Understanding ecosystems and resilience using DNA

Chief Scientist's Group report

June 2021

Version: SC190006/R

We are the Environment Agency. We protect and improve the environment.

We help people and wildlife adapt to climate change and reduce its impacts, including flooding, drought, sea level rise and coastal erosion.

We improve the quality of our water, land and air by tackling pollution. We work with businesses to help them comply with environmental regulations. A healthy and diverse environment enhances people's lives and contributes to economic growth.

We can't do this alone. We work as part of the Defra group (Department for Environment, Food & Rural Affairs), with the rest of government, local councils, businesses, civil society groups and local communities to create a better place for people and wildlife.

Published by:

Environment Agency
Horizon House, Deanery Road,
Bristol BS1 5AH

www.gov.uk/environment-agency

Further copies of this report are available from our publications catalogue: www.gov.uk/government/publications or our National Customer Contact Centre: 03708 506 506

Email: enquiries@environment-agency.gov.uk

Author(s):
Donald Baird, Alex Bush, Elizabeth Clare, Simon Creer, Helen Hipperson, Peter Kille, Eleni Matechou, Iain Perry, Edward Sibley, Tom Webb, and Douglas Yu

Keywords:
freshwater, marine, terrestrial, DNA, ecosystem, function, biodiversity, resilience, ecological, communities, metrics

Environment Agency's Project Manager:
Kerry Walsh

Project number:
SC190006

Citation:
Environment Agency (2021) Understanding ecosystems and resilience using DNA. Science Report SC190006/R, Environment Agency, Bristol.

Contents

Research at the Environment Agency

Scientific research and analysis underpins everything the Environment Agency does. It helps us to understand and manage the environment effectively. Our own experts work with leading scientific organisations, universities and other parts of the Defra group to bring the best knowledge to bear on the environmental problems that we face now and in the future. Our scientific work is published as summaries and reports, freely available to all.

This report is the result of research commissioned by the Environment Agency's Chief Scientist's Group.

You can find out more about our current science programmes at https://www.gov.uk/government/organisations/environment-agency/about/research

If you have any comments or questions about this report or the Environment Agency's other scientific work, please contact research@environment-agency.gov.uk.

Professor Doug Wilson
Chief Scientist

This project was led and funded by the Defra DNA Centre of Excellence in 2019/20, which aims to transform Defra group science to support policy and delivery by sharing and demonstrating the wider potential of existing methods; enabling the rapid development and trialling of new DNA based method applications; providing a focal point for leadership in Defra, UK and across OGDs and building shared capability including skill and facilities.

Executive summary

Climate change, biodiversity loss, population growth and failure to prevent environmental degradation to date, suggest we need to act differently in the future to understand and protect the services we need and the systems we value. The Climate Emergency and Biodiversity Crisis are major environmental challenges for the UK government, which sets out long-term objectives and targets to address these in its 25 Year Environment Plan (25YEP). Progress to reduce biodiversity loss by restoring degraded ecosystems, creating new habitats for wildlife and understanding how much the environment is changing, will be measured using indicators and performance measures underpinned by evidence-based metrics. For some indicators, metrics already exist, but could be improved, such as those used for measuring the ecological status of water bodies that use traditional morphological methods e.g., light microscopy. For other indicators such as those required to understand marine food web functioning, the functions of water and wetland ecosystems and measuring soil health, will require a change to how we analyse existing information or will require the development of novel metrics.

Ecosystems are complex with multiple pressures acting upon them. Understanding this complexity needs information at different levels of biological organisation (e.g., population, community) and scales (local, catchment, and regional) as well as new ways of analysing big data to answer environmental questions. There is a question mark over whether existing biological methods provide information at the scale and resolution needed. They can describe the state of the environment, but to prevent further degradation and harm we need to better understand what is causing harm and how ecosystems will change in response to our interventions. Current methods measure only a subset of the ecosystem (e.g., plants or invertebrates), are costly to execute at the scales needed to understand and manage ecosystems, are logistically challenging and resolution of data is compromised compared to molecular technologies that exploit an organisms genetic make-up (DNA).

Our existing methods have served their purpose, but investing in new technologies to support and build on these could unravel the impact complex ecosystems have on biodiversity. The species we assess have been dictated by what could be efficiently recovered from surveys and be readily identified. Many ecologically important species such as the microbiota (e.g., bacteria, fungi) and meiofauna (e.g., nematodes) have been overlooked completely in biomonitoring programmes and this untapped wealth of information should be explored.

Through a compilation of Think-Piece (TP) papers, this report describes potentially fruitful avenues and sets out opportunities from technological advances in DNA technologies to develop new indicators/metrics suitable for measuring ecological status and functions across aquatic and terrestrial systems. The TPs are detailed and specialised and we also provide a synthesis paper that captures the key areas for further exploration.

All TPs highlight the huge potential application of using DNA for biomonitoring. Its use in conservation management is varied and includes assessing trends of protected and

threatened species in response to anthropogenic pressures and restoration measures in a non-harmful way; assessing the impact of invasive non-native species; determining genetic variability and a species' capacity to adapt to climate change as well as understanding reproduction such as the timing and location of spawning events. Using DNA (or other genetic material such as RNA) opens up a whole new world of environmental monitoring due to its ability to assess what the eye can't see. Detecting viruses, bacteria and fungi allows us to track the dynamics of pathogens and antimicrobial resistance and importantly start to capture the important taxa that exert bottom-up influence on ecological processes such as nutrient cycling and that represent the foundations of our ecosystems. Despite the broad application of DNA-based technology for biodiversity assessment and the huge interest from government, progress has been slow to develop novel metrics and move the technology from research laboratory to field application. To speed this up, key priority focus areas include:

Establish a strategic funding pipeline for DNA focussing on technological and analytical refinement of methods and their standardisation and validation. This will be important for confidence building and ensuring consistency and comparability of data as well as robust species assignment.

On-going collaboration and co-design of projects and initiatives. Infrastructure exists through the UKDNA Working Group and Defra DNA Centre of Excellence. Skills and capacity in decision-making organisations needs to grow. There is a need to set clear direction for the research community to ensure the desired outcomes are met.

Establish large, intensive case studies within and across systems to develop and test novel metrics. These must address technical challenges, embrace citizen science to support sampling campaigns and test how data from complementary technologies (e.g. remote sensing) can be integrated with DNA data to better understand biodiversity and functional dynamics. Further develop new data analytics to support metric development including network assessments, machine learning and multi-and joint species distribution models.

Populating reference databases needs to be an ongoing priority, especially for priority UK taxa and in accordance with a consensus agreement.

Embrace technological advancement and explore environmental biobanking opportunities for future analysis. DNA-based technologies constantly evolve and improve, meaning DNA methodologies will change in the future and therefore approaches will require "future proofing'.

If we are to achieve the outcomes set out in the 25YEP then we need to seriously consider the priorities listed above. Doing so will help realise the benefits of advancements in DNA technology. Investment in development and refinement will speed up implementation and deliver fit for purpose biodiversity data and evidence to support ecosystem assessment. These technologies will transform current UK government biomonitoring programmes and decision making - but not without committed investment.

Introduction

A Defra DNA Centre of Excellence (CoE) was established in May 2019 to explore the use of DNA-based methods and facilitate their implementation into operational use and decision making to help government manage the natural environment. The government's 25 Environment Year Plan (25YEP: https://www.gov.uk/government/publications/25-year-environment-plan) to improve the environment, emphasises the need to take full advantage of technological advances and highlights the use of DNA-based methods as an area to further explore.

Many of the current biological monitoring and assessment methods used to understand the state of the environment generally use traditional biodiversity metrics and pressure-specific biological indices. For example, the indices used to measure macrophyte and phytobenthos quality in rivers is linked to eutrophication pressures, assumed to be caused by phosphorus. An indication of poor quality would require a measure to reduce phosphorus. This is a very simplistic view of pressures acting upon a system, when the quality of a system is governed by multiple pressures and therefore presents a restrictive approach to management which could lead to ineffective restoration measures. Pressure-specific indices provide limited information on the bigger picture at the ecosystem-level, despite the increasing need to take a more holistic, ecosystem-based approach to monitoring and to aid our understanding of the ways in which the environment functions. Such an approach could provide better insights into ecosystem health, resilience and the ways in which ecosystems can continue to provide the many services on which we now depend.

DNA-based methods have the potential to provide relevant information across different scales from the molecular level to the entire ecosystem and at different levels of organisation from the individual to population to community. They offer a range of possible uses that could help address key evidence needs including (but not limited to) soil health, pests and diseases and the impacts of invasive species and a changing climate. In some cases, the main benefit of DNA-based methods may be as a feasible means to identify and monitor functionally important taxa, but in others it may involve approaches that are quite different from traditional monitoring (e.g. assessing function and resilience using species interactions at different trophic levels).

In May 2019 Defra published the Outcome Indicator Framework, (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/925779/25-yep-indicators-2019.pdf) which is a comprehensive set of indicators describing environmental change that relates to the 10 goals within the 25YEP. The framework is made up of 66 indicators, all of which are underpinned by data from several metrics, and collectively will help us understand the effectiveness of policies and interventions to ensure progress towards the goals of the 25YEP. Some indicators and associated metrics require further research and development, for example these include:

1. B6 Natural functions of water & wetland ecosystems (freshwater systems): significant work is required to develop the indicator, building on existing monitoring methodologies and testing new approaches.
2. C8 Healthy seas: marine food webs functioning: although research is underway there is a need to further develop food web metrics.
3. C9 Healthy seas: seafloor habitats functioning: further development is needed for this indicator.
4. D7 species supporting ecosystem functions: significant further research and development is needed to include a range of species groups important for supporting ecosystem functions.
5. E7 Healthy soils: more work is needed to develop appropriate soil metrics and a scientifically sound national monitoring programme to measure healthy soils that underpin multiple functions.

All of the above require ecosystem “function” or “functioning” to be measured across different systems and the ability of existing metrics to predict ecosystem functions remains tenuous.

This report comprises a series of individual Think-Piece (TP) papers and a TP synthesis, commissioned by the CoE to explore whether DNA-based approaches could provide solutions to understanding the ways in which ecosystems function and how resilient they are to environmental change. Could advances in DNA-based technology alone or when integrated with other methods, have the potential to provide ecosystem-level metrics, building on the understanding we have gained from traditional ecological studies? Academics were invited to give their perspectives and critical analysis of how new metrics based on data derived from DNA-based methods could help us monitor aquatic and terrestrial ecosystems, providing new insights into the functioning and stability of these systems. TP papers include:

Think-Piece 1: Development of new DNA-based metrics to understand ecosystem functioning, and the resilience of **freshwater systems** in response to environmental change

Think-Piece 2: Development of new metrics incorporating DNA-based methods to understand **marine** ecosystem functioning

Think-Piece 3: DNA biomonitoring in the **terrestrial** biome

Think-Piece 4: The contribution of DNA-based methods to achieving **socio-ecological resilience**

Think-Piece 5: DNA-based methods: **Technology** solutions to evaluate ecosystem function

Authors were asked to consider the following:

- Definition of ecosystem functioning.

- The Indicator Outcome Framework report, and how any form of DNA-based methods could provide information on indicators D7 (key species/groups – all TPs), B6 (specifically freshwater TP), C8/C9 (specifically marine TP) and E7 (specifically terrestrial TP).
- Different attributes that should be used for example, but not limited to, genetic diversity, functional diversity, phylogenetic diversity and at what scale e.g. functional diversity at the community level, food web complexity at the ecosystem scale.
- How we might use DNA-based methods to understand the functioning and overall status of aquatic and terrestrial ecosystems.
- The spatial and temporal scales for the analysis and evaluation of ecosystem function.
- Any major constraints for operational implementation.

The synthesis lead-in paper summarises the main ideas and opinions across individual TPs, identifies key messages and common themes and provides a strategic vision to develop a research and development roadmap and priorities for the next 25 years, not only for the CoE but for the scientific community as a whole.

Think-Piece Synthesis: developing DNA-based biodiversity metrics for monitoring ecological status and ecosystem function over the next 25 years

Simon Creer

Molecular Ecology and Evolution Group, School of Natural Sciences, Bangor University, United Kingdom

Contents

Introduction

The following DEFRA DNA Centre of Excellence (COE) 'Think Pieces' (TP1-5) were commissioned to engage experts from the academic community to consider how best we can progress from the current revolution in primarily DNA-based biodiversity analyses to develop effective and complimentary biomonitoring of ecological status and ecosystem function across freshwater, marine and terrestrial habitats. The purpose of this synthesis piece is to draw on their key messages, draw attention to highlights, common themes and provide the necessary strategic vision to develop a research and development roadmap, priorities and likely trajectories for the next 25 years.

Communication, outreach and effective co-design of 21st Century biomonitoring strategies

One of the key influences in the lead up to the Think Pieces has been the maturation of the unique exemplar of the UK DNA Working Group, founded in 2014, that is now accompanied by the DEFRA COE. The latter developments have, and continue to be, highly effective and instrumental vehicles to facilitate multi-way engagement, synergies and outreach between the UK DEFRA and devolved nations environmental stakeholder groups and academia. We are not alone, since the annual UK DNA Working Group meetings now have international attendance and the UK community is complemented by a plethora of additional networks, including notably, the EU COST Action DNAqua-Net and their legacy work recently streamed to over 1400 global attendees via the inaugural DNAqua-Net International virtual meeting. Networking with and learning from mature initiatives such as the International Barcode of Life and phase two 'BIOSCAN' (https://ibol.org/), LIFEPLAN (https://www2.helsinki.fi/en/projects/lifeplan), TARA Oceans (https://oceans.taraexpeditions.org/en/m/about-tara/les-expeditions/tara-oceans/) and the Earth Microbiome Project (Thompson et al., 2017) will all be hugely beneficial for future developments (Clare, TP3). There is considerable interest and a multitude of excellent examples in the application of DNA-based biodiversity assessment for biomonitoring, but how do we move from the academic and industrial laboratory, to routine biomonitoring ecological status and function? Before proceeding, academics are very good at designing and publishing the results of interesting scientific experiments, but some experiments may not address the questions the end users need answers to. It is therefore vital to stress the importance of continuing working together to co-design solutions and ensure that all parties, including legal and regulatory bodies, management and field operatives are engaged in realising shared goals from project inception to completion. Yu and Matechou's (TP4) coverage of Dietz et al.'s (2003) thesis on the five pillars of socio-economic resilience provides an insight into how huge gains have been made in the field of biomonitoring using DNA approaches regarding (i.) knowledge generation and (ii.) capacity building, but we now need to make progress in (iii.) political bargaining, (iv.) enforcement and (v.) institutional design and adaptive governance. Perry and Kille (TP5) also echo a post-Brexit move from research skills to validation, regulatory and industrial implementation, leveraged by novel funding pipelines. Failure to do so will result in many interesting scientific papers, but limited impact to the field of regulatory biomonitoring.

Key messages

Getting it right, good study design

We can accurately analyse DNA molecules from a multitude of biological sources, ranging from individuals, bulk samples and environmental DNA sources (Creer et al., 2016). The Think Pieces cover specific definitions of source DNA, but in order for a study focused on particular species to succeed, some simple considerations need to be met. The study should (a.) use primers and probes that are complimentary to the target species genome, (b.) use a region of the genome that will resolve particular species (c.) use a database that features the target species, (d.) effectively capture and preserve the DNA from the field and (e.) employ appropriate molecular biological and informatic workflows to analyse the data. If these simple steps are followed, using DNA to trace species will work, but if any one of these conditions are not met, some, or all of the taxa of interest will not be detected to the desired taxonomic or ecological level. Many accounts of DNA biomonitoring experiments not working will usually be attributable to one or more of the above points not having been met. In general, oversights are usually fixable and methods can evolve, tracking technological innovation.

Target taxa, pressures and ecological status

A key difference between existing biomonitoring and DNA-based approaches are that existing biomonitoring approaches are restricted to a small number of *a priori* defined, easily identified species. An analogy can be drawn to the field of ecotoxicology, where toxicodynamic studies are performed on a small suite of laboratory model organisms, but with limited success in extrapolating tolerances to wild populations (Perry and Kille, TP5). Conversely, the taxonomy ‘lens’ of DNA-based approaches is much wider and can encapsulate 100s-1000s of taxa from across the tree of life, from microbes, to megafauna. It is inefficient to try and replicate existing multi-species metrics using multi-taxon DNA tools and large amounts of data are ignored. That is analogous to buying a Tesla electric car and not pressing down hard on the accelerator pedal to see how fast it will go? Existing ecological indicator species have been proposed over many years of research effort via association with environmental reference conditions/ecological status (Hering et al., 2018). There are no conceptual reasons why the same process cannot happen with a much larger taxonomy pool derived from DNA evidence, that contain many fold more valuable indicator taxa, that will respond differentially to a broad array of ecosystem pressures (Seymour et al., 2020) and importantly, ecological change forced by anthropogenic environmental forces. A multivariate, multitaxon DNA ‘lens’ could provide both baseline and also pressure-derived metrics across intensive temporal and extensive spatial axes and would provide a step change in our environmental analytical abilities in relation to management decisions. The evolution of electric vehicle design has accompanied the recent genomic revolution and so if Tesla cars are available, you may as well see how fast they can go, self-drive and park themselves? It is the same situation with environmental biomonitoring; the technology is there, we just need to explore all possibilities of what can be achieved by using the technology. Perry and Kille (TP5) also

provide an excellent perspective that many approaches used for environmental biodiversity identification (e.g. qPCR, metabarcoding) have validated applications in the healthcare industry – why should the environment be any different?

Which ecosystem functions?

