identifying those that are biologically active within a given ecosystem [7].

Microeukaryotic assemblage profiling (using regions of the 18S rRNA gene) is increasingly gaining recognition as an indicator system for aquatic biomonitoring: for example, recent studies have revealed shifts in protistan communities at the base of the food web associated with degraded coral

Table 1. Examples of studies including microbial, Tree of Life, and holobiont eDNA/eRNA

Title/description	Taxa	Habitat	Refs
Targeted microbe (PCR/qPCR) (individual taxa)			
High-resolution melt curve qPCR for eDNA detection of white-clawed and signal crayfish, and crayfish plague agent in water samples	*Austropotamobius pallipes, Pacifastacus leniusculus, Aphanomyces astaci*	Freshwater	[22]
Environmental DNA from multiple pathogens is elevated near active Atlantic salmon farms	Eukaryotic, bacterial, and viral pathogens	Marine	[81]
Microbial community studies (metabarcoding/metagenomics) (individual groups)			
Diatom eDNA metabarcoding and morphological methods for bioassessment	Diatoms	River (freshwater)	[1,4]
Ciliates (Alveolata, Ciliophora) as bioindicators of environmental pressure: a karstic river case	Ciliates	River (freshwater)	[2]
Benthic foraminiferal metabarcoding and morphology-based assessment around three offshore gas platforms: congruence and complementarity	Foraminifera	Marine benthos	[3]
eDNA metabarcoding to detect nematode and platyhelminth parasites in New Zealand lakes	Nematodes, Platyhelminthes	Freshwater	[24]
Conversion of a high-altitude temperate forest for agriculture reduced alpha and beta diversity of the soil fungal communities as revealed by a metabarcoding analysis	Fungi	Soil	[32]
Environmental RNA outperforms eDNA metabarcoding in assessing impact of marine pollution: a chromium-spiked mesocosm test	Foraminifera	Marine benthos	[53]
More broadly-targeted assemblages/communities (amplicon and shotgun sequencing studies)			
Bacterial 16S rRNA-based index (microgAMBI) tracking organic enrichment near aquaculture cages	Bacteria	Marine	[10]
Response of planktonic microbial assemblages to disturbance in an urban subtropical estuary	Microeukaryotes, bacteria	Estuary	[12]
Diversity and ecology of protists revealed by metabarcoding	Protists (microeukaryotes)	Various (review)	[19]
Characterisation of protist communities across varying fisheries management schemes in the Coral Triangle	Microeukaryotes	Marine	[30]
Molecular characterisation of harmful algal blooms in the Bohai Sea, China	Eukaryotic phytoplankton	Marine	[33]
Monitoring alder encroachment with metabarcoding shows changes in functional gene structures of soil bacteria	Bacteria, fungi	Soil	[35]
A multi-omics study on quantifying antimicrobial resistance in European freshwater lakes	Bacteria (AMR genes)	Lake	[36]
Changes in soil microbial communities in post mine ecological restoration	Bacteria, fungi	Soil	[45]
Microbial eukaryote diversity and activity in the water column of the South China Sea based on DNA and RNA high-throughput sequencing	Microeukaryotes	Marine	[52]
Using co-extracted eDNA and eRNA data to assess offshore oil production impacts on benthic communities	Bacteria, eukaryotes	Marine	[55]
Evaluating the role of bacterial diversity in supporting soil ecosystem functions under anthropogenic stress	Bacteria	Soil	[61]
Next-generation restoration metrics: using soil eDNA bacterial community data to measure trajectories towards rehabilitation targets	Bacteria	Soil (mine site)	[69]
Lake ecosystem robustness and resilience inferred from a climate-stressed protistan plankton network	Microeukaryotes	Lake	[91]
Functional gene transcription variation in bacterial metatranscriptomes in large freshwater lake ecosystems: implications for ecosystem and human health	Bacteria	Lake	[96]
Functional repertoire convergence of distantly related eukaryotic plankton lineages abundant in the sunlit ocean	Microeukaryotes	Marine plankton	[97]
Multi-marker metabarcoding/Tree of Life studies			
eDNA metabarcoding to assess river stressors through metazoan and protist community structure	Prokaryotes, microeukaryotes, metazoans	Riverine	[14]
Metabarcoding the Antarctic Peninsula biodiversity using a multi-gene approach	Prokaryotes, eukaryotes	Marine	[29]

Table 1. (continued)

Title/description	Taxa	Habitat	Refs
Benthic monitoring of oil and gas offshore platforms in the North Sea using environmental DNA metabarcoding	Eukaryotes	Marine benthos	[31]
Gene-to-ecosystem impacts of a catastrophic pesticide spill: testing a multilevel bioassessment approach in a river ecosystem	Bacteria (with morphological assessment of diatoms, arthropods & vertebrates)	Marine	[46]
Tree of Life eDNA metabarcoding reveals a similar taxonomic richness but dissimilar evolutionary lineages between seaports and marine reserves	Prokaryotes, eukaryotes	Marine	[48]
Holistic pelagic biodiversity monitoring of the Black Sea via eDNA metabarcoding approach: from bacteria to marine mammals	Prokaryotes, eukaryotes	Marine	[58]
Multi-group biodiversity distributions and drivers of metacommunity organization along a glacial-fluvial-limnic pathway on the Tibetan plateau	Cyanobacteria, diatoms, invertebrates, and vertebrates	Riverine	[59]
Comparing metabarcoding with metagenomics for marine biomonitoring	Prokaryotes, eukaryotes, viruses	Marine	[98]
Air-quality networks collect environmental DNA with the potential to measure biodiversity at continental scales	Eukaryotes (review)	Aerial	[109]
Holobionts and microbial consortia			
The *Tara* Pacific expedition – a pan-ecosystemic approach of the '-omics' complexity of coral reef holobionts across the Pacific Ocean	Microeukaryotes, bacteria, phages	Marine (coral holobiont)	[27]
Profiling walnut fungal pathobiome associated with walnut dieback using community-targeted DNA metabarcoding	Fungi	Walnut trees	[28]
Green alder (*Alnus viridis*) encroachment shapes microbial communities in subalpine soils and impacts its bacterial or fungal symbionts differently	Bacteria, fungi	Soil	[35]
Microscopic marine invertebrates are reservoirs for cryptic and diverse protists and fungi	Marine invertebrates, protists, fungi	Marine	[70]
Microbiomes of microscopic marine invertebrates do not reveal signatures of phylosymbiosis	Marine invertebrates, bacteria	Marine	[71]
Coral and seawater metagenomes reveal key microbial functions to coral health and ecosystem functioning	Coral, bacteria	Animal host and marine	[74]
Trophic interactions in microbiomes influence plant host population size and ecosystem function	Microeukaryotes, bacteria, phages	Duckweed *Lemna minor* microbiome	[77]
Impact of marine aquaculture on the microbiome associated with nearby holobionts: *Patella caerulea* near sea bream aquaculture cages	Bacteria	Marine water, sediment, *Patella*, sea bream	[78]
The seagrass holobiont: review of current knowledge and potential use as an ecological indicator	Bacteria, seagrass	Marine (seagrass holobiont)	[80]
Different soil fungi assemblages in ancient semi-natural woodland, plantations, and adjacent grassland	Ascomycete and basidiomycete fungi	Woodland, grassland	[83]
High-throughput sequencing of litter and moss eDNA reveals a positive correlation between the diversity of Apicomplexa and their invertebrate hosts across alpine habitats	Apicomplexa, invertebrates	Moss, plant litter	[84]
Niche-dependent sponge hologenome expression profiles and the host–microbes interplay: a case of the Hawaiian demosponge *Mycale grandis*	Demosponge *Mycale grandis*, bacteria	Marine	[87]
Viruses			
Genomic screening of 16 UK native bat species through conservationist networks uncovers coronaviruses with zoonotic potential	Coronaviruses	Bat faeces	[39]
A global atlas of soil viruses reveals unexplored biodiversity and potential biogeochemical impacts	DNA and RNA viruses, phages	Soil	[40]
Virus diversity and activity is driven by snowmelt and host dynamics in a high-altitude watershed soil ecosystem	DNA and RNA viruses, phages	Soil	[41]
Evaluating the transmission risk of SARS-CoV-2 from sewage pollution	SARS-CoV-2	Wastewater, surface water, sediment	[44]