In reference to five indicators (B6, C8, C9, D7 and E7) of the 25 Year Environmental Plan (https://www.gov.uk/government/publications/25-year-environment-plan), requiring further research and development (R&D) with a focus on 'functions' and 'functioning', the Think Pieces all refer to different forms of ecosystem functions but ecosystems are complex, multifunctional entities (Hector & Bagchi, 2007; Zavaleta, Pasari, Hulvey, & Tilman, 2010). In order to reduce such complexity, stakeholders, in collaboration with industry and academia need to identify which functions are priorities (e.g. nutrient and organic matter fluxes, decomposition, food webs etc.) and co-design effective metrics associated with the target function. Irrespective of specific ecosystem functions, functionality is mediated by biodiversity structure and composition across multiple scales (Bush and Baird, TP1). The beauty of the DNA 'lens' is that biodiversity targets are broad, that novel metrics and indicator frameworks could be co-designed along desired gradients of pressures, functions and tipping points, just as environmental metadata is incorporated into hypothesis testing in macroecological studies.

Populating DNA reference libraries

The need for adequately populated reference databases were a common theme across all Think Pieces, ranging from standardised, single locus DNA barcodes, to plastid/mitochondrial genome 'skims' (i.e. superficial genome coverage usually yielding plastid and mitogenomes), or full reference genomes. Knowledge is power and a fully populated library of reference genomes would be hugely useful, but for metabarcoding applications, widely acknowledged as the current standard in high throughput biodiversity information, the simple and inordinately cheaper DNA barcode is still hugely powerful. For eukaryotes, the DNA barcode provides the link between genotype, phenotype, body mass, trophic mode and inferred ecological function, whereas metagenomes and sequenced microbial genomes leverage functional attributes of the prokaryotic biosphere. Irrespective of the level of genomic coverage, a standardised barcode approach will also leverage the opportunity to assess phylogenetic diversity by phylogenetic placement amongst larger datasets. One of the most effective routes to a fully populated reference database of varying genetic/genomic depth is effective engagement with the natural history community, from (e.g.) societies, museums, taxonomists, county recorders, citizen scientists and stakeholders to accelerate the acquisition of DNA friendly (e.g. preserved in ethanol or other suitable buffers, not formalin), reliably identified voucher specimens. Future funding streams can then be used to generate barcode data, linked to global data repositories. Stakeholder 'bioblitzes' are also very powerful routes to data acquisition, whereby experts are invited to collate, identify and sort diverse sample types from different localities and habitats.

New ways of looking at ‘big data’ biodiversity information

The average high-throughput biodiversity study is usually underexploited from an analytical and often taxonomic perspective. All Think Pieces are unanimous in acknowledging that the breadth of digital biodiversity information available is very large, yielding opportunities for network assessments, machine learning, multi- and joint species distribution and downstream predictive modelling (Cordier et al., 2017; Fruhe et al., 2020). Note also that beyond the human eye lies the most biodiverse size fractions of life, including prokaryotes, fungi, protists and meiofauna that are brimming with putative indicator species that will be sensitive to diverse ecological and functional pressures (George et al., 2019; Makiola et al., 2020). Moreover, since DNA approaches can be deployed in a high-throughput format, biodiversity shifts in nestedness, or turnover can be detected in the context of national and international metacommunities; insights that are essential for understanding large scale change, the distribution of non-native invasive and exploited species (Bush and Baird, TP1). It is also crucial to note that not all nodes of a high-throughput biodiversity inventory will be instantly linked to a Linnaean binomial species name, although with some low richness groups, such as river/lake fish, the framework is already mature (Hänfling et al., 2016). Nevertheless, global DNA databases are already sufficiently populated to annotate most taxa effectively to phylum/family, or even genus levels. The digital nature of DNA sequences means that species and environmentally derived DNA sequence data will eventually be linked as reference libraries are populated more in the future; a type of reverse taxonomy approach (Blaxter et al., 2005). Yu and Matechou (TP4) discuss operational taxonomic units (OTUs), but it is also important to acknowledge that individual DNA sequences, or amplicon sequence variants, that are sometimes combined to form OTUs, will be the traceable common currency of future biodiversity assessments. Both Clare (TP3) and Webb et al. (TP4) also highlight that DNA data, especially from a trophic perspective, transcend usual biome boundaries (e.g. bats foraging on freshwater macroinvertebrates, seabirds feeding on fish and export of carbon between habitats), leading to a joined up synthesis of ecosystem level biodiversity.

Limitations of DNA data: lots of quality vs. less quantity

The perennial challenge of integrating DNA based biodiversity data into standardised monitoring, or ecological hypothesis testing is uncertainty regarding the quantitative nature of the data (Creer et al., 2016). Yu and Matechou (WP4) present a very considered synthesis of the subject. Very good progress has recently been made with qPCR analyses, eDNA metabarcoding occupancy studies and with high-throughput pollen analyses, complimented by spike-in experiments and genome skimming. The very simple cellular structure of pollen (2-3 cells in most flowering plants) means that counting copies of genomic markers will be highly simplified compared to other multicellular life. Improving quantification is clearly an area for further research. Yet, the reliance on multi-copy taxonomy gene marker targets, residing in organelles that will differ in densities between species and the obvious fact that multicellular eukaryotes have different developmental stages and hence biomass, cannot be ignored. A valuable area for future research would be extensive testing of presence/absence DNA data vs. traditional data derived from

existing monitoring strategies to see which methods yield the most ecologically sensitive information. If biomass data are critical to management decisions, lab based/mesocosm style analyses will only yield so much information. Instead, taxon-targeted, field scale, intensive sampling in space (including depth in the marine/freshwater environments) and time investigations will most likely answer if DNA evidence is associated with the appropriate ecological signals, including spawning events and population dynamics, to inform effective management decisions (Webb et al. TP2). The synthesis presented throughout the Think Pieces provide compelling evidence that DNA data are ecologically relevant, both in time and space. Caveats exist associated with legacy environmental DNA adsorbed to older sediment deposits (Thomsen & Willerslev, 2015) and so should we move to focusing on RNA? It is tempting, but limiting environmental nucleic acid analyses to only RNA (i.e. to aim to only study the living community) conceptually reduces the ecological breadth and signal of DNA-based studies (Lejzerowicz et al., 2015) and also adds logistical challenges to field surveys due to the highly labile nature of RNA.

Opportunities for technological innovation

The past 20 years has witnessed a paradigm shift in DNA sequencing technologies, ramping up throughput to bewildering levels. Yet, technological advancements will continue and it is important to validate and embrace new technologies where appropriate and not be paralysed by them (Clare TP3). Irrespective of which laboratory or mobile analytical platform we use to characterise genomes, or biodiversity, the standardised barcode will usually anchor DNA data to its species, or genus identity, even if the barcode in the future is accompanied by organellar skims or whole genomes. Perhaps a noteworthy addition and learning from developments from ancient DNA analyses, is that standard metagenomic, or shotgun sequencing approaches are an inefficient way to analyse trace amounts of eDNA (e.g. aqueous eDNA) since the genomic signal of macrobial life is swamped by microbial genomes (Stat et al., 2017). Sampling intensity and efficiency for aqueous eDNA is also an area that could potentially benefit from improvement. Filtering turbid waters regularly clog filters, leading to small volume analyses. Moreover, filtering captures only a snapshot of the available eDNA and is field time intensive. Numerous studies have revealed how natural samplers (e.g. scavengers, or sponges) accumulate macrobial eDNA (Mariani, Baillie, Colosimo, & Riesgo, 2019; Siegenthaler et al., 2019), but incorporating natural harvesters into a regulatory context would likely be difficult to standardize. Subsequently, it has been proposed that 3D printed matrices, with high affinity for DNA (Verdier, Konecny, Marquette, & Lefebure, 2021), or automated samplers (Stern et al., 2015) could be used as *in situ* eDNA field collation tools, analogous to the Autonomous Reef Monitoring ARMS plates for monitoring substrate dwelling marine cryptic fauna (Leray & Knowlton, 2015). Adopting *in situ* field sampling devices could increase the temporal representation of eDNA signals and dramatically cut down field visits if seasonal representation is important to regulatory questions.

UK next generation biomonitoring: 25-year research and development roadmap.

Communication and collaboration

The UK DNA Working Group and DNA Centre of Excellence are the UK founding organizations of a globally networked group of stakeholders, industry representatives and academics. Maintaining such a relevant collaborative network will be essential for connectivity and co-shared successful design for future DNA biomonitoring, whilst also reducing wasted research time on poorly designed, disjointed studies. The community will also need clear direction as to which ecosystem functions, or aspects of multifunctionality that are to be the focus of biomonitoring over the next 25 years. If the latter is not possible, then ensuring that species, community, phylogenetic and proxies of functional biodiversity feature in novel metrics would be a conservative route forwards.

Standardization

Kat Bruce, CEO of Nature Metrics and Co-Leader of qua Working Group “Lab and field protocols” reminisced recently that the initial working group discussions regarding standardization of DNA based approaches to biomonitoring resulted in dozens of different opinions on what comprised good practice. Different approaches are part of the evolution of methods-based research, but where the group made considerable gains, was in identifying consensus on different aspects of analyses. Consequently, a standardized approach for sampling aqueous eDNA is currently being considered by the European Committee for Standardization (CEN), TC230/WG28 "DNA and eDNA methods” group and a validation scale to determine the readiness of targeted environmental DNA assays for routine species monitoring has also resulted from expert consensus opinion (Thalinger et al., 2021). Kristian Meissner, also from DNAqua-Net and convenor of the CEN " DNA and eDNA methods” group also recently offered sage advice pleading for the community to “stop writing papers and start writing standards’, since standards are analogous to taking safe products to market in commercial terms. Standardizing metabarcoding workflows are also achievable (c.f Earth Microbiome project (Thompson et al., 2017)) and is certainly necessary. In order to prioritise specific applications, this would be a fruitful area of development facilitated via the COE, complemented by inter-laboratory comparisons and ring testing (https://aca.pensoft.net/article/65142/download/pdf/).

Lobbying for, creating and deploying novel funding pipelines

From a UK perspective, many UKRI funding streams are focussed on scientific excellence and it is often challenging to successfully acquire competitive funding for methods development and standardization of existing methodologies. Thus, funding headway needs to be made in leveraging the appropriate resources from both end user and stakeholder groups, industry, academia and funding councils to conduct the necessary R&D, validation and standardization that is needed.

Realising novel metrics derived from large, intensive and integrated analytical programs focused on management decisions/outcomes

Whether considering freshwater, marine, terrestrial or airborne biodiversity, the real gains for environmental biomonitoring will be from the analysis of multiple suites of taxa derived from large sampling campaigns, designed around key stressors, or ecosystem functional axes at appropriate spatial and temporal scales. The molecular lab workflows and analyses are the easy part. The principal challenge is the field sampling and collection of environmental metadata that could be augmented via collaboration between agencies, industry and academics, but also via suitably trained citizen scientists. Importantly, if novel metrics are the focus of any such analysis, DNA extracts should be interrogated for microbial, meiofaunal and macrobial life forms, with a similar rhetoric applied to the illumination of food webs and ecological networks. With the appropriate study design, such studies would answer a raft of questions. Importantly, the data would also leverage opportunities for intensive data exploration, training and test sets for machine learning approaches and multi- and joint predictive distribution species modelling, complemented by earth observation approaches for certain habitats (Yu and Matechou, TP4). DNA approaches will not replicate existing metrics (Cordier et al., 2020) but the intensity of biodiversity and associated ecological niche space that will be measured will accurately reflect ecological status depending on management outcomes. The latter approach was first exemplified in terrestrial ecosystems in Ji et al. (2013) and has since been proven in marine environments (Fruhe et al., 2020) (https://aca.pensoft.net/article/65421/) focusing on a range of macro and microbial taxa. Perry and Kille (TP5) also refer to ‘Specific Measurable Achievable Relevant and Time-constrained’ (SMART) metrics, principles that could guide future development. Targeted species biomonitoring could follow a similar, but revised strategy as for the Great Crested Newt program (Biggs et al., 2015), noting that it is still, at the time of writing, adhering to outdated and inefficient ethanol precipitation DNA extraction methodologies. Moreover, multiplexing lower numbers of suites of taxa would clearly enhance throughput if targeted biomonitoring was desirable for a number of key indicators, or non-native species, accompanied by controls for assigning confidence to certainty and uncertainty.

Populating reference libraries

Acquiring DNA barcode references for all 70,000 UK eukaryotic species should be a simple task and, depending on which approach used, should also be affordable, with a lower estimate of £70,000 and an upper estimate of £700,000 for different sequencing approaches. Individual labs will be concurrently skimming and publishing genome data and consortia like the Darwin Tree of Life and Earth BioGenome project will be creating chromosomal level, polished genomes. Note however, that Yu and Matechou (TP4) correctly state that ‘the era of ubiquitous genome sequence data lies some years ahead’. Irrespective of the nature of the genetic reference data, as long as DNA vouchering proceeds against the backdrop of the next generation of biomonitoring, eventually the two datasets will talk to each other seamlessly. For the purpose of UK biomonitoring, species gap analyses need to be conducted (Weigand et al., 2019), to identify which are the UK’s

priority taxa that need reference data. Subsequently, depending on resource availability, species need to be barcoded at the minimum, including vouchering DNA and metadata, with DNA extracts cryopreserved for future skimming or genome level sequencing where funds are available. Collaboration between end users, museums, taxonomists, citizen scientists, academics and Barcode of Life initiatives will enable rapid progress in this endeavour.

Priorities and likely trajectories

Assuming continuation of the UK DNA Working Group/CEO collaborative nature of the development of next generation biomonitoring in the UK, funding has to be the first priority, since without resources, we will not progress the field. Once funding has been acquired, the generation of novel metrics according to management outcomes should then be the next immediate priority, followed by inter laboratory validation and standardization. The construction of priority reference libraries needs to be concurrent and so a small proportion of yearly resources could be diverted to the generation of the necessary databases, prioritised according to consensus agreement. The way that we analyse DNA, whether via high-throughput biodiversity assessments, or genome sequencing will evolve, since innovators will always be pursuing longer sequences, mobile technologies and chromosomal level mapping, primarily driven by market interests in providing accurate sequencing solutions for human healthcare. Thus, longer length barcoding approaches are already here (Callahan et al., 2019; Hebert et al., 2018), which means primer regions and primers will change in the future, but any standardized framework would have to follow tried, tested and available methodologies. Nevertheless, another small proportion of budget resource could be allocated to future proofing approaches and (e.g.) ten yearly reviews could assess if emerging technologies are fit for purpose by reanalysing the cryopreserved DNA extracts from the forthcoming metrics assessments. Such an approach would mean that the DNA 'lens' would become larger and uncover more (phylo)genetic/genomic information from the target communities to potentially enhance our ability to optimise community resilience to forthcoming change.

The UK is at a potentially exciting post-Brexit crossroads in how we perform environmental biomonitoring. When, Kerry Walsh and Doug Wilson of the Environment Agency, amongst others, convened the first DNA Working Group meeting at FERA in York, 2014, there was a tangible lack of confidence in DNA approaches from some members of the stakeholder audience. Seven years on and after witnessing so many successful case studies of how to use DNA approaches effectively in biomonitoring, opinions have changed. Our challenge now is to realise our collective ambitions to transform aspects of UK regulatory biomonitoring. Before writing this synthesis, I read all the Think Pieces and would recommend all readers to move on to discover the collective opinions of the visionary writings that follow from Baird and Bush, Webb et al., Clare, Yu and Matechou and Perry and Kille that cover in finer detail freshwater, marine, terrestrial, socio-economical, analytical and technical perspectives concerning the next generation of UK biomonitoring. Consideration of their collective thoughts will be key to identifying the appropriate route forwards and securing SMART approaches to UK environmental biomonitoring and optimising ecological status, functional and socio-economically beneficial biodiversity for the 21st Century.

References

BIGGS, J., Ewald, N., Valentini, A., Gaboriaud, C., Dejean, T., Griffiths, R. A., . . . Dunn, F. (2015) Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (*Triturus cristatus*). Biological Conservation, 183, 19-28. doi:10.1016/j.biocon.2014.1011.1029.

BLAXTER, M., Mann, J., Chapman, T., Thomas, F., Whitton, C., Floyd, R., & Abebe, E. (2005) Defining operational taxonomic units using DNA barcode data. Philosophical Transactions of the Royal Society B-Biological Sciences, 360(1462), 1935-1943. doi:10.1098/rstb.2005.1725.

CALLAHAN, B. J., Wong, J., Heiner, C., Oh, S., Theriot, C. M., Gulati, A. S., . . . Dougherty, M. K. (2019) High-throughput amplicon sequencing of the full-length 16S rRNA gene with single-nucleotide resolution. Nucleic Acids Research, 47(18). doi:10.1093/nar/gkz569.

CORDIER, T., Alonso-Saez, L., Apotheloz-Perret-Gentil, L., Aylagas, E., Bohan, D. A., Bouchez, A., . . . Lanzen, A. (2020) Ecosystems monitoring powered by environmental genomics: A review of current strategies with an implementation roadmap. Molecular Ecology. 00, 1-22. doi:10.1111/mec.15472.

CORDIER, T., Esling, P., Lejzerowicz, F., Visco, J., Ouadahi, A., Martins, C., . . . Pawlowski, J. (2017) Predicting the Ecological Quality Status of Marine Environments from eDNA Metabarcoding Data Using Supervised Machine Learning. Environmental Science & Technology, 51(16), 9118-9126. doi:10.1021/acs.est.7b01518.

CREER, S., Deiner, K., Frey, S., Porazinska, D., Taberlet, P., Thomas, W. K., . . . Bik, H. M. (2016) The ecologist's field guide to sequence-based identification of biodiversity. Methods in Ecology and Evolution, 7, 1008-1018. doi:10.1111/2041-210X.12574.

DIETZ, T., Ostrom, E., & Stern, P. C. (2003) The struggle to govern the commons. Science, 302(5652), 1907-1912. doi:10.1126/science.1091015.

FRUHE, L., Cordier, T., Dully, V., Breiner, H. W., Lentendu, G., Pawlowski, J., . . . Stoeck, T. (2020) Supervised machine learning is superior to indicator value inference in monitoring the environmental impacts of salmon aquaculture using eDNA metabarcodes. Molecular Ecology. 00, 1-19. doi:10.1111/mec.15434.

GEORGE, P. B. L., Lallias, D., Creer, S., Seaton, F. M., Kenny, J. G., Eccles, R. M., . . . Jones, D. L. (2019) Divergent national-scale trends of microbial and animal biodiversity revealed across diverse temperate soil ecosystems. Nature Communications, 10. doi:10.1038/s41467-019-09031-1.

HEBERT, P. D. N., Braukmann, T. W. A., Prosser, S. W. J., Ratnasingham, S., dewaard, J. R., Ivanova, N. V., . . . Zakharov, E. V. (2018) A Sequel to Sanger: amplicon sequencing that scales. BMC Genomics, 19. doi:10.1186/s12864-018-4611-3.

HECTOR, A., & Bagchi, R. (2007) Biodiversity and ecosystem multifunctionality. Nature, 448(7150), 188-U186. doi:10.1038/nature05947.

HERING, D., Borja, A., Jones, J. I., Pont, D., Boets, P., Bouchez, A., . . . Kelly, M. (2018) Implementation options for DNA-based identification into ecological status assessment under the European Water Framework Directive. Water Research, 138, 192-205. doi:https://doi.org/10.1016/j.watres.2018.03.003.

HÄNFLING, B., Lawson Handley, L., Read, D. S., Hahn, C., Li, J., Nichols, P., . . . Winfield, I. J. (2016) Environmental DNA metabarcoding of lake fish communities reflects long-term data from established survey methods. Molecular Ecology, 25(13), 3101-3119. doi:10.1111/mec.13660.

JI, Y. Q., Ashton, L., Pedley, S. M., Edwards, D. P., Tang, Y., Nakamura, A., . . . Yu, D. W. (2013) Reliable, verifiable and efficient monitoring of biodiversity via metabarcoding. Ecology Letters, 16, 1245-1257. doi:10.1111/ele.12162.

LEJZEROWICZ, F., Esling, P., Pillet, L., Wilding, T. A., Black, K. D., & Pawlowski, J. (2015) High-throughput sequencing and morphology perform equally well for benthic monitoring of marine ecosystems. Scientific Reports, 5. doi:10.1038/srep13932.

LERAY, M., & Knowlton, N. (2015) DNA barcoding and metabarcoding of standardized samples reveal patterns of marine benthic diversity. Proceedings of the National Academy of Sciences of the United States of America, 112(7), 2076-2081. doi:10.1073/pnas.1424997112.

MAKIOLA, A., Compson, Z. G., Baird, D. J., Barnes, M. A., Boerlijst, S. P., Bouchez, A., . . . Bohan, D. A. (2020) Key Questions for Next-Generation Biomonitoring. Frontiers in Environmental Science, 7. doi:10.3389/fenvs.2019.00197.

MARIANI, S., Baillie, C., Colosimo, G., & Riesgo, A. (2019) Sponges as natural environmental DNA samplers. Current Biology, 29(11), R401-R402. doi:10.1016/j.cub.2019.04.031

SEYMOUR, M., Edwards, F. K., Cosby, B. J., Kelly, M. G., de Bruyn, M., Carvalho, G. R., & Creer, S. (2020) Executing multi-taxa eDNA ecological assessment via traditional metrics and interactive networks. Science of the Total Environment, 729. doi:10.1016/j.scitotenv.2020.138801.

SIEGENTHALER, A., Wangensteen, O. S., Soto, A. Z., Benvenuto, C., Corrigan, L., & Mariani, S. (2019) Metabarcoding of shrimp stomach content: Harnessing a natural sampler for fish biodiversity monitoring. Molecular Ecology Resources, 19(1), 206-220. doi:10.1111/1755-0998.12956.

STAT, M., Huggett, M. J., Bernasconi, R., DiBattista, J. D., Berry, T. E., Newman, S. J., . . . Bunce, M. (2017) Ecosystem biomonitoring with eDNA: metabarcoding across the tree of life in a tropical marine environment. Scientific Reports, 7. doi:10.1038/s41598-017-12501-5.

STERN, R. F., Picard, K. T., Hamilton, K. M., Walne, A., Tarran, G. A., Mills, D., . . . Edwards, M. (2015) Novel lineage patterns from an automated water sampler to probe marine microbial biodiversity with ships of opportunity. Progress in Oceanography, 137, 409-420. doi:10.1016/j.pocean.2015.04.015.

THALINGER, B., Deiner, K., Harper, L. R., Rees, H. C., Blackman, R. C., Sint, D., . . . Bruce, K. (2021) A validation scale to determine the readiness of environmental DNA assays for routine species monitoring. Environmental DNA, 00, 1-14. doi:https://doi.org/10.1002/edn3.189.

THOMPSON, L. R., Sanders, J. G., McDonald, D., Amir, A., Ladau, J., Locey, K. J., . . . Earth Microbiome Project, C. (2017) A communal catalogue reveals Earth's multiscale microbial diversity. Nature, 551(7681), 457. doi:10.1038/nature24621.

THOMSEN, P. F., & Willerslev, E. (2015) Environmental DNA - An emerging tool in conservation for monitoring past and present biodiversity. Biological Conservation, 183, 4-18. doi:10.1016/j.biocon.2014.11.019.

VERDIER, H., Konecny, L., Marquette, C., & Lefebure, T. (2021) Towards a simple way to collect eDNA using a 3D-printed passive sampler. ARPHA Conference Abstracts, 4, e65499.

WEIGAND, H., Beermann, A. J., Ciampor, F., Costa, F. O., Csabai, Z., Duarte, S., . . . Ekrem, T. (2019) DNA barcode reference libraries for the monitoring of aquatic biota in Europe: Gap-analysis and recommendations for future work. Science of the Total Environment, 678, 499-524. doi:10.1016/j.scitotenv.2019.04.247.

ZAVALETA, E. S., Pasari, J. R., Hulvey, K. B., & Tilman, G. D. (2010) Sustaining multiple ecosystem functions in grassland communities requires higher biodiversity. Proceedings of the National Academy of Sciences of the United States of America, 107(4), 1443-1446. doi:10.1073/pnas.0906829107.

Think-Piece 1: Development of new DNA-based metrics to understand ecosystem functioning and the resilience of freshwater systems in response to environmental change

Alex Bush [1] and Donald Baird [2]

[1] Lancaster Environment Centre, Lancaster University, United Kingdom

[2] Canadian Rivers Institute, Department of Biology, University of New Brunswick, Canada

Contents

Tables

Summary

DNA-based techniques have the potential to dramatically alter the way we approach environmental monitoring because they largely remove the taxonomic constraints that dictated the practicality of many applications in the past. We have arguably become accustomed to using proxies and indicators, rather than directly measuring ecological features of interest or testing the relationship between them. Using DNA-based methods we can now afford to take a more holistic view of the ecosystem.

Beyond the development of more powerful models to detect shifts in occurrence of taxa or communities, understanding how those patterns relate to ecological functioning remains challenging. Not only are ecological functions and services often difficult to measure themselves, but the differences in the relative spatial and temporal scale of functions and services, as well as with ecological communities is rarely testable with current survey designs. For example, the ability to detect the “activity” of functional genes in microbial communities is an exciting development, but how representative those observations are of processes relevant to human activity is much less certain. Finally a major issue in any discussion of ecosystem function is that any single facet will only be one perspective of a multidimensional system, and that ecosystems do not maximize all functions simultaneously.

There are very few cases in which ecologists have been able collect sufficient high-quality data to describe dynamics of natural ecological communities (all biomes, not just freshwater). This has compromised our ability to draw conclusions and react to any changes observed through biomonitoring. There are a variety of ways in which we can enrich our description of the communities present on the basis of functional traits and phylogenetic relationships, but this is often limited to a few localities (alpha diversity). Interpretation requires a broader understanding of context, and hence appreciation of how diversity changes between sites (beta-diversity) and is drawn from regional species pools (gamma-diversity). Therefore, we argue a key focus for improving our understanding of how taxa contribute to functioning should be a **focus on quantifying the structure of metacommunities** in which they operate.

In addition to better understanding how communities evolve over time and space, DNA-based surveys also offer the ability to start collecting data across multiple trophic levels. Methods to collate networks from traits data are already available for macroinvertebrates, and a growing scientific awareness of this field has been accompanied by increasing numbers of diet-studies that will refine further development. An exciting area for future research is therefore likely to be studying **whether there are predictable characteristics of networks that can be used to reflect landscape management and environmental change?**

A more holistic understanding of metacommunity structure and assemblage-structure (interaction networks) will improve how we interpret the status of “nodes” within those networks, be they particular sites or species. However, what is crucial about each of these concepts is the nature of stability, and the processes that govern which communities can

support high-biodiversity locally (alpha), and regionally (beta and gamma). Decades of theory and experimental studies support the notion that high alpha diversity will support higher functionality. Whether it is best reflected by taxonomic, functional or phylogenetic diversity is not yet confirmed. Spatial and temporal variability introduces further diversity to an ecosystem, providing resilience to disturbances. Therefore, **by focusing on how we can promote ecological stability, we implicitly retain biodiversity and the critical processes required to support ecological functionality**.

Introduction

Only 1% of the earth's surface is covered by freshwaters but they are habitat for over 10% of all animals and over 35% of all vertebrates (McRae et al., 2008). Freshwater biodiversity is both a valued resource that is dependent on the state of the system, but also makes a substantial contribution to the provision of other ecosystem functions which are beneficial to society. Resilient freshwater systems are thus vital to supporting healthy human populations and achieving sustainable development, and this is recognised in the UK Government's 25 Year Environment Plan (25YEP). Providing clear understanding of how environmental condition is defined, and how this responds to management actions is key to effective intervention, and the Outcome Indicator Framework includes numerous targets that directly or indirectly concern freshwater. We recognise that further work will be required before all targets concerning 'functioning' can be defined, but emphasise we can immediately begin to define basic properties of the ecosystems structure. This document will focus on the challenges of describing, interpreting and quantifying metrics of ecosystem resilience and function in freshwater ecosystems, and suggesting how the necessary data for new metrics could be generated, by taking advantage of recent technological advances in high-throughput DNA-based sequencing.

Ecosystem function: mediated by biodiversity

The Outcome Indicator Framework identifies 66 indicators in 10 themes that will be used to support a conceptual framework based on natural capital accounting, identify pressures acting upon natural capital assets, assess the condition of assets, and highlight the services and/or benefits that are provided by natural capital. The focus of this document is on the opportunities provided by DNA-based techniques to deliver indicators in freshwater ("theme B") and for wildlife ("theme D"), in particular indicator B6 on sustaining the functioning of freshwater and wetland ecosystems, and indicator D7 on the status of species supporting ecosystem functions. Although the focus of this document is on metrics of functioning, we wish to make it clear that the data required to address functioning inherently overlap with data required to characterise freshwater condition in a number of other indicators, such as B3 ("State of the water environment"), as well as multiple indicators in theme D (e.g., D1, D2 and D4). Indeed, it is because DNA-based techniques have shown such promise in delivering data for these other metrics of environmental health that there is interest in their use for addressing ecosystem function. In this Think-Piece we also address why existing methods of data collection and ecological research have not been suited to developing insights into functioning at large scales.

Ecosystem functions are ecological processes that control the fluxes of energy, nutrients and organic matter through an environment (e.g. primary production converts inorganic matter into biological tissue; and decomposition which breaks down organic material to be recycled). Biodiversity is important for generating and stabilising ecosystem functions, and thus ensures the provisioning of numerous ecosystem services to society, but there are mismatches in the spatial and temporal scales at which the relationships between anthropogenic drivers, biodiversity, ecosystem functioning and ecosystem services are best understood (see Figure 1 in Isbell et al., 2017). At the small scale of empirical studies,

species losses often decrease the efficiency with which ecological communities capture essential resources such as nutrients, and ultimately produce biomass. At intermediate scales pertinent to land-use decisions, management decisions typically prioritise production of food and other materials at the expense of other ecosystem services such as regulation. The concern is that multiple ecosystem services are now potentially being undermined at the landscape scale because we have not accounted for how anthropogenic drivers affect functions, which in turn are mediated by biodiversity.

Hundreds of theoretical, experimental, and observational studies across different types of ecosystems and biomes (Cardinale, et al., 2012) have confirmed positive effects of local-scale biodiversity on ecosystem functions (BEF). Most evidence is for plants and the linkages between local species richness (α-diversity) and biomass productivity, but increasingly research has expanded the focus on productivity, and simultaneously considers the effects of diversity on multiple ecosystem functions. In freshwater ecology there has been a strong focus on litter decomposition with generally similar conclusions (Gessner et al., 2010; Hooper et al., 2012). Despite this significant scientific effort, it remains difficult to predict the extent to which anthropogenic changes in biodiversity will alter ecosystem services, especially at the spatial and temporal scales relevant to policy and conservation. This difficulty stems from a scale mismatch in our knowledge of the state of biodiversity and hence its response and influences on functions at larger scales, and ultimately how this influences ecosystem services valued by society.

There is now greater recognition of this mismatch, and that as well as alpha-diversity, community distinctiveness (β-diversity), and the richness of the regional metacommunity (γ-diversity) are important. This is particularly true once multiple functions are considered, and for maintaining functionality within heterogeneous landscapes (Mori, et al., 2018; Pasari, et al., 2013; Winfree et al., 2018). Sustaining resilient freshwater systems whose biodiversity can continue to support associated ecosystem functions is an incredibly challenging issue because virtually all systems are impacted, or are vulnerable to, multiple stressors. Just as no single characteristic of quality could describe the complexity of an ecosystem's physical and biological processes; no single metric is likely to reflect an ecosystems resilience to all threats. Instead, ***ecosystem functioning is mediated by biodiversity structure and composition across multiple scales (α, β, γ)***. This differs from many metrics that consider only a narrow aspect of an ecosystem in isolation, but fails to address their inter-relationships. In our view, it is now critical to address this critical knowledge gap regarding how ecological structure (including taxonomic, functional, and phylogenetic characteristics), and functioning of ecosystems varies in response to the increased spatial and temporal heterogeneity of landscape-level stressors. Together, these features underpin ecosystem function (either by facilitating a process, or by damping extreme fluctuations), encompassing scales of organisation from local to national.

Freshwater as a distinct system

Despite the critical importance of healthy freshwater ecosystems to sustaining a range of services to society, the global loss of biodiversity has been most rapid and most widespread in freshwater ecosystems (Hooper et al., 2012). In the 30 years between 1970

and 2000, populations of more than 300 selected freshwater species declined by ~55% while those of terrestrial and marine systems each declined by ~32% (McRae et al., 2008). Given our incomplete and fragmented knowledge of freshwater fauna and flora, those estimates are likely to be significantly underestimated. Furthermore, international conventions addressing global biodiversity loss do not appear to have reduced the rate of decline (WWF, 2018). The rate of loss in freshwater is due to numerous stressors extending from habitat loss and degradation, overexploitation, pollution, flow modification through to invasion by exotic species. The effects of global processes such as climate change and altered nutrient cycles are further superimposed upon local stressors (Steffen et al., 2015). As human populations increase and the intensity of landscape management rises so has the impact on freshwater systems.

Although freshwater systems still face significant challenges to their ecological integrity, significant recovery of UK freshwater habitats has occurred in the past 20-30 years (Durance and Ormerod, 2009). This is a direct result of directives, enforced by standards to control the discharge of contaminants into surface waters, from urban wastewater, sewage treatment facilities and agricultural practices. These improvements are believed to have delayed or mitigated the severity of ecosystem responses that could have been expected as a result of observed climate warming (Durance and Ormerod, 2009; Vaughan and Gotelli, 2019). In addition, demands upon freshwater ecosystems are likely to remain strong, or intensify in the future, and new metrics are needed to indicate the early stages of ecosystem goods and services decline, and to support evidence-based policies to protect, mitigate and, if needed, restore loss of function.

Freshwater ecosystem functions and management goals

It is beyond our scope to review the literature for ecosystem services and functions related to freshwater, but there are some key principles we should bear in mind. First is the acknowledgement that just as ecosystem functions operate at different spatial and temporal scales to that of biodiversity attributes, ecosystem services may not always align with ecosystem functions (e.g. Nicholson et al., 2019). Secondly, a related consequence of differences in scaling is that impacts on functioning may not be immediately apparent, or may appear at a distance from where biodiversity loss or gain was observed (e.g. increase in flood risk and downstream soil erosion due to burning and clearance of UK uplands; Douglas et al., 2015; Orr, et al., 2008). Third, trade-offs between functions are inevitable, and therefore it may not always be prudent to describe changes in functioning as either “good” or “bad”, based only on observations of a single function.

The anthropocentric view that we can manage ecosystems for an “optimum” degree of functioning is thus conceptually distinct from traditional bioassessment based on preserving a defined reference state. Yates et al. (2019) recently reviewed this topic for rivers, identifying a variety of potential metrics of functioning. The study suggests a number of indicators that could directly or indirectly provide proxies for functioning based on observed states of microbial, algal, invertebrate or plant communities. Nonetheless, we must stress that surrogates still have their limits, and without understanding the context of

their ecology, these metrics do not identify how managers should respond to signs of depressed or declining functioning.