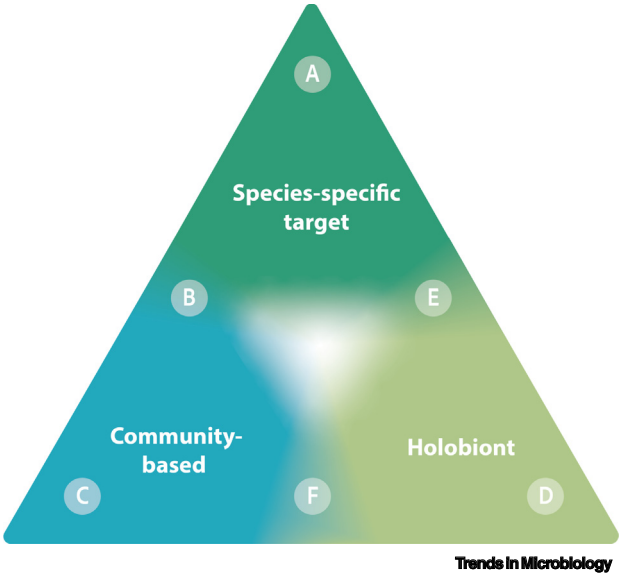

Figure 1. The environmental nucleic acid (eNA) triangle, showing the relationship and integration between three different eNA perspectives. (i) Single-species targets (e.g., the crayfish plague agent, *Aphanomyces astaci* and crayfish hosts); indicated by region A. (ii) Taxon-specific assemblages (B; e.g., fishes, nematodes, diatoms) or phylogenetically broader groups (C; e.g., microeukaryotes, bacteria, animals, plants). (iii) Holobiont hosts and associated symbionts (D; e.g., gut microbiomes, coral holobionts, pathobiomes). Region E can be exemplified by host-associated microbial assemblages detected by eNA independently of their host (e.g., plant-root symbionts in soils), and F by co-sampled host–symbiont assemblages (e.g., zooplankton and their symbionts).

rubble fields resulting from fishing activity [30], environmental impacts of offshore construction [31], fungal beta diversity shifts under land-use change [32], and the potential for tracking algal blooms [33].

Alongside taxonomic markers that relate to assemblage or community structure, genes of known metabolic function can be targeted in environmental samples, tracking specific microbial processes such as N-cycling and CO_2 fixation [5,34], in many cases using quantitative PCR assays. Metabarcoding of such genes can provide complementary taxonomic–functional insights [35]. For example, sets of antimicrobial-resistance genes (ARGs) and their host bacterial lineages can be targeted by both shotgun and amplicon-based eNA methods, in tandem with multi-omic approaches, to identify potential impacts from dense human populations on nearby freshwater habitats [36]. This illustrates how combined analyses of taxonomy with functional metrics can start to address the growing interest in 'one health' impacts of microbes on both humans and the environment, and vice versa.

Metabarcoding is also used for environmental studies of viruses, but because viruses do not have universal marker genes such as the ribosomal rRNA gene array regions used for bacteria and eukaryotes, PCR-based approaches usually focus on specific (often known pathogen) targets or phylogenetically restricted assemblages [37,38]. In contrast, shotgun sequencing can recover whole or partial DNA and RNA viral genomes from environmental or organismal samples, without phylogenetic bias imposed by PCR primer choice, and providing information from functional (e.g., virulence-related) genes and for phylogenomic analyses [39–41].

While viruses remain far less understood than many other microbial groups, they too offer great potential for extracting complementary and data-rich ecological signals for eNA ecosystem

Glossary

eDNA: the DNA component of 'eNA'.
eNA: nucleic acids extracted from environmental samples, such as water, soil and sediments, or air, including both intracellular and extracellular NAs, and whole (micro-)organisms. As organisms are represented in the environment by both DNA and RNA, we refer to these collectively as 'eNA', unless only one form is being referred to.
eRNA: the RNA component of 'eNA'.
Holobiont: a unit formed by a host and the associated species living within and on it (its **symbiome**).
Hologenome: the totality of the host genome, symbiont genomes, and their combined functional attributes.
Metabarcoding: a DNA-based identification method using high-throughput sequencing technologies to generate large numbers of amplicons of a 'barcoding' (= taxonomic marker) gene such as regions of the 16S or 18S rRNA genes, ITS rDNA, mitochondrial genes (e.g., COI, 12S), chloroplast genes (e.g., rbcL), etc.
Microbial consortium: stable coexistence of two or more microbial lineages exhibiting a jointly optimal metabolic division of labour, which has arisen via competitive population dynamics.
Microbiome: a term used variably, from (implicitly) including only bacteria and Archaea, to a more inclusive usage incorporating bacteria, Archaea, viruses, protists, and fungi.
Pathobiome: the set of host-associated organisms (encompassing prokaryotes, eukaryotes, and viruses) associated with reduced (or potentially reduced) health status, as a result of interactions between members of that set and the host.
Shotgun sequencing: generation of (often very) large high-throughput sequence data without preselection by PCR; theoretically unbiased sequencing of all DNA or RNA in a sample. Also referred to as (meta)genomic (DNA) and (meta)transcriptomic (RNA) sequencing.
Symbiome: all organisms, including eukaryotes, bacteria, and viruses, associated with hosts, encompassing negative, neutral, and positive interactions, or any combination of these interactions (recognising that the nature of the association can be context-dependent, and can occur on any timescale, including transient interactions). The nature and duration of