Stability

"Nature likes to overinsure itself. Layers of redundancy are the central risk-management property of natural systems." by Nassim Taleb, from Antifragile: Things That Gain From Disorder

Yates *et al.* (2019) show how we can begin to match certain metrics directly and indirectly to particular functions. But this approach is piecemeal, and is inherently blind to possible trade-offs. How then are we to manage for functioning if we cannot yet identify all the relevant functional pathways? A more holistic alternative, with parallels across all fields of natural sciences, is to manage for stability. The pressing concern for environmental managers is when widespread loss of biodiversity will begin to seriously hinder ecological functioning (and services), and how we can track its decline or improvement. Accepting that biodiversity mediates ecosystem functioning, and that in many cases, greater biodiversity promotes more efficient functioning, it is prudent, even within this anthropocentric approach to seek to retain biodiversity locally and regionally. Understanding how to retain a greater proportion of biodiversity, or even just a critical subset is a question of stability, and a more thorough understanding of its nature will inform both the strength of the relationships between biodiversity and functioning, as well as how different stressors need to be mitigated. Thus metrics of stability could confer more effective, or less variable, functioning, without pertaining to a specific function per se.

Stability in ecology is complex, but perhaps the main reason why our understanding of stability remains limited by unclear and conflicting results is that the vague definition of stability itself (Donohue et al., 2016). For example studies have shown that biodiversity can enhance stability (e.g. Cardinale et al., 2013), others have found the opposite result (Yodzis, 1981). In fact these studies do not directly contradict one another because stability is multidimensional and can be described by different metrics, not all of which correlate positively with biodiversity (Hillebrand, Langenheder, et al., 2018). For example compositional stability can promote or hinder functional stability. Empirical and theoretical studies show more diverse assemblages maintain stable productivity over time because they allow for more rapid changes in species composition through compensatory dynamics (Cottingham, et al., 2001). Therefore at a local scale, functional and compositional stability are negatively correlated, because the increase in functional stability is a direct consequence of high compositional turnover (Allan et al., 2011). By contrast, composition and functioning are more often strongly linked when recovering from press disturbances (rather than fluctuations) (de Boer, et al., 2014), because functional recovery depends on the recovery of the pre-disturbance composition (Guelzow, et al., 2017).

Five components of ecological stability are in common use (Pimm, 1984):

1. **Asymptotic stability (0/1);** does the system tend towards an equilibrium?
2. **Variability (CV, %);** spatial and temporal variability, the inverse of stability.

3. **Persistence:** length of time before a system changes state. State can be defined many ways, including invasion of new species or loss of native species.
4. **Resistance:** ratio of system variable after, vs. before some pulse disturbance.
5. **Resilience:** the rate at which a system returns to its equilibrium after a pulse disturbance. Rate of recovery is most influenced by the *least* abundant species included in a description of the ecosystem.

The multidimensionality of stability is crucial. It means we could significantly underestimate the impacts of disturbances if we only chose to monitor a single measure of stability, or a metric that reduced the complexity of stability to a single dimension. For example, many policy documents have described stability as ‘a measure of the persistence of systems and of their ability to absorb change and disturbance and still maintain the same relationships between populations or state variables’ (Holling, 1973). This combines persistence, resistance and the existence of not just one local asymptote, but multiple stable equilibria. If there is a limit beyond which a system cannot return directly to its former state, this is termed a tipping point.

The threat of tipping points sparked interest in developing metrics that could serve as Early Warning Signals (EWS). Features of EWS include increasing temporal autocorrelation and rising variance that indicate longer recovery times from small disturbances, known as “critical slowing down” (CSD; Scheffer et al., 2009). Indicators of CSD could suggest a system is close to a tipping point, and further small disturbances could push the ecosystem into alternative stable states (Dakos et al., 2012). A major drawback of such EWS, however, is that long-term, uninterrupted, and high resolution data records are needed (Scheffer et al., 2009). In addition, to apply EWS, ecosystems must have multiple stable states, and yet most systems do not display such transitions, and not all regime shifts are preceded by CSD (Dakos, et al., 2015). EWS theory and simulations also make a number of assumptions that simplify the variability of landscapes and species (Dakos et al., 2019; Nijp et al., 2019). For example spatial heterogeneity of real-world landscapes can improve resilience (i.e. the reversibility of regime shifts), and spatial environmental variability, as well as anthropogenic factors, can affect how assemblages respond. Thus to summarise, EWS could capture some aspects of stability, particularly variability, but as they an emergent property of complex shifts in ecological structure, relying on them is risky because they do not account for other dimensions of stability.

Ecosystems are not random assemblages, they are highly structured networks in which species are the nodes of energy and resources, and biotic interactions result in fluxes exchanged between them. Thus ecological networks, and trophic networks in particular, have a strong impact on the functional efficiency of an ecosystem. Indeed a common paradigm within ecosystem functioning research has been to break down the view of biogeochemical pathways into elements of biodiversity that contain reserves of energy and resources (e.g. Naeem, et al., 2012). How networks promote stability and thereby maintain biodiversity has been a long-standing central theme of food-web research (May, 1972). However, despite a substantial increase in the number of network studies, ecological structure is still difficult to quantify in natural systems, and perhaps more importantly, there

is still a lack of theory to guide how we should interpret changes to food webs for ecosystem functioning (Barnes et al., 2018; Gravel, et al., 2016).

Based on turnover of community composition and structure across natural and anthropogenic gradients, as well as the balance between stochastic and deterministic variability, we can begin to quantify when deviations in structure are likely to represent meaningful departures from natural variability in ecosystem state. The “equilibrium” of the components of stability is harder to define community composition than say productivity or contaminant removal, but many methods now exist to reduce the complexity and compare shifts in n-dimensional hypervolume (Jarvis et al., 2019). Ecosystem stability at large-scales is therefore a function of assembly processes that allow communities to maintain the persistence of all species overall so that ecosystems and functions can recover and adapt. This includes recolonisation and recovery of local diversity following disturbances (assuming a driver of change has been removed).

As well as providing context necessary for interpreting specific functional metrics, supporting processes that maintain stability is likely to indirectly support numerous other functions too.

DNA as data

The variety of techniques available to analyse DNA has been reviewed many times before (e.g. Leese et al., 2018; Porter and Hajibabaei, 2018), and we will not compare all their relative benefits here. Nonetheless, we would assert that the most promising new technique currently available for supporting new management metrics is DNA metabarcoding. The reference libraries are far more developed for amplicon sequencing than for other techniques, and metabarcoding is considerably cheaper. As we will discuss, our ability to detect changes in ecosystem structure over large spatial scales, or even in the variation of ecosystem structure, depends upon being able to process more samples, and hence cost is a critical factor. Although PCR-free metagenomic techniques could remove a source of error, and provide more quantitative data, it is currently cost-prohibitive for routine application. Other methods such as transcriptomics or whole-genome sequencing are yet more expensive, as well as being narrowly focused on individuals of a single taxa. Although there are instances in which we will wish to survey for single species (e.g. invasive species or non-destructive sampling for rare taxa), the most likely metrics to inform functioning, and biomonitoring in general, are community-based. Nonetheless, techniques other than metabarcoding are still important for improving biomonitoring, particularly for refining our knowledge of species-response traits. DNA provides a common currency for comparison across these fields of study, and, if the appropriate metadata are recorded, new techniques can be introduced to supersede metabarcoding in the future.

Of course metabarcoding is also a diverse field, and studies using metabarcoding for environmental surveys can be further broken down by the medium from which DNA is extracted. Extraction can be performed directly from the sampled biological material, hereafter referred to as bulk sampling, or from the fixative used to preserve a sample, or as eDNA from water (i.e. without intentionally sampling living organisms)(Blackman et al.,

2019). Inherently the biomass with bulk-samples overwhelms the likelihood of detecting eDNA, and therefore arguably provides the closest match to organisms collected at the time and place of the survey (Hajibabaei et al., 2019). The release of DNA from different tissues and taxa is unknown, but if there is interest in post-hoc analysis of sampled material, fixative-DNA is potentially a viable alternative to bulk-DNA (Erdozain et al., 2019; Hajibabaei, et al., 2012). Lastly, the spatio-temporal ambiguity of eDNA samples is well documented (Cristescu and Hebert, 2018; Deiner et al., 2017), and while there is still great interest in reducing this uncertainty (Harrison, et al., 2019), it is likely to remain a significant concern regarding its use.

Given the above trade-offs, we will instead refer to DNA-based techniques in general terms, as tools to generate taxonomic lists from samples taken at particular locations and times. The main properties of interest when describing the data generated from DNA are 1) taxonomic resolution, 2) sample similarity, 3) taxon misidentification, and 4) taxon abundance. We review these issues in detail in Bush et al. (2019), and therefore only summarise the key outcomes here.

Taxonomic Resolution: A major benefit of DNA-based techniques is the ability to consistently identify any group to a fine taxonomic resolution. Ecological theory is built around an understanding of species, and decades of debate and studies have clearly indicated that the use of coarse resolution taxonomy blurs our impression of ecosystem state (Jones, 2008). Fine (i.e. genus/species) taxonomic resolution could be less important if niches are conserved among related taxa, but this is an oversimplification of the complexity of niche space, and an increasing number of studies have shown substantial differences in the sensitivity of closely related species (Beermann, et al., 2018; Macher et al., 2016).

Resolution with metabarcoding depends upon the primers used and no single primer or primer set currently exists which can support detection of all species in an ecosystem equally. The term “universal primers” is used to describe primers with broad taxonomic coverage, but in all cases they fail to encompass all taxa. Thus, taxonomic bias introduced by primer selection, and therefore the choice of primers is a critical feature of any attempt to interpret multiple surveys.

Sample Similarity: Imperfect detection is a universal problem in ecology, and since the development of the first hierarchical models to account for these issues, there has been a rapid growth in our understanding of its consequences (Guillera-Arroita, 2017). Models are not improved by adding more data, they must acknowledge the inherent biases in the data collection itself. Without doing so our confidence in our inference is potentially misplaced. We show in Bush et al. (2019) that DNA-based macroinvertebrate surveys can be *more efficient* than traditional techniques (i.e. higher probability of detecting taxa), and the same is true of eDNA surveys for fish (reviewed by Ruppert, et al., 2019). Accounting for sampling error demonstrates that the efficiency of DNA-based techniques translates to greater statistical power to detect change (Bush et al. *in review* https://www.biorxiv.org/content/10.1101/819714v1). More detailed studies are beginning to show how the probability of detection could act as a common currency to compare DNA-

based studies that differ in their specific techniques (et al, 2018)(Griffin et al. 2019 *in press*).

Misidentification: This point specifically relates to the ***consistency*** with which identification can be achieved using DNA-based tools. This overlaps with the issues of taxonomic resolution above, because the likelihood of errors increases with taxonomic resolution. However, even ignoring the opportunity to generate finer resolution data, the rate of misidentification expected using DNA metabarcoding for aquatic macroinvertebrates is already lower than we would expect to occur in routine surveys at the same level (Bush et al., 2019). Misidentification is still possible of course, but the performance reflects the quality of reference libraries, and these continue to expand. The likelihood of misidentification is quantifiable, prioritising areas for improvement, and means it can be accounted for by hierarchical models (Davis et al., 2018). Finally, changes to taxonomy cannot be easily applied to old data, but because identification is now recorded in bioinformatics pipelines, it can be updated and rerun to use new algorithms and improvements to reference libraries.

Quantitative Data: Despite the substantial advantages outlined above, DNA metabarcoding has been criticised for its inability to return quantitative (count) data. Since primers with useful taxonomic resolution do not amplify the DNA templates of all taxa equally, the proportion of sequence reads generated for a particular taxon is not a dependable indication of biomass/abundance within a sample. However, it is also true that a single estimate of abundance/biomass is typically a very poor indicator of the true value for an ecosystem. A robust estimate of abundance for a single stream invertebrate will require so many samples to be collected that it would be impractical for routine application (Elliott, 1971). Lists of taxa generated by metabarcoding are therefore best interpreted conservatively as presence/absence observations. In some circumstances where observed composition of taxa is stable and therefore any bias due to primers is consistent, it may be possible to compare the *relative* number of sequences for a given taxa among samples (Ji et al., 2019). Stable composition is possible in closed systems like lakes, or if species-specific primers are used (Levi et al., 2018), but even this semi-quantitative measure is not always reliable (Rice, et al., 2018); and this approach does not allow for comparison among taxa. Given each estimate of abundance/biomass in a sample is so inherently variable, we would argue its value for biomonitoring is currently insufficient to justify the reduction in coverage elsewhere, as well as additional financial cost of recovering this information.

Ecosystem approach

Traditionally metrics have considered narrow subsets of the ecosystem, justified on the basis of increasing the signal to noise ratio, but more often dictated by what data could be efficiently recovered from ecological surveys. Indicators are by their nature meant to act as a surrogate for broader more complex themes, and although they may seem intuitive, these relationships are rarely tested. As DNA-based techniques have largely removed the taxonomic constraints for processing samples, we can now afford to take a more holistic view of the ecosystem (Mueller and Geist, 2016). Therefore, where the form of BEF relationships is of particular concern, co-locating measures of function with routine monitoring will help calibrate the form we expect BEF relationships to take.

Local assembly (α)

Functional diversity

On their own, taxon richness and taxonomic turnover do not contain information about how the structure of assemblages relates to resource exploitation, niche partitioning and coexistence mechanisms (Thompson et al., 2012). Thus the diversity of taxa that fulfil particular functional roles may be a good proxy for some functions (Yates et al. 2019), and we can overlay trait information on DNA-based detections. Nonetheless, while trait-based functional diversity indicators are appealing, they may explain very little of the residual variation between taxonomic diversity and ecological functions (van der Plas et al., 2019). In addition, different functions would be expected to relate to different traits. For this reason we would recommend we keep an open mind to what traits are most important (Van den Berg et al., 2019), and further study of phylogenetic relationships (see below) may help suggest at what scale these are important.

A challenge to adopting many potential metrics (e.g. Yates et al. 2019) is that we anticipate effective functioning will be driven by overall biomass or abundance of individual taxa, or groups of taxa, not their diversity. For example filter feeders are expected to function in broadly similar ways, and therefore their role in water filtration is expected to align with total abundance, rather than their diversity. Given DNA-based techniques are currently poor proxies for either biomass or abundance, then their use may not be seen to offer an advantage in this scenario. However we argue it is highly unlikely that community composition would remain static amid substantial changes in the abundance of dominant taxa. Therefore although an increase in a particular taxon may be hard to identify with data from DNA-based techniques, there is a strong possibility we could observe a shift in composition that is indirectly associated with higher abundance among a subset of the taxa. A more direct, mechanistic interpretation may be possible from network structure inferred from community composition (see below).

The primary limitation of trait-based approaches is the availability of trait data themselves. There is a wealth of literature that can be mined to extract trait information (Compson et al., 2018) but just as the identification of many taxonomic groups is challenging, by the same token trait analyses are restricted to well-studied taxa, or only assigned at a coarse

taxonomic resolution, even when studies are aware taxon-specific responses may be being confounded (Elbrecht et al., 2016).

Phylogenetic diversity

Functional diversity can capture more of the underlying similarities and differences between taxa that can inform ecological processes, but often traits are derived from morphological features and it is hard to justify which level of aggregation, such as trophic or functional groups, is optimal for understanding the relevance to assembly or function. The alternative is to perform analyses at multiple levels of aggregation, from all species separately up to a single value of diversity or connectance. This approach has been suggested for measures of phylogenetic (Chalmandrier, et al., 2015) and trait informed phylogenies would offer additional options for functional metrics. Some studies have shown strong phylogenetic associations between species' sensitivity to contaminants (Guénard, et al., 2014), although relationships with something as complex as function are likely to be far weaker. Testing for phylogenetic signal in ecotoxicology is challenging because the range of taxa being tested is rather modest (Moore et al., 2019). Likewise, the lack of suitable trait data limits what relationships species' functioning might have with phylogenetic relatedness (Van den Berg et al., 2019).

We should not underestimate the time it might take to develop a robust phylogeny of some groups, but some studies have already shown how new technology may be harnessed to accelerate this process (Krehenwinkel, et al., 2018). An ability to understand the dominance and occupancy patterns of species in community assembly and interaction networks from an evolutionary context, and in a form that directly complements the information collected by DNA-based surveys, is surely worth further investigation.

Genetic diversity

Genetic diversity is of course closely related to phylogenetic diversity, and there have already been attempts to infer structure within-species ranges based on haplotype diversity (Elbrecht, et al., 2018). However, genetic diversity is normally considered as a proxy for phenotypic variation within a species that could translate to functional redundancy at the level of species. Genetic diversity is also important for increasing species' capacity to adapt to climate change (Catullo, et al., 2015). We may therefore be interested in whether DNA-based techniques could also monitor genetic diversity of economically important species' (e.g. salmonids), or species of conservation concern (e.g. natterjack toads, freshwater eels, pearl-mussel). However, with respect to functioning, it would be more important to know the genetic diversity of the dominant taxa in the system. An example that shows the difficulty of such a judgement is the amphipod genus *Gammarus*. *Grammars* make a significant contribution to litter decomposition in streams and rivers in the UK and Europe, and have been the subject of numerous ecotoxicological studies. Despite being well studied, DNA metabarcoding has recently revealed that *Gammarus fossarum*, a European species, has been present in the UK for more than 50 years (Blackman et al., 2017). In addition, the results of many lab studies on *G.fossarum* had to be revisited after DNA tests showed there were in fact multiple cryptic species (Altermatt et al., 2014 and references therein). As a positive, the presence of "new"

species like *G.fossarum* might provide added functional redundancy, should other “native” species decline under climate change, but it suggests that we may not want to pursue genetic proxies until we have a firmer grasp of species composition. Finally, while few would argue with the principle of conserving species’ genetic diversity, it is hard to imagine a link to functioning that would mean it exceeded the priority of retaining species diversity.

Trophic metrics

Loss of diversity across trophic levels has the potential to influence ecosystem functions even more strongly than diversity loss within trophic levels (Cardinale et al., 2012). Loss of diversity among primary producers can cascade “upward”, influencing the stability of multiple insect trophic levels (Haddad, et al., 2011), or manipulations of apex predators like fish can cascade “downward” to influence ecosystem properties (Carey and Wahl, 2011). While random interaction networks become unstable with higher diversity and complexity, natural networks are stabilised by non-random structure, a skewed distribution of interaction strengths and body-mass structure. There is now a huge range of metrics to describe the web of interactions (Majdi et al., 2018), but more work is needed to understand how networks respond to environmental gradients and various disturbances. Simulations are an effective tool for exploring multiple facets of a system simultaneously, but at some point these need to be validated with data from large numbers of natural food-webs.

Empirically quantifying food web structure is complex and time consuming, and therefore impractical at scale. Increasingly comparisons are being inferred based on the most probable interaction networks (Morales-Castilla, et al, 2015). This inference is based firstly on observations of what taxa are present, and then traits like body-size and feeding guilds are used to define plausible interactions. The resulting network can be further refined if more information on taxa is available (e.g. assimilation and energy flux: Barnes et al., 2018), although often an increasing number of assumptions must be made to extend inferences to an entire food web. Trait data is notoriously patchy for many taxonomic groups, and this is compounded in taxa for whom basic taxonomy is already a challenge.

Compson et al. (2018) demonstrated how this could be overcome by mining scientific literature to increase coverage of trait database. Using lists of taxa generated by DNA metabarcoding enables more complete reconstruction of trophic networks, and in theory, multiple trophic groups could be studied in parallel. Nonetheless, as DNA metabarcoding is not suitable for inferring counts or abundance, we currently lack a mechanism to gauge interaction strengths in the proposed networks. This constrains the kinds of tools we can apply to infer stability and energy flux. In spite of these challenges, the early development of this approach has already shown the inferred food-web structure strongly aligns (r^2 = 0.6-0.78) with traditional empirical measures of trophic structure based on stable isotopes (Compson et al., 2019). Complexity of the proposed networks also appears to relate to the estimates of niche breadth based on stable isotopes. In addition, a further study has shown changes in the food web structure of wetland invertebrates is linked to changes in rates of litter decomposition (Baird lab unpublished data). The most interesting aspect of this advance is that functioning did not relate most strongly to the diversity of detritivores, but to the loss of top predators, contributing to an overall degradation of the community.

Metacommunity structure (β)

Diverse communities are more efficient decomposers or recycling nutrients because they contain key species that have a large influence on productivity, and differences in functional traits among organisms increase total resource capture. Greater biodiversity allows for greater species turnover and compensatory growth as environments change, lowering system variability through time. For example, using controlled plant plots Pasari et al. (2013) were able to show that although α diversity had strong positive effects on most individual functions, the positive effects of β and γ diversity only emerged when multiple functions were considered simultaneously. The review by Mori et al. (2018) provides further examples, and outlines how beta-diversity could be partitioned into a nested and turnover components.

The nested component relates to the dominance, or occupancy, of different taxa. Dominant taxa are potentially key drivers of functions over large scales, or numerous functions, although their contribution is typically weaker than that of higher richness. Richness is considered a poor indicator of biodiversity change because it does not register the changes in species identity (Hillebrand, Blasius, et al., 2018). However, changes in the mean occupancy of functional groups pre-determines the identity of taxa considered, and recognises the positive effects of α diversity at larger scales. This can include both specialist (net-spinners), and generalist (e.g. filter feeding macroinvertebrates) groups. While to begin with a linear decline in functioning with occupancy of functional taxa may be a reasonable assumption, further research to identify the importance of common taxa in particular will identify at what stage there is likely to be rapid decline.

The turnover component of diversity is associated with complementarity effects for multifunctionality, and is synonymous with concepts like compensatory dynamics, biological insurance, and the portfolio effect. Using a dynamic metacommunity model (Wang and Loreau, 2016) also found that higher β diversity reduced the variability in multifunctionality across ecosystems through spatial insurance effects. Homogenisation (i.e. greater spatial similarity in community composition), could destabilise ecological networks because population fluctuations become synchronised at large scales (France and Duffy, 2006; Gouhier, Guichard, and Gonzalez, 2010; Olden and LeRoy Poff, 2004). Based on this, measures of how beta diversity reduces ecosystem variability from local to regional scales could be related to functioning, and any factor contributing to increasing beta variability provides spatial insurance to regional ecosystems (Wang and Loreau, 2014). This concept appears to be mathematically tractable for partitioning variability in productivity of biomass at local and regional scales, but given there is now empirical evidence to support the theory (Wang, et al., 2019), it could be reasonable to use compositional turnover as an indirect measure of regional stability.

Macroecological structure (γ)

The regional pool of species is relevant to interpreting many of the metrics discussed as soon as multiple sites are included, but we have reserved this final section specifically for possible metrics of function that relate to distribution and diversity of species at a national

(macroecological) scale. How much biodiversity should we retain? Current trajectories are likely to result in the loss of taxa that have a role in ecosystem services, but we may also wish to preserve a higher proportion to safeguard against tipping points, and to provide the capacity to adapt to global changes. An example of a viable metric would be the Bioclimatic Ecosystem Resilience Index, which combines species-area curves, models of compositional turnover across environmental gradients, and landscape connectivity to estimate the expected retention of biodiversity under scenarios of climate and land-use change (Ferrier et al. 2019: https://www.biorxiv.org/content/10.1101/795377v1).

Measures of total biodiversity (γ) are also relevant to understanding how resilient communities are to invasion, and what roles new species fill when they become established in the UK. If more diverse assemblages are less likely to be colonised by new species, this would suggest similar functions are already being performed by native species, and further expansion of new species will be characterised as invasion. On the other hand, if new species do not displace existing taxa their status may be considered more positively, particularly for adding redundancy to functional diversity.

Perspective

Ecosystem function is mediated by biodiversity, and the components of ecological stability all relate to species' composition, function and the dynamics of communities in some way. The data generated from DNA allow in many cases for higher taxonomic resolution, but also improved detection and hence greater statistical power than we can otherwise achieve. DNA-based survey techniques are therefore going to be critical if we want to track biodiversity, change and relate those to function and functional stability (Table 1).

We feel we cannot stress enough that in order to understand what factors influence ecological functions, when changes are significant, and what actions might be taken to manage for certain functions we initially need to identify the biodiversity (taxonomic, structure, functional, phylogenetic) we expect to observe. Existing reference condition models follow the same principle but are too coarse to make any inference about changes in the dynamics of the ecological community, or at the appropriate scale. This point is similar to describing the variability dimension of stability, but is far more fundamental. BEF relationships will be strongest if metacommunity structure is dominated by species sorting, whereas they may easily appear weak in cases where patch dynamics, multiple equilibria or high dispersal prevail (Thompson et al. 2019 https://www.biorxiv.org/content/10.1101/832170v1).

Are there alternative metrics that would not rely on a "observed vs. expected", reference-condition approach? One could apply an indicator related to function such as the diversity of filter feeding invertebrates and conclude that areas with lower diversity have less capacity to filter water. However, we are well aware such an abstraction is flawed, especially without further context on species dominance. For example, communities invaded by zebra and quagga mussels (Aldridge, et al., 2014) have high filtration capacity but are species poor. Even in the absence of invasive species, we lack sufficient understanding of the ***potential*** of ecosystems, and hence the performance of such metrics and how we rank a site's ecosystem function has no context. Given functions will vary with abiotic conditions, the capacity of different habitats and assemblages will vary. Is the low diversity of filter feeders a commonly observed phase in community transition? Is there a limit to the diversity of filter-feeders we would expect when there is a complex and otherwise intact food-web? Conversely, once a reference model is available, these same metrics of functional performance can be interpreted relative to the predicted ecological condition. The outliers in this relationship will be far more informative for management purposes than the metrics alone.

The examples above assume that DNA-based monitoring can recover taxonomic identity, acknowledging of course the opportunity for errors depending on the sequencing technique, bioinformatics pipeline and reference libraries. Taxonomic identity is useful because it allows DNA-based monitoring to connect with historical monitoring, makes some results easier to communicate, and is the point of reference for overlaying other sources of data (e.g. trait databases and controlled experiments). Taxonomy is not however a barrier to adopting a more holistic metacommunity perspective (i.e. "taxonomy free"; Vasselon, et al., 2017). Assuming samples are collected in a consistent, or at least

comparable manner, we can develop models that define what diversity we would expect to observe in a new sample, and our confidence in that prediction. As a result, a strategy based on metacommunity dynamics that defines ecosystem stability on the basis of compositional change in space and time can be applied to any ecosystem, not just freshwater, and any taxonomic group. The theory and evidence for how to generalise metrics of stability or resilience with the different scales of ecological life histories could be a productive avenue of future research.

Applying an anthropocentric description of ecosystems as functional entities, will inevitably lead to a demand for performance and metrics of ecosystem function to be defined for circumstances that then align to human boundaries (a farm, council, watershed, region, national). However, the most appropriate way to describe attributes of an ecosystem is at the scales they are organised, the ecological hierarchy (α, β, γ), and these may not always match. Although they are very important to many functions, the intensity of sampling required to capture the rapid turnover of microbial communities will constrain the scale at which they can be applied. Pinning down what is the most appropriate spatial scale for analysis of a community is also important for understanding how BEF relationships are scaled, and Frishkoff, et al. (2019) show how this can be done and account for imperfect detection. If the rapid turnover of microbial communities does prove impractical for large-scale monitoring, perhaps dedicated testing focused on the surrogacy and complementarity of other taxonomic groups like invertebrates or benthic algae could reveal robust associations with ***mean*** microbial functionality. As above, validating such relationships could provide an avenue for integration across scales and biomes.

Table 1 Metrics associated with aquatic ecosystem function or ecosystem stability that could be supported by DNA-based surveys

Metric type	Taxa	Relationship to functioning	Relationship to stability	Data required for implementation
Taxonomic diversity	All: microbial, algae, invertebrate, fish and macrophyte	↑ diversity of coexisting taxa = ↑ complementary niches = ↑ efficient functioning	All dimensions but variability is most intuitive	Initial investment to develop reference metacommunity model Model parameters initially lack priors but model performance will improve with further data collection
Genetic diversity	Salmonid fish Dominant invert shredder Potential vectors for future pathogens	↑ genetic diversity of functional taxa = ↑ persistence and ↑ resilience (faster recovery)	Resistance (persistence) to press disturbance like climate change	Multi-gene haplotype diversity across distribution of target species
Phylogenetic diversity	All: microbial, algae, invertebrate, fish and macrophyte	↑ phylogenetic diversity = ↑ functional diversity (and possibly ↓ invasibility)	Resilience (recovery) of community to pulse or fluctuating disturbance Resistance (persistence) to invasion	Requires multi-locus phylogenetic tree tied to reference amplicon database. Need further research on functional complementarity of related taxa (e.g. litter decomposition)

Trait-based functional diversity	Limited to trophic morphology in fish and inverts (filter feeders, net-spinners, shredders, top predators)	↑ diversity of functionally-relevant taxa = ↑ efficient functioning	All dimensions but variability is most intuitive	Primary constraint is the quality, coverage and relevance of available trait data
Network metrics (centrality, no. of links, connectance, modularity)	Limited to well-studied groups with response / effects traits libraries and known functional mechanism. Invertebrates and potentially fish	↑ complex trophic networks = ↑ capacity to retain, recycle and transfer energy and nutrients ↑ connected consumers = ↑ redundancy	Resilience of different trophic metrics e.g. trophic height, following disturbance to reflect nutrient cycling and 1° consumer biomass	Algorithms to detect change require many networks to be built, and continually improves with refinements to metacommunity model above Still require development of theory to interpret differences
Taxonomic β diversity	All – but is difficult to sustain for taxa like microbes with rapid turnover	↑ spatial turnover = ↑ redundancy and ↓ spatial synchrony	Variability, and potentially resilience	Is a functionally relevant outcome of a metacommunity model that is easily transferable across scales DNA-metabarcoding improves detection and consistency of observation to make comparisons at scale

Occupancy of functional groups	Limited to well-studied groups with response / effects traits libraries and known functional mechanism	May reveal systematic decline of functional diversity that is non-significant relative to variability at α-scale	Persistence/resistance Resilience	Easy to implement but statistical power heavily reliant on scale of sampling
Regional / watershed γ	All	↑ pool of functional diversity = ↑ redundancy	Resilience Knowledge of γ also increases detection of unknown invasive spp	Product of metacommunity model Sampling sufficiency dictated by rate of species accumulation (mean detectability)
Bioclimatic Ecosystem **Resilience Index**	All	↑ connectivity to environmentally similar habitats = ↑ long-term stability of function regionally/nationally	Asymptotic stability Persistence/resistance	Species-area curves need some adaptation for aquatic environments, but otherwise the parameters for turnover are available from sampling needed to identify α, β and γ of the metacommunity

Conclusions

Decades of BEF research support the view that ecosystem functioning will be maximised if landscapes maintain high α diversity in each ecosystem, and high spatial β diversity (and consequently high γ). We still expect the exact form of the relationship between biodiversity ecosystem function (BEF) to vary for different functions, and as environmental conditions or the identity of species changes. Nonetheless, this basic principle is likely to be a robust strategy to support multifunctionality. Further research can identify the specific nature of BEF relationships where biodiversity is already heavily degraded (e.g. in agricultural areas), and are most likely to be having a functional impact. **Therefore the starting point for improving our understanding of ecosystem functions is to firstly understand how ecological communities are structured**. Specific indices of trait-diversity and network structure can then be built upon this framework, and interpreted with respect to temporal turnover (i.e. rates of local extinction and recolonization), and likewise, how distinct those communities are within the landscape (related to dispersal limitation and environmental gradients).

As part of the Ecobiomics projects (Edge et al., 2019), we have been developing a reference condition model of aquatic invertebrates using DNA metabarcoding for the Atlantic provinces of Canada. Early evidence from several projects has highlighted the importance of multiple sources of uncertainty on our power to discriminate condition, and further work is needed to assess its efficiency for broad-scale monitoring. The reference metacommunity model is a critical tool for understanding the limits of our observations, and although this represents a relatively steep initial investment, it is no more than other large research grants, and ongoing sampling is then able to become far more strategic. In Atlantic Canada we are now able to start analysing patterns of α, β (including occupancy) and γ, and trait data (body-size and feeding morphology) are available to focus on functionally relevant taxa, or infer structural network metrics. There are many ways an equivalent exercise in the UK could benefit from the start made in Canada.

Finally, we should ask whether we need to monitor freshwater systems with distinct metrics from those used in the terrestrial or marine sphere? In the past ecologists have argued that the substrate (land or water) is immaterial, and that at a more theoretical level, all systems occur within a continuum (Wiens, 2002). Others argue the overriding importance of hydrology, and the connectivity provided by water flow within a strict hierarchical structure mean lotic (riverine) systems are fundamentally distinct from terrestrial systems (Poole, 2002). These different perspectives are to some extent a matter of resolution (spatial and temporal) with which a system is considered. While this document focuses on prospects for monitoring freshwater ecosystems, we should seek common ground with scientists working in marine and terrestrial systems in defining the stability and functioning of ecosystems.

As indicated at the beginning of this document, our focus has been on how to further our understanding of functioning, but naturally the information collected by DNA provides enormous opportunities to address other indicators of freshwater habitat condition

identified in the Outcome Indicator Framework. At this stage, Indicator B6 “Natural functions of water and wetland ecosystems” presents some of the greatest conceptual challenges because the potential contributions of DNA require some definition of the functions of interest. We argue that regardless of the system, ecosystem functioning cannot be evaluated by a single process, and therefore management supporting the retention of biodiversity at local and regional scales is a rational strategy for which we can identify metrics. Persistence under pressure from multiple stressors is particularly acute in freshwater systems and hence understanding how we define and monitor ecosystem stability and resilience are key to promoting their overall health, and guiding policies or management actions.

To inform functioning we recommend the development of DNA-based biomonitoring in aquatic systems initially focuses on developing a detailed understanding of community structure, but at large scales. Can we predict or identify mechanisms for how local communities are assembled from regional metacommunities, and importantly, how communities change in time and space in response to environmental change and disturbances? Establishing this baseline, quantifying natural variability and the biases in our sampling methods is critical to understanding the confidence we place in observed changes (Bush et al., 2019; Evans, et al., 2016; Guillera-Arroita and Lahoz-Monfort, 2012; Southwell et al., 2019). Further inference of functioning can then be built upon that taxonomic framework, either as trait-based metrics, food-webs, or other derived indicators (Pawlowski et al., 2018).

References

ALDRIDGE, D. C., Ho, S., and Froufe, E. (2014) The Ponto-Caspian quagga mussel, Dreissena rostriformis bugensis (Andrusov, 1897), invades Great Britain. *Aquatic Invasions*, 9(4), 529-535.

ALLAN, E., Weisser, W., Weigelt, A., Roscher, C., Fischer, M., and Hillebrand, H. (2011) More diverse plant communities have higher functioning over time due to turnover in complementary dominant species. *Proceedings of the National Academy of Sciences*, 108(41), 17034-17039. doi:10.1073/pnas.1104015108.

ALTERMATT, F., Alther, R., Fišer, C., Jokela, J., Konec, M., Küry, D., . . . Westram, A. M. (2014) Diversity and Distribution of Freshwater Amphipod Species in Switzerland (Crustacea: Amphipoda). *PLoS One*, 9(10), e110328. doi:10.1371/journal.pone.0110328.

BARNES, A. D., Jochum, M., Lefcheck, J. S., Eisenhauer, N., Scherber, C., O'Connor, M. I., . . . Brose, U. (2018) Energy Flux: The Link between Multitrophic Biodiversity and Ecosystem Functioning. *Trends in Ecology and Evolution*, 33(3), 186-197. doi:10.1016/j.tree.2017.12.007

BEERMANN, A. J., Zizka, V. M. A., Elbrecht, V., Baranov, V., and Leese, F. (2018) DNA metabarcoding reveals the complex and hidden responses of chironomids to multiple stressors. *Environmental Sciences Europe*, 30(1), 26. doi:10.1186/s12302-018-0157-x.