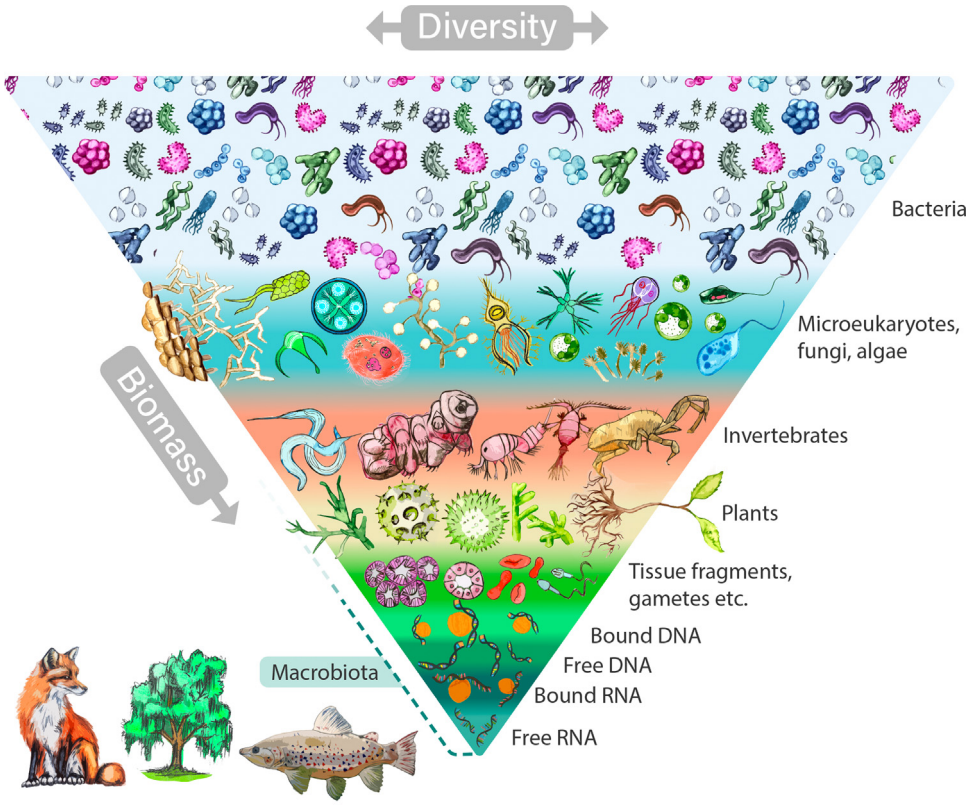

Figure 2. An illustration of biological diversity in a generalised hypothetical water, sediment, or soil sample. The biomass of organisms represented increases roughly logarithmically from top to bottom, but the actual material sampled (cells, gametes, organelles, free and bound nucleic acids, etc.) does not increase in size in the same way. Microbes are sampled whole, as viable or unviable cells, parts of cells, or free nucleic acids, whereas macroorganisms are only represented indirectly via fragments, gametes, faeces, and free and bound nucleic acids. Viruses are not shown as they will be co-sampled with other organismal material, and the nature of their 'free' states is poorly known.

the association is not specified by the use of 'symbiont'; indeed, in many cases both are unknown.

Symbiont: a member of the symbiome (including parasites and pathogens).

Tree of Life: phylogenetic representation of the diversity and evolutionary relationships all prokaryotic and eukaryotic organisms, and viruses.

assessments due to their vast diversity and host associations [41–43]. For example, soil viral communities influence many soil processes via release of nutrients from microbial hosts and altering host metabolism via auxiliary metabolic genes. Their ecology may be partly decoupled from other microorganisms, with community turnover occurring over shorter temporal and spatial scales [40]. Much eNA environmental monitoring of viruses has focused on human disease, including analysis of water or air samples to assess the environmental impact and disease spread from waste-treatment plants and wet markets into wild, farmed or artificial ecosystems – in the case of SARS-CoV-2 for instance (e.g., [37,44]). eNA methods have also recently been used to assess environmental reservoirs and spillover hosts of wildlife viruses (e.g., bird flu, turtle fibropapilloma virus, and new coronaviruses with zoonotic potential in bats [39]). In the broader sense of considering microbes and macroorganisms as an interconnected system, viruses are major interactors with larger biota, and via their roles as phage – pathogens of bacteria – they have the potential to (re) shape entire ecosystems and holobionts. Our understanding of viruses/phages as environmental indicators is embryonic compared with most other taxa and remains a huge knowledge gap to be addressed.

Tree of Life eNA: linking biodiversity, biomass, and function

When scaling up beyond a solely microbial focus, bacterial 16S and eukaryotic 18S metabarcoding can be used in combination with other markers such as the mitochondrial cytochrome *c* oxidase subunit I (COI) and the chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) genes to reveal anthropogenic and other environmental impacts and gradients across the full community of both microbes and macroorganisms (e.g., [11,19,26,31]). These approaches enable tracking of assemblages with particular functional roles that map onto the negative trajectories of ecological impacts, as well as those of recovery and restoration [45-48]. This broader incorporation of both the microbial and macrobiotic branches within the tree of life in this way can reveal unexpected responses to stressors, such as the consequences of pesticide spills on gene-to-ecosystem impacts. This more holistic approach can be especially useful for tracking combinations of acute and chronic impacts as well as direct and indirect effects as they ripple through the food web [46–48].