BLACKMAN, R. C., Constable, D., Hahn, C., Sheard, A. M., Durkota, J., Hänfling, B., and Handley, L. L. (2017) Detection of a new non-native freshwater species by DNA metabarcoding of environmental samples – first record of Gammarus fossarum in the UK. *Aquatic Invasions*, 12(2), 177-189. doi: https://doi.org/10.3391/ai.2017.12.2.06.

BLACKMAN, R. C., Mächler, E., Altermatt, F., Arnold, A., Beja, P., Boets, P., . . . Deiner, K. (2019) Advancing the use of molecular methods for routine freshwater macroinvertebrate biomonitoring – the need for calibration experiments. *Metabarcoding and Metagenomics*, 3, e34735.

BUSH, A., Compson, Z. G., Monk, W. A., Porter, T. M., Steeves, R., Emilson, E., . . . Baird, D. J. (2019) Studying Ecosystems With DNA Metabarcoding: Lessons From Biomonitoring of Aquatic Macroinvertebrates. *Frontiers in Ecology and Evolution*, 7(434). doi:10.3389/fevo.2019.00434.

CARDINALE, B. J., Duffy, J. E., Gonzalez, A., and Hooper, D. U. (2012) Biodiversity loss and its impact on humanity. *Nature*, 486, 59-67.

CARDINALE, B. J., Gross, K., Fritschie, K., Flombaum, P., Fox, J. W., Rixen, C., . . . Wilsey, B. J. (2013) Biodiversity simultaneously enhances the production and stability of community biomass, but the effects are independent. *Ecology*, 94(8), 1697-1707. doi:10.1890/12-1334.1.

CAREY, M. P., and Wahl, D. H. (2011) Fish diversity as a determinant of ecosystem properties across multiple trophic levels. *Oikos*, 120(1), 84-94. doi:10.1111/j.1600-0706.2010.18352.x

CATULLO, R. A., Ferrier, S., and Hoffmann, A. A. (2015) Extending spatial modelling of climate change responses beyond the realized niche: estimating, and accommodating, physiological limits and adaptive evolution. *Global Ecology and Biogeography*, 24(10), 1192-1202. doi:10.1111/geb.12344.

CHALMANDRIER, L., Münkemüller, T., Lavergne, S., and Thuiller, W. (2015) Effects of species' similarity and dominance on the functional and phylogenetic structure of a plant meta-community. *Ecology*, 96(1), 143-153. doi:10.1890/13-2153.1.

COMPSON, Z. G., Monk, W. A., Curry, C. J., Gravel, D., Bush, A., Baker, C. J. O., . . . Baird, D. J. (2018) Linking DNA Metabarcoding and Text Mining to Create Network-Based Biomonitoring Tools: A Case Study on Boreal Wetland Macroinvertebrate Communities. In *Advances in Ecological Research* (Vol. 59, pp. 33-74): Academic Press.

COMPSON, Z. G., Monk, W. A., Hayden, B., Bush, A., O'Malley, Z., Hajibabaei, M., . . . Baird, D. J. (2019) Network-Based Biomonitoring: Exploring Freshwater Food Webs With Stable Isotope Analysis and DNA Metabarcoding. *Frontiers in Ecology and Evolution*, 7(395). doi:10.3389/fevo.2019.00395.

COTTINGHAM, K. L., Brown, B. L., and Lennon, J. T. (2001) Biodiversity may regulate the temporal variability of ecological systems. *Ecology letters*, 4(1), 72-85. doi:10.1046/j.1461-0248.2001.00189.x.

CRISTESCU, M. E., and Hebert, P. D. N. (2018) Uses and Misuses of Environmental DNA in Biodiversity Science and Conservation. *Annual Review of Ecology, Evolution, and Systematics*, 49(1), null. doi:10.1146/annurev-ecolsys-110617-062306.

DAKOS, V., Carpenter, S. R., Brock, W. A., Ellison, A. M., Guttal, V., Ives, A. R., . . . Scheffer, M. (2012) Methods for Detecting Early Warnings of Critical Transitions in Time Series Illustrated Using Simulated Ecological Data. *PLoS One*, 7(7), e41010. doi:10.1371/journal.pone.0041010.

DAKOS, V., Carpenter, S. R., van Nes, E. H., and Scheffer, M. (2015) Resilience indicators: prospects and limitations for early warnings of regime shifts. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 370(1659), 20130263. doi:10.1098/rstb.2013.0263.

DAKOS, V., Matthews, B., Hendry, A. P., Levine, J., Loeuille, N., Norberg, J., . . . De Meester, L. (2019) Ecosystem tipping points in an evolving world. *Nature Ecology and Evolution*, 3(3), 355-362. doi:10.1038/s41559-019-0797-2

DAVIS, A. J., Williams, K. E., Snow, N. P., Pepin, K. M., and Piaggio, A. J. (2018) Accounting for observation processes across multiple levels of uncertainty improves

inference of species distributions and guides adaptive sampling of environmental DNA. *Ecology and Evolution*, 8(22), 10879-10892. doi:doi:10.1002/ece3.4552.

de BOER, M. K., Moor, H., Matthiessen, B., Hillebrand, H., and Eriksson, B. K. (2014) Dispersal restricts local biomass but promotes the recovery of metacommunities after temperature stress. *Oikos*, 123(6), 762-768. doi:10.1111/j.1600-0706.2013.00927.x.

DEINER, K., Bik, H. M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., . . . Bernatchez, L. (2017) Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. *Molecular Ecology*, 26(21), 5872-5895. doi:10.1111/mec.14350.

DONOHUE, I., Hillebrand, H., Montoya, J. M., Petchey, O. L., Pimm, S. L., Fowler, M. S., . . . Yang, Q. (2016) Navigating the complexity of ecological stability. *Ecology letters*, 19(9), 1172-1185. doi:10.1111/ele.12648.

DOUGLAS, D. J. T., Buchanan, G. M., Thompson, P., Amar, A., Fielding, D. A., Redpath, S. M., and Wilson, J. D. (2015) Vegetation burning for game management in the UK uplands is increasing and overlaps spatially with soil carbon and protected areas. *Biological Conservation*, 191, 243-250. doi:https://doi.org/10.1016/j.biocon.2015.06.014.

DURANCE, I., and Ormerod, S. J. (2009) Trends in water quality and discharge confound long-term warming effects on river macroinvertebrates. *Freshwater Biology*, 54(2), 388-405.

EDGE, T. A., Baird, D. J., Bilodeau, G., Gagné, N., Greer, C., Konkin, D., . . . Macklin, J. (2019) The Ecobiomics project: Advancing metagenomics assessment of soil health and freshwater quality in Canada. *Science of The Total Environmen*t, 135906. doi:https://doi.org/10.1016/j.biocon.2015.06.014.

ELBRECHT, V., Beermann, A. J., Goessler, G., Neumann, J., Tollrian, R., Wagner, R., . . . Leese, F. (2016) Multiple-stressor effects on stream invertebrates: a mesocosm experiment manipulating nutrients, fine sediment and flow velocity. *Freshwater Biology*, 61(4), 362-375. doi:10.1111/fwb.12713.

ELBRECHT, V., Vamos, E. E., Steinke, D., and Leese, F. (2018) Estimating intraspecific genetic diversity from community DNA metabarcoding data. PeerJ, 6, e4644. doi:10.7717/peerj.4644

ELLIOTT, J. M. (1971) Some methods for the statistical analysis of samples of benthic invertebrates (Vol. 25). Ambleside, Westmorland, U.K.: Freshwater Biological Association.

ERDOZAIN, M., Thompson, D. G., Porter, T. M., Kidd, K. A., Kreutzweiser, D. P., Sibley, P. K., . . . Hajibabaei, M. (2019) Metabarcoding of storage ethanol vs. conventional morphometric identification in relation to the use of stream macroinvertebrates as ecological indicators in forest management. *Ecological Indicators*, 101, 173-184. doi:https://doi.org/10.1016/j.biocon.2015.06.014.

EVANS, D. M., Kitson, J. J. N., Lunt, D. H., Straw, N. A., and Pocock, M. J. O. (2016) Merging DNA metabarcoding and ecological network analysis to understand and build resilient terrestrial ecosystems. *Functional Ecology*, 30(12), 1904-1916. doi:10.1111/1365-2435.12659.

FRANCE, K. E., and Duffy, J. E. (2006) Diversity and dispersal interactively affect predictability of ecosystem function. *Nature*, 441(7097), 1139-1143. doi:10.1038/nature04729.

FRISHKOFF, L. O., Mahler, D. L., and Fortin, M.-J. (2019) Integrating over uncertainty in spatial scale of response within multispecies occupancy models yields more accurate assessments of community composition. *Ecography*, 42(12), 2132-2143. doi:10.1111/ecog.04365.

GESSNER, M. O., Swan, C. M., Dang, C. K., McKie, B. G., Bardgett, R. D., Wall, D. H., and Hättenschwiler, S. (2010) Diversity meets decomposition. *Trends in Ecology and Evolution*, 25(6), 372-380. doi:https://doi.org/10.1016/j.tree.2010.01.010.

GOUHIER, Tarik C., Guichard, F., and Gonzalez, A. (2010) Synchrony and Stability of Food Webs in Metacommunities. *The American Naturalist*, 175(2), E16-E34. doi:10.1086/649579.

GRAVEL, D., Albouy, C., and Thuiller, W. (2016) The meaning of functional trait composition of food webs for ecosystem functioning. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 371(1694). doi:10.1098/rstb.2015.0268.

GUELZOW, N., Muijsers, F., Ptacnik, R., and Hillebrand, H. (2017) Functional and structural stability are linked in phytoplankton metacommunities of different connectivity. *Ecography*, 40(6), 719-732. doi:10.1111/ecog.02458.

GUÉNARD, G., Carsten von der Ohe, P., Carlisle Walker, S., Lek, S., and Legendre, P. (2014) Using phylogenetic information and chemical properties to predict species tolerances to pesticides. *Proceedings of the Royal Society B: Biological Sciences*, 281(1789). doi:10.1098/rspb.2013.3239.

GUILLERA-ARROITA, G. (2017) Modelling of species distributions, range dynamics and communities under imperfect detection: advances, challenges and opportunities. Ecography, 40(2), 281-295. doi:10.1111/ecog.02445.

GUILLERA-ARROITA, G., and Lahoz-Monfort, J. J. (2012) Designing studies to detect differences in species occupancy: power analysis under imperfect detection. *Methods in Ecology and Evolution*, 3(5), 860-869. doi:doi:10.1111/j.2041-210X.2012.00225.x.

HADDAD, N. M., Crutsinger, G. M., Gross, K., Haarstad, J., and Tilman, D. (2011) Plant diversity and the stability of food webs. *Ecology letters*, 14(1), 42-46. doi:10.1111/j.1461-0248.2010.01548.x.

HAJIBABAEI, M., Porter, T. M., Robinson, C. V., Baird, D. J., Shokralla, S., and Wright, M. T. G. (2019) Watered-down biodiversity? A comparison of metabarcoding results from DNA extracted from matched water and bulk tissue biomonitoring samples. *PLoS One*, 14(12), e0225409. doi:10.1371/journal.pone.0225409.

HAJIBABAEI, M., Spall, J. L., Shokralla, S., and van Konynenburg, S. (2012) Assessing biodiversity of a freshwater benthic macroinvertebrate community through non-destructive environmental barcoding of DNA from preservative ethanol. *BMC ecology*, 12, 28-28. doi:10.1186/1472-6785-12-28.

HARRISON, J. B., Sunday, J. M., and Rogers, S. M. (2019) Predicting the fate of eDNA in the environment and implications for studying biodiversity. *Proceedings of the Royal Society B: Biological Sciences*, 286(1915), 20191409. doi:10.1098/rspb.2019.1409.

HILLEBRAND, H., Blasius, B., Borer, E. T., Chase, J. M., Downing, J. A., Eriksson, B. K., . . . Ryabov, A. B. (2018) Biodiversity change is uncoupled from species richness trends: Consequences for conservation and monitoring. *Journal of Applied Ecology*, 55(1), 169-184. doi:doi:10.1111/1365-2664.12959.

HILLEBRAND, H., Langenheder, S., Lebret, K., Lindström, E., Östman, Ö., and Striebel, M. (2018). Decomposing multiple dimensions of stability in global change experiments. Ecology letters, 21(1), 21-30. doi:10.1111/ele.12867.

HOLLING, C. S. (1973) Resilience and Stability of Ecological Systems. *Annual Review of Ecology and Systematics*, 4(1), 1-23. doi:10.1146/annurev.es.04.110173.000245.

HOOPER, D. U., Adair, E. C., Cardinale, B. J., Byrnes, J. E. K., Hungate, B. A., Matulich, K. L., . . . O/'Connor, M. I. (2012) A global synthesis reveals biodiversity loss as a major driver of ecosystem change. Nature, 486(7401), 105-108. doi:http://www.nature.com/nature/journal/v486/n7401/abs/nature11118.html#supplementary-information.

ISBELL, F., Gonzalez, A., Loreau, M., Cowles, J., Díaz, S., Hector, A., . . . Larigauderie, A. (2017) Linking the influence and dependence of people on biodiversity across scales. *Nature*, 546(7656), 65-72. doi:10.1038/nature22899.

JARVIS, S. G., Henrys, P. A., Keith, A. M., Mackay, E., Ward, S. E., and Smart, S. M. (2019) Model-based hypervolumes for complex ecological data. *Ecology*, 100(5), e02676. doi:10.1002/ecy.2676.

JI, Y., Huotari, T., Roslin, T., Schmidt, N. M., Wang, J., Yu, D. W., and Ovaskainen, O. (2019) SPIKEPIPE: A metagenomic pipeline for the accurate quantification of eukaryotic species occurrences and intraspecific abundance change using DNA barcodes or mitogenomes. *Molecular Ecology Resources*, 0(ja). doi:10.1111/1755-0998.13057.

JONES, F. C. (2008) Taxonomic sufficiency: The influence of taxonomic resolution on freshwater bioassessments using benthic macroinvertebrates. *Environmental Reviews*, 16, 45-69.

KREHENWINKEL, H., Kennedy, S. R., Rueda, A., Lam, A., and Gillespie, R. G. (2018) Scaling up DNA barcoding – Primer sets for simple and cost efficient arthropod systematics by multiplex PCR and Illumina amplicon sequencing. *Methods in Ecology and Evolution*, 9(11), 2181-2193. doi:doi:10.1111/2041-210X.13064.

LEESE, F., Bouchez, A., Abarenkov, K., Altermatt, F., Borja, Á., Bruce, K., . . . Weigand, A. M. (2018) Chapter Two - Why We Need Sustainable Networks Bridging Countries, Disciplines, Cultures and Generations for Aquatic Biomonitoring 2.0: A Perspective Derived From the DNAqua-Net COST Action. In D. A. Bohan, A. J. Dumbrell, G. Woodward, and M. Jackson (Eds.), *Advances in Ecological Research* (Vol. 58, pp. 63-99): Academic Press.

LEVI, T., Allen, J. M., Bell, D., Joyce, J., Russell, J. R., Tallmon, D. A., . . . Yu, D. W. (2018). Environmental DNA for the enumeration and management of Pacific salmon. bioRxiv. doi:10.1101/394445.

MACHER, J. N., Salis, R. K., Blakemore, K. S., Tollrian, R., Matthaei, C. D., and Leese, F. (2016) Multiple-stressor effects on stream invertebrates: DNA barcoding reveals contrasting responses of cryptic mayfly species. Ecological Indicators, 61(Part 2), 159-169. doi:https://doi.org/10.1016/j.ecolind.2015.08.024.

MAJDI, N., Hette-Tronquart, N., Auclair, E., Bec, A., Chouvelon, T., Cognie, B., . . . Perga, M.-E. (2018) There's no harm in having too much: A comprehensive toolbox of methods in trophic ecology. *Food Webs*, 17, e00100. doi:https://doi.org/10.1016/j.fooweb.2018.e00100.

MAY, R. M. (1972) Will a Large Complex System be Stable? *Nature*, 238(5364), 413-414. doi:10.1038/238413a0.

McRAE, L., J., L., Bubb, P. J., Baillie, J. E. M., Kapos, V., and Collen, B. (2008) The Living Planet Index - Guidance for National and Regional Use. . Retrieved from Cambridge, UK: https://www.unep-wcmc.org/system/dataset_file_fields/files/000/000/129/original/Living_Planet_Index._Guidance_for_national_and_regional_use.pdf?1398687590.

MOORE, D. R. J., Priest, C. D., Galic, N., Brain, R. A., and Rodney, S. I. (2019) Correcting for Phylogenetic Autocorrelation in Species Sensitivity Distributions. *Integrated Environmental Assessment and Management*, n/a(n/a). doi:10.1002/ieam.4207.

MORALES-CASTILLA, I., Matias, M. G., Gravel, D., and Araújo, M. B. (2015) Inferring biotic interactions from proxies. *Trends in Ecology and Evolution*, 30(6), 347-356. doi:10.1016/j.tree.2015.03.014.

MORI, A. S., Isbell, F., and Seidl, R. (2018) β-Diversity, Community Assembly, and Ecosystem Functioning. *Trends in Ecology and Evolution*. doi:10.1016/j.tree.2018.04.012.

MUELLER, M., and Geist, J. (2016) Conceptual guidelines for the implementation of the ecosystem approach in biodiversity monitoring. *Ecosphere*, 7(5), e01305. doi:10.1002/ecs2.1305.

NAEEM, S., Duffy, J. E., and Zavaleta, E. (2012) The Functions of Biological Diversity in an Age of Extinction. *Science*, 336(6087), 1401-1406. doi:10.1126/science.1215855.

NICHOLSON, C. C., Ward, K. L., Williams, N. M., Isaacs, R., Mason, K. S., Wilson, J. K., . . . Ricketts, T. H. (2019) Mismatched outcomes for biodiversity and ecosystem services: testing the responses of crop pollinators and wild bee biodiversity to habitat enhancement. *Ecology letters*, n/a(n/a). doi:10.1111/ele.13435.

NIJP, J. J., Temme, A. J. A. M., van Voorn, G. A. K., Kooistra, L., Hengeveld, G. M., Soons, M. B., . . . Wallinga, J. (2019) Spatial early warning signals for impending regime shifts: A practical framework for application in real-world landscapes. *Global Change Biology*, 0(ja). doi:doi:10.1111/gcb.14591.

OLDEN, J. D., and LeRoy Poff, N. (2004) Clarifying biotic homogenization. *Trends in Ecology and Evolution*, 19(6), 282-283. doi:10.1016/j.tree.2004.03.024.

ORR, H. G., Wilby, R. L., McKenzie Hedger, M., and Brown, I. (2008) Climate change in the uplands: a UK perspective on safeguarding regulatory ecosystem services. *Climate Research*, 37(1), 77-98.

PASARI, J. R., Levi, T., Zavaleta, E. S., and Tilman, D. (2013) Several scales of biodiversity affect ecosystem multifunctionality. *Proceedings of the National Academy of Sciences*, 110(25), 10219-10222. doi:10.1073/pnas.1220333110.

PAWLOWSKI, J., Kelly-Quinn, M., Altermatt, F., Apothéloz-Perret-Gentil, L., Beja, P., Boggero, A., . . . Kahlert, M. (2018) The future of biotic indices in the ecogenomic era: Integrating (e)DNA metabarcoding in biological assessment of aquatic ecosystems. *Science of The Total Environment*, 637-638, 1295-1310. doi:https://doi.org/10.1016/j.scitotenv.2018.05.002.

PIMM, S. L. (1984) The complexity and stability of ecosystems. *Nature*, 307(5949), 321-326. doi:10.1038/307321a0

POOLE, G. C. (2002) Fluvial landscape ecology: addressing uniqueness within the river discontinuum. Freshwater Biology, 47(4), 641-660. doi:10.1046/j.1365-2427.2002.00922.x.

PORTER, T. M., and Hajibabaei, M. (2018) Scaling up: A guide to high-throughput genomic approaches for biodiversity analysis. *Molecular Ecology*, 27(2), 313-338. doi:doi:10.1111/mec.14478.

RICE, C. J., Larson, E. R., and Taylor, C. A. (2018) Environmental DNA detects a rare large river crayfish but with little relation to local abundance. *Freshwater Biology*, 63(5), 443-455. doi:10.1111/fwb.13081.

RUPPERT, K. M., Kline, R. J., and Rahman, M. S. (2019) Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. *Global Ecology and Conservation*, 17, e00547. doi:https://doi.org/10.1016/j.gecco.2019.e00547.

SCHEFFER, M., Bascompte, J., Brock, W. A., Brovkin, V., Carpenter, S. R., Dakos, V., . . . Sugihara, G. (2009) Early-warning signals for critical transitions. *Nature*, 461(7260), 53-59. doi:10.1038/nature08227.

SOUTHWELL, D. M., Einoder, L. D., Lahoz-Monfort, J. J., Fisher, A., Gillespie, G. R., and Wintle, B. A. (2019) Spatially explicit power analysis for detecting occupancy trends for multiple species. *Ecological Applications*, 29(6), e01950. doi:10.1002/eap.1950.

STEFFEN, W., Richardson, K., Rockström, J., Cornell, S. E., Fetzer, I., Bennett, E. M., . . . Sörlin, S. (2015) Planetary boundaries: Guiding human development on a changing planet. *Science*, 347(6223), 1259855. doi:10.1126/science.1259855.

THOMPSON, R. M., Brose, U., Dunne, J. A., Hall, R. O., Hladyz, S., Kitching, R. L., . . . Tylianakis, J. M. (2012) Food webs: reconciling the structure and function of biodiversity. *Trends in Ecology and Evolution*, 27(12), 689-697. doi:https://doi.org/10.1016/j.tree.2012.08.005.

VAN DEN BERG, S. J. P., Baveco, H., Butler, E., De Laender, F., Focks, A., Franco, A., . . . Van den Brink, P. J. (2019) Modeling the Sensitivity of Aquatic Macroinvertebrates to Chemicals Using Traits. *Environmental Science and Technology*, 53(10), 6025-6034. doi:10.1021/acs.est.9b00893.

VAN DER PLAS, F., Schröder-Georgi, T., Weigelt, A., Barry, K., Meyer, S., Alzate, A., . . . Wirth, C. (2019) Plant traits are poor predictors of long-term ecosystem functioning. bioRxiv, 859314. doi:10.1101/859314.

VASSELON, V., Rimet, F., Tapolczai, K., and Bouchez, A. (2017) Assessing ecological status with diatoms DNA metabarcoding: Scaling-up on a WFD monitoring network (Mayotte island, France). *Ecological Indicators*, 82, 1-12. doi:https://doi.org/10.1016/j.ecolind.2017.06.024.

VAUGHAN, I. P., and Gotelli, N. J. (2019) Water quality improvements offset the climatic debt for stream macroinvertebrates over twenty years. *Nature Communications*, 10(1), 1956. doi:10.1038/s41467-019-09736-3.

WANG, S., Lamy, T., Hallett, L. M., and Loreau, M. (2019) Stability and synchrony across ecological hierarchies in heterogeneous metacommunities: linking theory to data. *Ecography*, 42(6), 1200-1211. doi:10.1111/ecog.04290.

WANG, S., and Loreau, M. (2014) Ecosystem stability in space: α, β and γ variability. *Ecology letters*, 17(8), 891–901. doi:10.1111/ele.12292.

WANG, S., and Loreau, M. (2016) Biodiversity and ecosystem stability across scales in metacommunities. *Ecology letters*, 19(5), 510-518. doi:10.1111/ele.12582.

WIENS, J. A. (2002) Riverine landscapes: taking landscape ecology into the water. *Freshwater Biology*, 47(4), 501-515. doi:10.1046/j.1365-2427.2002.00887.x.

WINFREE, R., Reilly, J. R., Bartomeus, I., Cariveau, D. P., Williams, N. M., and Gibbs, J. (2018) Species turnover promotes the importance of bee diversity for crop pollination at regional scales. *Science*, 359(6377), 791-793. doi:10.1126/science.aao2117.

WWF. (2018) Living Planet Report - 2018: Aiming Higher. Retrieved from Gland, Switzerland.: https://s3.amazonaws.com/wwfassets/downloads/lpr2018_summary_report_spreads.pdf.

YATES, A. G., Culp, J. M., Armanini, D. G., Baird, D. J., Jardine, T. D., and Orlofske, J. M. (2019) Enhancing bioassessment approaches: development of a river services assessment framework. Freshwater Science, 38(1), 12-22. doi:10.1086/701674.

YODZIS, P. (1981) The stability of real ecosystems. *Nature*, 289(5799), 674-676. doi:10.1038/289674a0

Think-Piece 2: Development of new metrics incorporating DNA-based methods to understand marine ecosystem functioning

Tom Webb, Helen Hipperson, and Edward Sibley

Department of Animal and Plant Sciences, University of Sheffield

Contents

Tables and Figures

Summary

Lay summary

Understanding how marine ecosystems function is essential for managing our use of the UK's seas. In particular, monitoring the health of key species, the stability of food webs and the condition of seafloor habitats can provide us with the information needed to continue benefiting from marine ecosystems without overexploiting them. Though manually acquiring the data necessary to achieve this has traditionally proven logistically challenging, the use of DNA technologies promises to revolutionise measuring features such as species abundance and diversity, food web interactions and habitat structure. DNA technologies also allow us to track changes in these features as human impacts on our oceans increase. Here, we review the applications of DNA technologies in marine ecosystem monitoring and look at how they have contributed to five main areas of research: biodiversity assessments, food web interactions, reproduction and species populations, human impacts on marine ecosystems, and ecosystem functioning. We also describe the various challenges associated with DNA technologies, underlining the need to develop uniform approaches for their use in marine ecosystems. To finish, we discuss the future outlook for DNA technologies, in particular their place in future marine ecosystem research as the technologies develop. Given the uncertain future of many marine ecosystems, further development and use of DNA technologies to provide deeper insight into their function is essential to preserve the benefits of healthy oceans.

Executive summary

- Functioning marine ecosystems underpin the immense biological, economic, social and cultural value of the UK's seas, and such measures of ecosystem functioning are integral to indicators of marine environmental status, including the Outcome Indicator Framework. Specific indicators considered here are *D7 Species supporting ecosystem functions*, *C8 Marine food webs functioning*, and *C9 Seafloor habitats functioning*.
- Metrics to support these indicators require quantification of: the presence, abundance, population dynamics, conservation status, and trophic interactions of key species (such as top predators); the diversity of functionally important groups (e.g. benthic invertebrates) and their responses to multiple stressors; and direct measures of functioning in sediment communities.
- All these parameters are expensive and logistically challenging to monitor using traditional techniques, and so the use of DNA technologies to contribute to quantifying these indicators is attractive. In particular, eDNA and similar technologies can be used for biodiversity assessment across gradients of human pressure, and to measure trophic relationships; methods such as high-throughput sequencing of ribosomal RNA can directly reveal microbial activity and function within sediments.
- We review previous applications of DNA-based methods in marine environments for assessing biodiversity, measuring trophic interactions, quantifying reproductive

behaviour and population dynamics, tracking biological responses to anthropogenic pressures, and for directly measuring ecosystem functions. We relate these back to potential applications within the Outcome Indicator Framework.

- We address the challenges associated with applying these technologies to marine ecosystem functioning, specifically addressing the need for standardised approaches to facilitate the generation of long-term and comparative datasets.
- Finally, we present a horizon scan of the opportunities that DNA-based methods are expected to present for measuring marine ecosystem functioning as the technology develops, including direct DNA sequencing and autonomous sampling, both of which will expand possibilities for marine ecosystem monitoring.
- We have an exceptional opportunity to harness developments in the field of environmental DNA to implement new assessments of ecological functioning, such as faster evaluations of species presence–absence in marine ecosystems, rapid and comprehensive gut content analysis allowing more comprehensive quantification of food webs with finer spatial and temporal resolution, plus an increased ability to estimate biomass and abundance. Given the current rate of environmental change and the opportunity to implement new environmental monitoring legislation, it is timely to put these tools into effect.

Introduction

The concept of ecosystem functioning is central to the vision of clean, healthy, safe, productive and biologically diverse oceans and seas outlined in the UK's marine strategy. Functioning marine ecosystems underpin the immense biological, economic, social and cultural value of the UK's seas. As such, measures of ecosystem functioning are integral to indicators of marine environmental status, including the Outcome Indicator Framework (Defra 2019). Measuring ecosystem functioning is therefore a key component of marine environmental management, and the potential of new technologies to improve the efficiency and effectiveness of marine monitoring is of considerable strategic interest. In this think piece, we consider the role that DNA-based methods can play in both qualitative and quantitative assessments of marine ecosystem functioning, with a focus on the Outcome Indicators D7 (*Species supporting ecosystem functions*), C8 (*Marine food webs functioning*), and C9 (*Seafloor habitats functioning*). We first provide a brief overview of marine ecosystem functioning, how it is traditionally measured (and the limitations of these methods), and how it relates to various measures of biodiversity. We then describe the three focal Outcome Indicators (D7, C8 and C9) and how they are interrelated. We identify five key areas in which DNA-based methods can help us to understand marine ecosystems, and explain how each can contribute to operationalising metrics for measuring the three indicators. Finally we consider the challenges and opportunities that these technologies present in marine applications, including a brief horizon scan of likely technological developments.

What is marine ecosystem functioning, and why does it matter?

The term ‘ecosystem functioning’ is widely used to encompass phenomena including stocks of energy and materials in the ecosystem, fluxes of energy and materials, and relative stability over time (Paterson et al. 2012). In a marine context ecosystem functions are typically quantified by measuring rates of ecosystem processes (e.g. primary and secondary production, respiration, decomposition, nutrient cycling, and flows of energy through food webs), functional pathways (e.g. nutrient fluxes and uptake, sediment mixing and stabilisation), or frequently by combining information on the functional traits of species with traditional surveys of species abundance and distribution. All of these functions can be measured either directly or using proxies (see Table 2 for examples), but this is often logistically complicated and expensive to do at scales relevant to marine ecosystem management, making the search for new and more efficient methods for assessing marine ecosystem function especially relevant.

Understanding marine ecosystem functioning is important because of the immense value - biological, economic, social, and cultural - embedded within the UK’s marine environment (e.g. Beaumont et al. 2008). Functioning ecosystems are fundamental to this value. For example, productive and diverse soft sediment communities underpin hugely important nutrient cycling and fisheries. Particular functions within these communities, such as bioturbation - particle reworking and ventilation resulting from the actions of benthic macroinvertebrates - can drive changes in abiotic conditions (e.g. increasing sediment oxygen concentrations) which in turn increase the biomass of organisms, rates of organic matter decomposition, regeneration of nutrients, and rates of primary productivity (e.g. Solan et al. 2004).

Table 2 Examples of marine ecosystem functions that are particularly relevant to Outcome Indicator C9 *Seafloor habitats functioning*, how they are typically measured, and the focal groups of organisms. Adapted and extended from Paterson et al. (2012)

Ecosystem Function	Measured via	Organism group	Potential for DNA technology
Net Primary Productivity	Biomass	Microphytobenthos, Algae	eDNA to estimate abundance of primary producers
Nutrient flux	Nutrient analysis	Sediment Infauna	Molecular diet studies
Bioturbation	Bioturbation potential, biogenic mixing depth	Sediment Infauna	eDNA to detect presence of key bioturbating taxa
Decomposition	Biomass	Fungi, Bacteria	eDNA to detect presence of cryptic decomposer taxa
Carbon storage and carbon flux	Fraction of sediment carbon from coastal sources	Macroalgae	Metabarcoding of offshore sediments to identify coastal macroalgae

Measuring marine ecosystem functioning

As Table 2 shows, measuring ecosystem functioning *in situ* and at management-relevant scales typically involves using proxies which often involves quantifying the biomass of one or more taxonomic or functional group. Sometimes, data need to be analysed at a finer taxonomic scale. For instance, assessing community-level bioturbation potential involves supplementing taxonomically-resolved benthic surveys with information on relevant functional traits (e.g. body size, burrowing habit, mobility, mode of sediment mixing) of the constituent species (Solan et al. 2004). Ultimately, then, conventional community surveys need to be conducted, with samples analysed to identify and quantify the organisms present.

Many metrics of ecosystem functioning tend to increase with increasing biodiversity, when quantified simply as species richness (e.g. Cardinale et al. 2006). Often these biodiversity-ecosystem functioning (BEF) relationships are sensitive to species identity - for instance, the presence or absence of a single abundant and functionally important species determines the rate at which bioturbation changes with species richness in a NE Atlantic soft sediment community (Solan et al. 2004). Equally, functionally unique rare species contribute substantially to functional diversity (Mouillet et al. 2013), emphasising that bulk measures of biomass or counts of species number are not sufficient to fully quantify ecosystem function. As we discuss in detail below, molecular methods are already widely used to identify species present in environmental samples, and methods to quantify species abundance are becoming increasingly robust, suggesting a clear pathway for the incorporation of DNA technologies into metrics of marine ecosystem function.

Marine ecosystem functioning in the Outcome Indicator Framework

We focus on three Outcome Indicators that are especially relevant to marine ecosystem functioning: D7 (*Species supporting ecosystem functions*), C8 (*Marine food webs functioning*), and C9 (*Seafloor habitats functioning*). As shown in Figure 1, there is considerable overlap in the components of these indicators, as related to marine ecosystems. In particular, D7 and C8 include a very similar set of concerns. Nonetheless, here we describe each indicator in turn, providing an overview of what it is and why it is important, together with some examples of key applications in a UK shelf sea context.