To interpret eNA signals deriving from different organismal types it is crucial to understand how the latter are represented across different environmental samples. Bacteria and microeukaryotes are typically both diverse and abundant in many environmental samples (e.g., water, soils, sediments) and are predominantly represented in the samples by whole, viable organisms. Consequently, their eNA signal therefore mostly directly represents their diversity and functional activity. In contrast, the macroorganismal eNA signal generally comprises parts of organisms (e.g., sloughed cells), exudates (e.g., mucous, faeces), or extracellular nucleic acids – either bound to particles or in solution – that do not necessarily directly indicate occurrence of whole organisms (Figure 2). Organisms may be represented in environmental samples by a range of life stages (e.g., gametes, cysts, spores, or dispersal stages). Therefore, a single species may be represented by particles spanning a wide size range in different sample types (Figure 3).

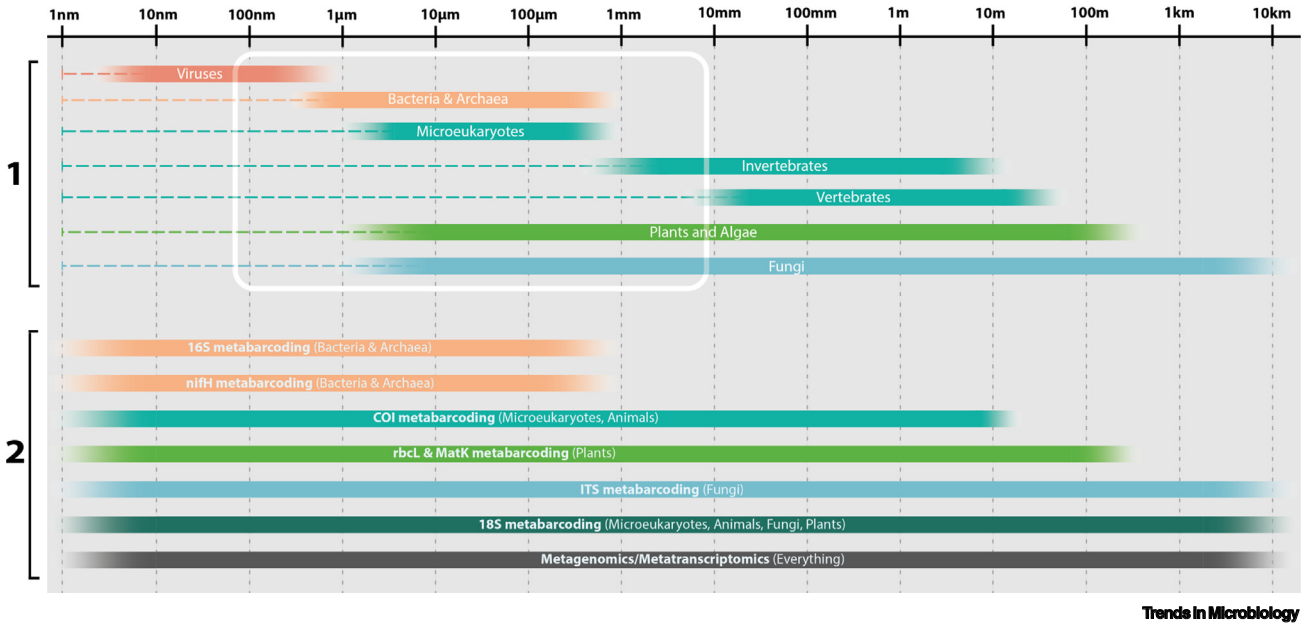

Figure 3. The size ranges of organisms detected in environmental nucleic acid (eNA) samples and the forms in which they may be represented. (1) Approximate individual organism size ranges of different organismal groups are indicated by wide unbroken bars. Broken lines indicate size ranges within which cells, gametes, organelles, etc., and extracellular nucleic acids (Nas) occur in the environment, and therefore may be captured in eNA samples. The white box indicates the particle size fraction captured in many environmental samples (not including larger fractions collected in bulk samples, e.g., malaise traps). (2) Genetic markers commonly used to detect different organismal groups, and the scales over which signal from those markers and shotgun sequencing approaches can be generated from eNA samples, whether from whole organisms, life-cycle stages, fragments, or free/bound nucleic acids. Figure adapted from [49].

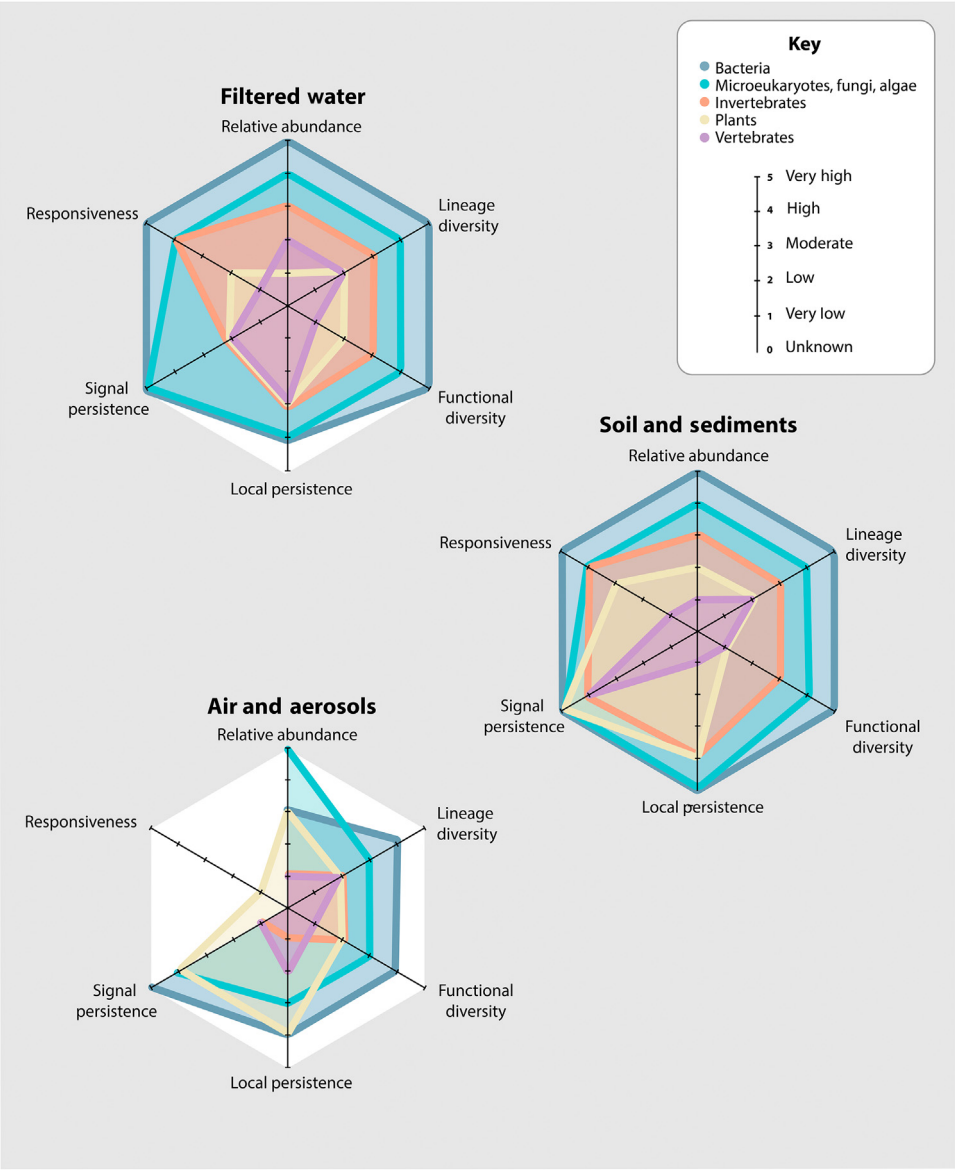

Figure 4. Properties of representation of different organismal categories in generalised water, soil/sediment, and air environmental nucleic acid (eNA) samples. Definitions of the six types of representation are given in the main text. Relative rankings of these properties between organismal groups are proposed, where 0 = unknown, and 1–5 represent a categorical ranking estimated from the authors' consensus interpretation of classical and molecular ecological studies. Note that the rankings are generalised within water, soil, and air sample types, but may vary according to ecological differences within each type. Viruses were excluded as they are insufficiently understood in this context, and will be influenced by their obligately symbiotic lifestyles. Note that these organismal attributes are not necessarily reflected by eNA data generated using currently available methods. For example, PCR primer biases may lead to low or no amplification of some taxa actually present, and indirect eNA detection of macroorganisms will be affected by many parameters, including taxon-specific nucleic acid shedding rates into the environment and subsequent degradation.

Appreciating the nature of this representation (whole organism, viable life-stage, dead fragments, or free nucleic acids) is critical for interpretation of eNA signals [50].

DNA-based analyses of microbial biomass do not necessarily always represent biological activity because cysts, spores, and dormant and dead cells are not discriminated from active, living cells. Ideally, eNA analyses addressing contemporaneous biomonitoring should focus on active material that is actually driving the traits and functions of interest. Using eRNA instead of – or in addition to – eDNA as the template for eNA assessments offers a more direct link to biological activity [51,52]. This use of alternative nucleic acid has been shown in some (but not all) cases to be more informative than DNA alone for assessing taxonomic turnover rate and response to environmental variables [51,53,54]. Combined DNA–RNA approaches have been recommended for biomonitoring [55] (and indeed are required for viruses, many of which have genomic RNA rather than DNA). The DNA–RNA combination enables not only discrimination between cellular and noncellular nucleic acids, but because it also distinguishes between 'living' and 'legacy' material, it enables comparisons between what is present and active in a given ecosystem versus what was there previously but is no longer living, due to local extinctions and/or species turnover [37,56]. Further refining this approach, a triple metabarcoding approach using rDNA, rRNA, and propidium monoazide-linked rDNA has recently been proposed to distinguish between active, dormant, and dead microeukaryotes [57]. Combined DNA–RNA analyses may allow us to address questions related to alpha, beta, and gamma diversity in the landscape, and potential versus realised functional biodiversity.

Microbes are frequently characterised by a close connection between their functional role and taxonomic identities. In fact, many are defined based on their function rather than their morphology, for example, methanogenic bacteria. Organisms at higher trophic levels – for example, larger animals – may serve as integrators of various stressors into a smaller set of indicator species over space, time, and the food web, making it difficult to disentangle stressor–response relationships (e.g., top predators often reflect the combined effects of stressors on the trophic levels they depend upon) and are therefore harder to connect to a particular function or individual stressor than is the case for the microbes (e.g., [46]). The response signals of higher trophic organisms are also diluted or dispersed in eNA studies as they are detected indirectly via smaller particles (Figure 3), are often more mobile than smaller organisms [58,59], and may be uniquely subject to 'top-down' pressures such as fishing or population shifts due to migrations, etc.

Responses across the Tree of Life will vary depending both on the nature of the stressors and the functional and trophic status of the taxa in the system. In a riverine pesticide spill case example [46], a range of microbial occurrence and gene expression responses were observed between impacted and control sites, but perturbations dissipated via interactions in the food web and had reduced impact at higher trophic levels [46]. Since microbes, macrophytes, macroinvertebrates, and larger animals respond differently to physicochemical and spatial variables, a holistic Tree of Life approach that considers them together allows us to gauge the impacts of a broader suite of potential stressors than would be possible by only considering a subset of these groups, which is still the prevailing approach (e.g., [14,15,29,31,48,58]).

Given the complementarity between eNA signals from micro- and macroorganisms, we propose a framework for comparing respective signals from bacteria, microeukaryotes, fungi, plants, invertebrates, and vertebrates, to guide their integration into holistic Tree of Life eNA studies. We illustrate proposed rankings of eNA signals from each of these organismal groups with respect to six fundamental ecological and evolutionary attributes: (i) relative abundance, (ii) lineage

richness, (iii) functional diversity, (iv) local persistence (long-term vs. transient presence of taxa in/near the sites being sampled), (v) persistence over time of eNA signal of organisms irrespective of changes in their actual diversity and abundance, and (vi) responsiveness (an indication of how quickly the community of each category of organisms responds to changes in their abiotic and biotic surroundings) (Figure 4). The relative rankings of these attributes are inferred from general knowledge of the biology of each organismal group in each environment type, rather than representing a systematic review; such supporting data are currently either unavailable or not easily accessible; further research is needed to validate these estimates more formally.

Relative abundance and lineage richness (Figure 4) can be derived and compared relatively consistently by eNA across all organismal groups in different sample types (e.g., [60]). Functional diversity (FD) of communities and the ways in which they respond to change is related to lineage richness, but also provides information that can ultimately link to measures of ecosystem functioning [61,62] making it an important element of biomonitoring and a bridge between community structure and process-based metrics.

Strengthening the link from biodiversity to ecosystem properties typically requires some form of quantification per unit area or volume, since how much biomass is 'doing the work' is often more important than simply the richness or relative abundance of the organisms involved. However, a common criticism that is levelled at eNA approaches is that while they can give us highly resolved taxonomic and FD measures, they often lack a truly quantitative dimension that maps directly onto abundance or biomass per unit area. Recent research suggests that this longstanding shortcoming can be overcome, with a range of quantitative approaches now emerging for metabarcoding data, including modelling the amplification process during metabarcoding to account for biases of PCR-driven variation [60,62–64], shotgun metagenomics [65], and combined approaches including quantitative (q)PCR [66]. For macroorganisms, analysing allometric relationships between eNA data and traditional metrics of abundance, and taking into account metabolic scaling coefficients, are significantly improving the use of eNA metabarcoding for inferring abundance both for a single species and between species [67]. For microbial taxa, quantitative molecular approaches can now be complemented by emerging high-throughput microscopy technologies, which provide a more direct assessment of (absolute) quantification and insight into functional traits via direct imaging [68].

The ecological and evolutionary attributes represented in Figure 4 are key for 'biosensing' environmental change, yet some measures are far easier to derive than others and may require repeated sampling to assemble the full picture, especially in relation to temporal dynamics. A core aim is to extract information on how the driver and the response of interest are coupled, how that relationship changes when it is put under stress (e.g., due to climate change, pollution, land use change or other perturbation), and how resilient is the system once that pressure has been removed (e.g., via a management intervention) relative to its operation under reference conditions [69].

Holobionts, consortia, and their potential for biomonitoring

Microbial and macroorganismal (co-occurrence) dynamics can provide further insight into ecosystem health and functioning via the recognition that even individual organisms are effectively ecosystems in their own right, as they are associated with a diversity of **symbionts** that also interact with the surrounding environment. The diversity and function of host-associated microbial taxa is a rapidly expanding field [54,70–72]. The term **holobiont** describes the assemblage of smaller organisms (the **symbiome**) associated with a larger host [73]. The ecological and/or evolutionary relationships represented by holobionts could be adopted into eNA biomonitoring to

augment traditional ecological assessments by incorporating organism health and symbiotic functioning [74], enrich network-based and trophic analyses (in which symbiotic interactions are under-represented [75]), and provide functional links integrating eDNA signals from microbes and macroorganisms [76]. For instance, predation by protists and parasitism of bacteria by bacteriophages within plant symbiomes can influence host population size and ecosystem functioning [77].

Host–symbiont associations can be strong, exemplified by core gut **microbiomes** or specific host–parasite relationships. Such associations are illustrated by region A of Figure 5. Although sampling microbiomes and parasites of host tissues is not considered 'environmental' sampling, it can be regarded as one end of an eNA sampling continuum proposed for pathogen eNA investigations [54], which has a logical continuity between sampling organisms directly for their symbionts and the wider environment in which they occur. Many host-associated microbes are rare or undetectable outside of their hosts (see Outstanding questions), but may be detectable in host exudates (e.g., mucus or excreta), or as other life-cycle stages, such as crayfish plague spores in water, which can be detected by eNA in parallel with their hosts [22].

Holobionts can be good indicators of health and environmental influences, for example the symbiome of the limpet *Patella* has been shown to acquire fish pathogens and lose heavy-metal-intolerant microbes when living near sea bream aquaculture cages, but conversely adapting by acquiring bacteria to mitigate the accumulation of pollutants [78]. The *Patella* symbiome comprises a non-neutral selection of microbes from the surrounding water; eNA signals of aquaculture impact in water and sediment surrounding the limpets were weak or not apparent. Similarly, direct subsampling of coral holobionts provides a more sensitive indication of their status and health than the surrounding water [79] (Figure 5, region B). Seagrasses modify their environment, for example by enriching nearby sediments with nitrogen-fixing and sulfur-cycling bacteria, with sediment microbial community complexity reflecting the ecological condition of the seagrass meadow (Figure 5, regions B,C). However, the influence of the seagrass holobiont on the surrounding water is less clear, the microbiome of which is perhaps more strongly influenced by currents and tides [80].

Although some holobiotic influences can be detected by eNA in marine systems independently of their hosts – for example detection of elevated salmon pathogen levels in environments around salmon farms [81] – in the other aquatic examples described, above, direct sampling of the holobionts was more informative about their responses to environmental change than the surrounding water. Many organisms selectively acquire microbes from their environment (even filter-feeding bivalves [82]), or are actively or opportunistically colonised by environmental microbes. The resulting holobiont therefore represents integrated responses of the host, symbiome, and surrounding environment. The **pathobiome** concept describes these combined influences in cases of reduced health status [72].

Holobiotic effects detectable by eNA independently of hosts may be more apparent when more physically constrained in sediments and soils – for example, tree range expansions being reflected by shifts in diversity of their bacterial and ectomycorrhizal symbionts in the soils being encroached [35], and mycorrhizal fungi assemblages detected by eNA of soils of different woodland types and grassland being highly distinct and reflecting known associations between plant type and fungus [83]. Thereby, eNA can be used to infer the occurrence of a particular holobiont in a sampled habitat, and to track its composition or status across a temporal/spatial or ecological gradient, or in response to an environmental impact. However, even with the most comprehensive sampling design and powerful co-occurrence analyses, taxa association signals in eNA

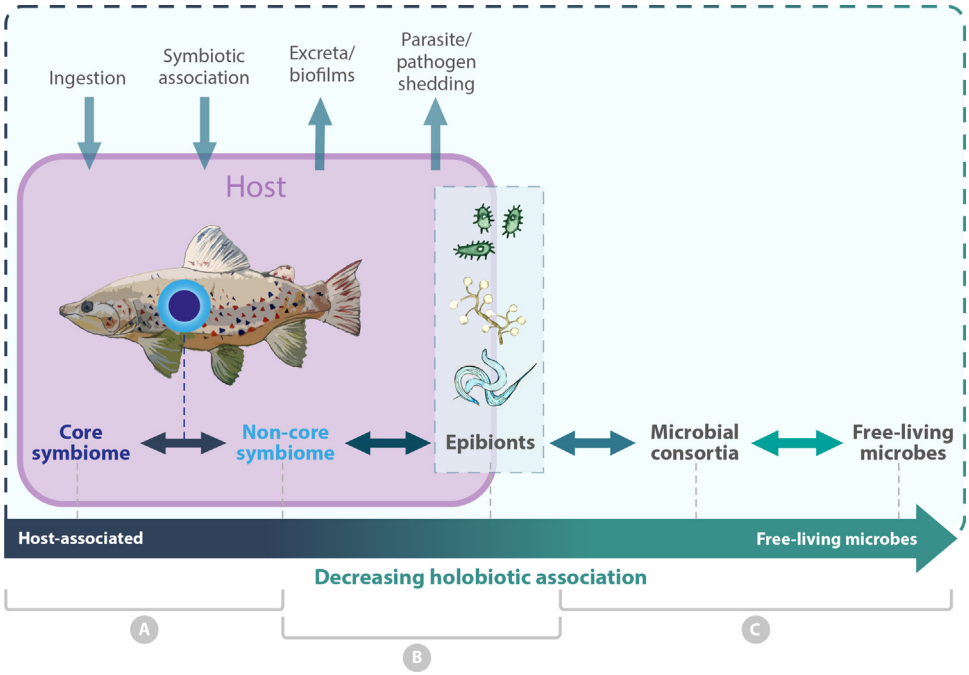

Figure 5. The holobiotic continuum. Associations between 'host' organisms and their symbionts can be considered on a scale from the core symbiome (e.g., bacterial taxa consistently found within the gut microbiome, or obligate parasites) to microbial consortia, and to free-living microbes that may only associate with larger species incidentally or not at all. In between are more transient members of the symbiome (e.g., opportunistic pathogens), epibionts growing on the surface of larger organisms, and taxa that associate with excreta or other material shed from them.

data may be overridden by other factors (e.g., water currents and tides [79,80]) and will be lost beyond certain spatial and temporal limits (see Outstanding questions).

Small holobionts can be sampled and analysed in their entirety, enabling direct linkage between hosts and symbionts due to the explicit co-occurrence of all entities in a given sample. Examples include (i) identifying a correlation between invertebrates and their apicomplexan parasites across an alpine gradient [84], (ii) single-cell investigations into interactions between microbial algae and their viruses [85], and (iii) the potential to measure shifts in diverse biogeochemical processes, such as methanogenesis, methylmercury production, and degradation of high-molecular-weight organic compounds by analysing highly abundant and environmentally responsive holobionts such as copepods (reviewed in [86]).

Although taxonomic profiles of holobionts can vary in response to surrounding environmental conditions [78–80], in some cases functional gene diversity and expression patterns correlate much more strongly with niche differences than taxonomic profiles alone, as illustrated by a **hologenome** study of the demosponge *Mycale grandis* [87]. Hologenomic systems have great potential as fully integrated models for biomonitoring. For example, the cladoceran *Daphnia magna*, central to fresh/brackish water food webs, is a model species used for ecological and ecotoxicological studies; the integration of its microbiota and their host interactions is already providing insight into the holobiont's disease and drug resistance, and its ability to withstand oxidative stress [88]. This holobiotic system has the potential for further development into a sensitive and dynamic tool for environmental assessment.

Microbes occurring in environmental samples apparently independently of larger hosts (Figure 5, region C) may in fact represent small holobiotic systems (microbial host–parasitic and endosymbiotic relationships – for example, phages and bacteria associated with phytoplankton [89]). Microbial consortia comprised of metabolically cohesive and complementary prokaryotic and microeukaryotic taxa have been proposed to be an intermediate level of organisation between species and community levels, and may offer new opportunities for understanding microbial ecology and evolution, and predicting ecosystem function [90]. Even 'free-living microbes' (right-hand end of Figure 5) interact in many ways (e.g., predator–prey relationships, decomposers, commensals, etc.), forming networks of relationships that respond to environmental change and contribute to ecosystem resilience and 'health' [91].

Microbial diversity, function, and ecosystem health

A fundamental question in microbial ecology, beyond whether and which aspects of microbial communities can be used as ecological indicators and for biomonitoring, is the relationship between microbial diversity and ecosystem health [61,91]. An important component of health is resilience to perturbation. Microbial diversity itself is a less sensitive measure of resilience to environmental change than biotic interactions as measured by network analyses and associated metrics [61,92]. Underpinning this resilience is the 'rare biosphere' – a large number of rare taxa characterising microbial communities – which can facilitate resilience even when more abundant species are exhibiting high, even chaotic, turnover [93], and DNA–RNA analyses have shown that whole bacterial communities – active and inactive subsets together – confer greater resilience than the active subset alone [94]. These findings further emphasise the importance of integrating the abundance, taxonomic and functional diversity, persistence, and responsiveness attributes shown in Figure 4 for understanding overall ecosystem dynamics, resilience and 'health'.

Additionally, microbial communities are highly functionally redundant, meaning that functional diversity of different communities is usually more stable than taxonomic composition might imply [95], leading to different responses of taxonomic versus functional (gene expression) profiles to environmental changes/gradients and phenology [51,96]. Networks of functions, rather than (or in addition to) taxa, may be more informative about the resilience and adaptive potential of a community (see Outstanding questions).

Other suggested microbial metrics of compromised environmental health include decreases in alpha diversity and specialist data, increases in beta diversity and generalists, and increases in pathogenicity and antimicrobial resistance (AMR) [13]. eNA metabarcoding is an excellent method for measuring some of these, but, being usually based on a single marker gene and with limited phylogenetic/taxonomic resolution, cannot effectively be used to investigate networks of biotic interactions and their contribution to ecosystem health. In contrast, shotgun sequencing of DNA or RNA can simultaneously capture pan-genome taxonomic and functional (metabolic capacity and ecological trait) data without primer or PCR biases, and with the ability to correlate read counts to biomass [65].

Recent metagenomic studies have demonstrated the value of shotgun sequencing, for example in functional analyses of uncultured marine planktonic communities [97], and marine monitoring [98], the latter study comparing the pros and cons of metabarcoding and metagenomic methods for eNA monitoring. Genomic data are increasingly used to elucidate metabolic interactions and complementarities between microbial taxa [99], evolutionary processes underlying microbial adaptation to evolutionary change [100], providing taxonomic resolution down to the level of biologically distinct strains (e.g., within bacteria species) and haplotypes [18,101], and for revealing patterns of differentiation and adaptation between microbial populations [102]. The importance

of diverse and flexible 'multi-omic' techniques for (a mechanistic understanding of) biomonitoring and ecological assessments is now very clear, and points the way to emerging techniques and multidisciplinary 'systems' approaches in which eNA applications will be indispensable.

Future perspectives and challenges

A core challenge for biomonitoring is the development of routinely used and biotic indices that inform on ecological status and change. Microbes provide a potentially rich source of indices, but the diversity of choice, combined with lack of baseline ecology and response data, can be inhibiting. We propose that the dynamic and responsive organismal interactions in holobionts, consortia, and Tree of Life networks provide the ecological context often missing from the perspective of individual taxa or even whole assemblages in standard environmental sample types, and therefore offer a richer and more informative source of bioindicators, whether taxon- or network-based. Holobionts, in particular, represent quasi-Tree of Life systems that integrate the collective and interactive responses of a large diversity of taxa, and are 'responsive biosensors', both participating and reflecting changes in their surrounding environment (see Outstanding questions).

Recent technical and computational advances are removing taxonomic limitations imposed by incomplete databases [103], and using deep learning to discern assembly rules and identify keystone species in any microbiome [104]. Analytical bottlenecks previously associated with large environmental datasets are being overcome through advanced network analyses, machine learning and artificial intelligence algorithms to reveal new biodiversity and functional metrics [20,105–107], on the basis of which new biotic indices can potentially be developed.

In parallel, the development of *in situ* samplers and biosensors [108,109] will ultimately both remove the need for physical sampling in many cases and vastly increase the temporal and physical resolution of eNA surveys, particularly when the sample-to-data workflow can be automated through emerging robotics and drone technologies. Such developments will accelerate the widespread and routine integration of eNA into the design of enhanced national surveillance programmes [110,111]. A growing repertoire of data collection and integration methods is poised to significantly extend the reach of eNA techniques. The incorporation of eNA data into Geographic Information Systems (GIS) and Earth Observation data enables multivariate and multiscalar environmental characterisation [110] and provides the basis for new forms of predictive modelling (see Outstanding questions).

Novel microbial-holobiotic perspectives are currently opening up new vistas of research into the potential for more proactive interventions that can halt or reverse declines in ecosystem integrity, for example bioengineering microbiomes to improve plant health and promote sustainable aquaculture [112,113]. Similar approaches are under development for a range of habitat restoration and biodiversity recovery efforts [114,115], to accelerate rewilding initiatives, and for boosting organismal resistance to toxins [116]. Integrated approaches, including synthetic microbial communities, functional gene and metabolomic analyses have been proposed to elucidate holobiont–environment interactions (e.g., between plants, their arbuscular mycorrhizal fungi, bacteria, and soils [117]) and inform the development or improvement of engineered microbiomes. A fundamental challenge for the development and refinement of eNA and next-generation/multi-omic monitoring methods is to facilitate the mechanistic understanding and measurement of ecosystem dynamics, and management of remedial interventions.

Future molecular approaches to enhance eNA capacity could potentially be adapted from cutting-edge technologies currently applied at the organismal level and with established medical

applications, for example single-cell multi-omics [118]. Single-cell (meta)transcriptomic analyses can associate ecophysiological properties and interaction characteristics directly with individual cells [119] or cellular consortia (e.g., alga–virus interactions [120]), with the potential to measure how such interactions are influenced by environmental factors. Although single-cell metatranscriptomics has not yet been applied to complex environmental biota samples to the best of our knowledge, the potential for investigating small-scale holobiotic interactions and their responses is clear, and represents a high resolution extension of eRNA methods for functional inference. Highly complementary to genetic 'omics, metabolomics holds great promise for a step change in understanding microbial interactions, community function, and environmental responses, for example, for climate change impact assessment [121] (see Outstanding questions). Similarly, metaproteomics is emerging as a complementary field that can integrate new insights into microbiome and holobiont taxonomic structure, function, and dynamics from the protein level to enhance environmental monitoring, food production, and biotechnology [122].

Concluding remarks

Microbes offer a more dynamic and distinct set of bioenvironmental metrics compared to the traditional macroorganism focus that has dominated the field to date, but will be most informative when these are analysed in combination and, increasingly, in the form of holobiont networks. The integration of microbial-macroorganismal holobionts that respond to – and shape – their environment offers revolutionary new ways to unite organismal, molecular, and multispecies ecology. Holistic Tree of Life and holobiont concepts, and multi-omic technical approaches hold great promise for the next generation of large-scale biomonitoring programmes, synergistically with other data types (e.g., abiotic/chemical, hydrological, meteorological, remote sensing, LiDAR (Light Detection and Ranging), bioacoustics, and socioeconomic, etc.) [123]. Cross-disciplinary systems-thinking is required for the 'planetary health' vision that is rapidly redefining humanity's interaction with the natural world, and our ability to measure, predict, and mitigate wider threats such as climate change, biodiversity loss, pollution, disease, and antimicrobial resistance [124].

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Declaration of interests

No interests are declared.

Outstanding questions

How concordant are indices based on microbial eDNA assessments with those from macrobiota alone?

Are ecosystem responses to environmental change better measured by analysing (quantitative) shifts in eNA taxonomic profiles, or by changes in functional or organismal interaction networks?

To what extent – and how – are eNA methods capable of detecting and analysing holobiotic relationships? Which holobiotic systems/environments and molecular approaches are best suited for this?

How informative are changes in intra-holobiont and intra-consortium interactions for measuring impacts of environmental change, for example, climate change?

Can molecular studies of holobionts or host-associated microbiomes provide more informative assessments of ecosystem health/environmental quality than purely environmental DNA/RNA studies?

How can holistic eNA methods for biomonitoring be standardised and validated?

Should eDNA and eRNA data routinely be analysed in combination, whether for amplicon-based or shotgun sequencing investigations?

What is the potential of air DNA/RNA monitoring for biomonitoring across the whole Tree of Life, and can air monitoring inform on diversity and function in subterranean and aquatic habitats?

To what extent can eNA signals from viruses be integrated into ecosystem assessment and functional studies?

How can microbial eNA signals be integrated into wider geospatial analyses using earth observation data and GIS, and integrating other data types, such as hydrological and meteorological?

What are the technical limitations to automated and *in situ* sampling,

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analyses, and streaming of eNA data, and how can these be overcome?

How can multi-omic approaches be mainstreamed to enable holistic analyses of environmental biodiversity profiles, functional diversity, and metabolic interactions?

How can sufficient microbial baseline data be acquired to inform new metrics for biomonitoring? Additional funding? Synergies with existing sampling programmes?

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