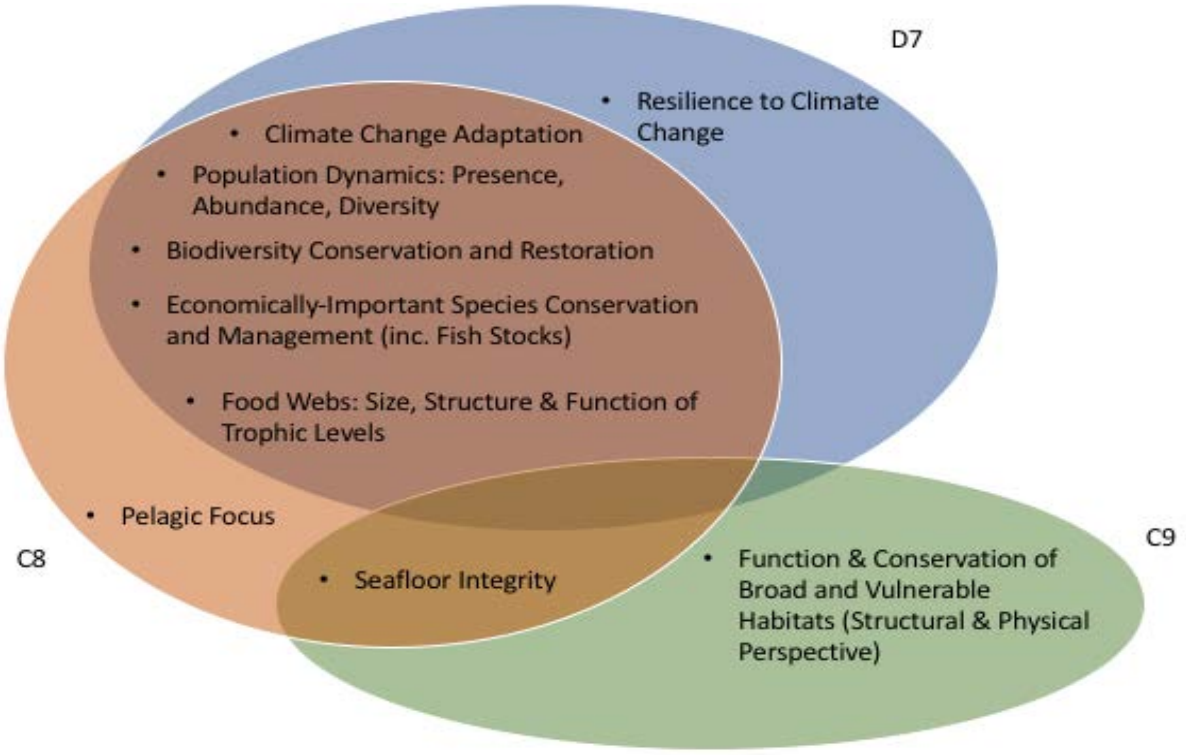

Figure 1 Schematic overview of the components of the three target Outcome Indicators considered in this document, and how they are interrelated. Indicators are D7 Species supporting ecosystem functions; C8 Marine food webs functioning; and C9 Seafloor habitats functioning

D7 Species supporting ecosystem functions

This indicator falls under headline theme 7 of the outcome indicator framework, *Changes in nature on land and water that support our lives and livelihoods*. The ultimate goal is thriving plants and wildlife, and progress towards this requires answers to a series of questions: What are the wider benefits provided to society by thriving plants and wildlife as a fundamental part of ecosystems that deliver multiple benefits, and endow resilience on natural systems? Are plants and wildlife that contribute to important ecosystem functions thriving? Are wildlife habitats increasing in their extent, quality and connectivity? Are wildlife habitats becoming less vulnerable to climate change impacts? Are the populations of widespread species that characterise our farmland, woodlands, wetlands and coastline

increasing? Addressing these questions in a marine context is largely based on quantifying the presence and/or abundance of key species (such as top predators) and the diversity of functionally important groups (e.g. benthic invertebrates). This recognises that all species have a functional role within ecosystems, and thus measuring the presence, abundance and diversity of species will be important in determining the resilience of ecosystems to environmental changes, as well as for reporting progress towards Convention on Biological Diversity Aichi Targets 7 & 8. The use of eDNA and similar technologies for biodiversity assessment (see below) is therefore particularly relevant for developing metrics to support this indicator.

C8 Marine food webs functioning

Indicator C8 contributes to headline theme 5 of the outcome indicator framework, *Changes in the health of our seas that affect our lives and livelihoods*. As for D7, this contributes towards the goal of thriving plants and wildlife, as well as that of using resources more sustainably and efficiently. Relevant questions include whether exploited fish and shellfish communities in our seas are in a healthy condition, and whether marine food webs are functioning well. This involves using metrics on the size, structure and function of different trophic levels in marine food webs to track the health of our seas, which in turn feed in to a range of related reporting structures (e.g. the OSPAR Convention, the UK Marine Strategy, the EU Marine Strategy Framework Directive, and the Convention on Biological Diversity Aichi Target 6). The focus of this indicator is on pelagic habitats and populations of key species groups within the food web which show whether ecosystems are healthy and are being used sustainably, and targets include making sure populations of key species are sustainable with appropriate age structures, reversing the loss of marine biodiversity, and ensuring that fish stocks are maintained at levels that can produce their maximum sustainable yield. This requires accurate methods to survey the presence and abundance of key species, their population dynamics and conservation, and their trophic interactions - all of which are key areas where DNA-based technologies can contribute.

C9 Seafloor habitats functioning

Indicator C9 is designed to show changes in the functionality and extent of seafloor habitats, with the target of ensuring seafloor habitats are productive and sufficiently extensive to support healthy, sustainable ecosystems. This is tightly linked to the UK interpretation of the EU MSFD Descriptor 6 *Seafloor Integrity*, and has relevance too for reporting requirements under the OSPAR Convention, the UK Marine Strategy, the EU Marine Strategy Framework Directive, and the EU Habitats and Birds Directives. It requires an understanding of the distribution and condition of individual broad habitat types and selected vulnerable habitats. Well-functioning seafloor habitats (physically and structurally) are both productive and sufficiently extensive to carry out natural functionality, including the necessary ecological processes which underpin ecosystem goods and services, and are capable of supporting a healthy and sustainable ecosystem for the long term. To the extent that seabed functioning is dependent on key species and functional groups, DNA-based biodiversity assessments can help to deliver relevant metrics. In

addition, direct measures of some aspects of the functioning of sediment communities can be derived from DNA and ’omics technologies as described below.

DNA-based methods to measure marine ecosystem functioning

A useful overview of the potential of DNA sequencing to monitor marine ecological status is provided by Goodwin et al. (2017). Here, we focus specifically on marine applications of key DNA-based methods that have the most potential to contribute to measuring the outcome indicators outlined above. These include the use of eDNA, metabarcoding, and metagenomics to monitor biodiversity, to quantify feeding relationships within food webs, to understand reproduction and population dynamics of key taxa, and to measure the impacts of human activities. Throughout, we refer to eDNA as any DNA that can be obtained through sampling an environmental source, such as water or sediment. This includes both extracellular DNA and DNA contained within whole cells, be they shed from multicellular or microbial organisms. Hence, eDNA studies can include the analysis of microbial DNA, which can be of great use in monitoring ecosystem functioning. We consider both metabarcoding and metagenomics, with the former referring to the method of amplifying specific regions of DNA (e.g. COI), often targeting specific taxonomic groups, from an environmental sample, and the latter referring to shotgun sequencing of all of the DNA obtained from an environmental sample.

Biodiversity Assessment

A first step in assessing marine ecosystem functioning is often to survey which species occur in an ecosystem, and in what numbers. The presence of key species is fundamental to Indicator D7, and understanding marine food webs (Indicator C8) also requires information on both key species of commercial and conservation interest, as well as on whole community composition and interrelationships. Given that certain species also display close affinities with particular seabed habitats, this information is also critical for Indicator C9. DNA methods have been widely used to identify the presence of individual species as well as to survey entire communities on the basis of eDNA, including numerous studies of fish and other vertebrates from a wide range of marine habitats, as well as sediment and plankton invertebrate communities.

DNA-based attempts to quantify community composition have had mixed success – often revealing previously unknown diversity within a region, whilst at the same time missing some taxa known to occur there, either from previous work or from concurrent traditional surveys. For instance, while analysis of eDNA from seawater samples of several habitats within the Qatari Arabian Gulf identified 191 taxa from 73 families of fish, birds, reptiles and mammals, including more than twice as many fish species than were found by visual censuses, only 15% of the fish species known to the region were detected, and almost a third of the 36 species recorded during visual censuses of the same habitats remained undetected by eDNA (Sigsgaard et al. 2019). Efforts to ground-truth DNA technologies have included attempting to quantify a known fish assemblage from a mesocosm, which has sometimes resulted in high levels of agreement (>93% of reads identified as fish species housed in the mesocosm), whilst also highlighting limitations such as a failure to distinguish between congeneric species (Miya et al. 2015). Other similar studies have

been less successful – e.g. detecting just four of nine vertebrate families present in a mesocosm (Kelly et al. 2014).

In general, however, molecular analysis can be far more efficient than visual censusing and other traditional survey methods for cataloguing marine communities. For instance, in an early Danish attempt to quantify fish communities from eDNA analysis of seawater, all conventional survey methods conducted were either matched or outperformed by molecular methods (Thomsen et al. 2012), and eDNA outperformed traditional methods in surveys of zooplankton communities on coral reefs in Florida (Djurhuus et al. 2018). Port et al. (2016) identified more than twice as many fish taxa from seawater samples taken from kelp forest habitats than were observed in concurrent visual surveys, and furthermore were able to distinguish communities from habitats as little as 60 m apart. In Antarctic soft sediments, metabarcoding identified nearly twice as many orders of benthic macrofauna compared to traditional morpho-taxonomy (Vause et al. 2019). As an indication of the potential efficiency of DNA-based methods, more fish species were identified from <100 eDNA samples that took <6 h to collect than from 140 underwater visual surveys conducted over 14 years in one Japanese system (Yamamoto et al. 2017). Even when detection is suboptimal, DNA methods present some advantages. For instance, Baker et al. (2018) were only able to detect orcas *Orcinus orca* from seawater samples associated with 68% of confirmed sightings; but the fact that positive detections were recorded up to 120 minutes after sampling could prove important for sampling of cryptic cetaceans, such as beaked whales (Baker et al. 2018). In addition, some groups are particularly well-suited to molecular surveys. For instance, gelatinous and larval zooplankton, which are difficult to detect in traditional plankton surveys, were effectively censused by eDNA analysis of plankton samples from Western Australia (Berry et al. 2019).

A particularly exciting development concerns the ability to infer not only the distribution (Knudsen et al. 2019) but also the abundance or biomass of some commercially important species such as Atlantic cod *Gadus morhua* from eDNA detection rates (Salter et al. 2019). Biomass quantification remains problematic (Thomsen et al. 2012, Knudsen et al. 2019) but these are promising developments for future fisheries monitoring. Already, eDNA has been used to identify a putative northern range expansion of Atlantic thread herring *Opisthonema oglinum* in Long Island Sound, US (Liu et al. 2019). However, the same study detected the Pacific sand lance *Ammodytes americanus* in complete contradiction to its known range (Liu et al. 2019), reflecting the continued importance of basic taxonomic study, particularly of problematic taxa such as the genus *Ammodytes*.

Lesser-known taxa and habitats can be particularly well-suited to DNA-based sampling. For instance, analysis of seawater samples from the surface to the deep ocean globally tripled the number of known marine virus populations, and doubled the number of bacterial and archaeal virus genera (Roux et al. 2016). Wang et al. (2014) were able to identify over 400 distinct marine fungal phylotypes from across the West Pacific Warm Pool, including two new marine fungal clades. Such approaches have enabled comparative studies of different broad habitat types, showing for instance that benthic bacterial communities are significantly more even and diverse than pelagic bacterial communities, most likely due to a higher density of bacteria in marine sediments, greater temporal stability of benthic

habitats, and a higher degree of niche diversity and resource partitioning in benthic waters (Zinger et al. 2011). In deep sea sediments, metabarcoding has successfully identified multiple metazoan phyla, with a dominance of nematodes considered to reflect both meiofauna-biased reference sequence inventories as well as the genuine ecological importance of meiofauna to deep sea sediment communities (Guardiola et al. 2016, Sinniger et al. 2016). Both DNA and RNA sequencing capture spatial variation in deep-sea sediment communities, although DNA reveals a more diverse community (Guardiola et al. 2016), as it is detectable in both living and dead sources of genetic material. All major eukaryotic groups have been identified from <1 g of deep-sea sediment sampled from the Arctic and Southern Oceans (Pawloski et al. 2011), although the lack of taxonomic resolution and predominance of taxonomically unassigned Operational Taxonomic Units reflects the limited reference databases, not just for microbial eukaryotes, but for deep-sea benthic taxa as a whole (Pawloski et al. 2011). Biases in reference databases may also be partially responsible for the dominance of prokaryotic sequence reads in other habitats too: for instance, on Ningaloo Reef, Western Australia, almost 95% of sequence reads that could be assigned to taxa were bacteria, and only 2.4% were eukaryotes (Stat et al. 2017). Nonetheless, the ability of eDNA to identify microbial and other poorly-sampled taxa has the potential to significantly increase their representation in global biodiversity assessments.

Basic taxonomic surveys can be extended to consider the functional roles of different taxa too. For instance, molecular analysis can track seasonal vertical migrations of photoautotrophic microplankton, as well as revealing higher functional richness of non-photosynthesising microbes in deeper waters (Bryant et al. 2016). Within sediment communities, molecular methods to quantify community composition can be compared with information on abiotic conditions to infer the functional attributes of individual taxa – for instance meiofauna living in known oxygen minimum zones are assumed to be tolerant of such conditions (Sinninger et al. 2016) – and the presence of fungal sequences in deep-sea and other sediments is strongly correlated with organic carbon content (evidence of saprophytic lifestyle), dissolved organic carbon (evidence for fungal metabolism of organic substrates or dead plant material), and concentrations of sulphide, nitrate, orthophosphate and silicic acid (which indicates anaerobic metabolism pathways and nutritional requirements; Orsi et al. 2013, Wang et al. 2014, Vuillemin et al. 2018). The relative roles of autochthonous and allochthonous carbon production in different habitats can also be inferred from fungal assemblage diversity (Wang et al. 2014), while surface water productivity correlates well with both pelagic and benthic bacterial communities (Zinger et al. 2011).

In summary, the consensus from the literature is that biodiversity assessments of marine communities using eDNA methods consistently identify more diverse assemblages than traditional methods (e.g. visual surveys, trawling, BRUVs) as eDNA methods can capture species that are cryptic, transient or uncommon. Application of both universal primers and targeted primers vastly increases the resolution afforded; universal primers capture the wider community at low taxonomic resolution, with targeted primers detecting biodiversity from particular taxonomic groups at high resolution. Inferences of functional diversity can be made through detection of particular taxa. However, biodiversity assessments can be

limited by unpopulated reference databases, particularly for eukaryotes. Indeed, a prior understanding of faunal composition is essential both in choosing suitable primers and to verify the eDNA detection of particular taxa, to ensure spurious identifications of species not actually present in the focus assemblage (i.e. false positives) can be removed. Furthermore, stringent sequence allocation thresholds must be employed to eliminate spurious reads and mitigate contamination. However, these thresholds can lead to rejection of taxa that are present in the assemblage yet are not amplified in eDNA sequencing due to low eDNA contribution or primer biases (i.e. false negatives). Despite these issues, profiling community composition using eDNA remains particularly relevant to the overlapping themes of indicators D7 and C8, notably the diversity-centric population dynamics and biodiversity conservation. Moreover, once methods are well established for a particular system (e.g. comprehensive reference libraries), there will be significant potential to assess spatio-temporal dynamics to monitor assemblage responses to variable environmental factors and climate change.

Measuring Trophic Interactions

Trophic interactions – the feeding relationships among organisms – are fundamental to Outcome Indicator C8 and relevant to D7 and C9 too. They are very difficult to quantify, as observational studies of feeding behaviour require intensive time investment, and remain limited in taxonomic, spatial, and temporal scope. For instance, seabird feeding behaviour has been extensively observed, but this is typically limited to provisioning of chicks in breeding colonies on land. Diets can also be assessed by examining the gut contents of organisms either caught in dedicated surveys (e.g. fish stomach contents; Pinnegar 2014) or opportunistically sampled (e.g. via the UK Cetacean Strandings Investigation Programme, http://ukstrandings.org). This requires the laborious examination of gut contents, and consequent identification of dietary components may be biased by differential preservation (e.g. organisms with hard parts such as crustaceans are better preserved than soft-bodied taxa like jellies), differential gut passage time of different dietary items, and – particularly in the case of stranded specimens – a reliance on abnormal, stressed, or unhealthy organisms for dietary studies. An alternative is to use patterns of coexistence – e.g. the presence of a predator and potential prey species in the same location is used to infer a trophic interaction. This approach, however, is fraught with uncertainty (e.g. Thurman et al. 2019). The use of DNA-based technologies, in particular metabarcoding, to rapidly and reliably identify diets from material including gut contents, faecal samples, and sediments, has therefore garnered considerable interest. Here, we review how these methods have been applied in marine ecosystems, and their strengths and weaknesses.

Metabarcoding has been widely applied to the analyses of faecal samples from marine top predators, in particular seabirds and marine mammals, as well as to the gut contents of fish and some invertebrate taxa. Sometimes this has confirmed known feeding preferences of species of conservation importance, such as the near-exclusively piscivorous diet of red-throated divers *Gavia stellata*, dominated by clupeids (Kleinschmidt et al. 2019), or the dominance of salmonids in the diets of Salish Sea orcas (Ford et al. 2016). Additional resolution is often possible too – for instance, almost all salmon

sequences from scats of harbour seals *Phoca vitulina* were assigned to salmon species and life stage, and corresponded well to known timings of the runs of different salmon species (Thomas et al. 2017). The fact that molecular analyses require smaller volumes of source material than morphological analyses means they can be applied to species where faecal samples are challenging to collect, such as cetaceans (Ford et al. 2016). Sampling the diets of both predators and their prey can help to detect instances of secondary predation too (i.e., prey items present in the gut of the prey of the top predator; e.g. Bowser et al. 2013), and putative trophic relationships inferred by species co-occurrences can be directly tested (e.g. Correia et al. 2019).

DNA-based methods have also frequently expanded the known dietary range of marine predators, including fish (e.g. three-spined stickleback *Gasterosteus aculeatus*; Jakubavičiūtė et al. 2017), shorebirds (e.g. semipalmated sandpiper *Calidris pusilla*; Gerwing et al. 2016), and coastal invertebrates (e.g. brown shrimp *Crangon*, Siegenthaler et al. 2019). Often the newly-identified dietary items are from taxonomic groups that are difficult to detect using traditional analysis, for instance the presence of gelatinous prey in the diets of little (*Eudyptula minor*) and Adélie (*Pygoscelis adeliae*) penguins and of black-browed (*Thalassarche melanophris*) and Campbell (*T. impavida*) albatrosses (Jarman et al. 2013, McInness et al. 2017a, Cavallo et al. 2018), and the presence of rapidly-digested elasmobranchs by Australian fur seals *Arctocephalus pusillus doriferus* (Deagle et al. 2009). At a broader scale, molecular methods have revealed jellyfish to be common among fish species in the Irish Sea, especially by commercially important species herring *Clupea harengus* and whiting *Merlangius merlangus* (Lamb et al. 2017). Only occasionally have molecular methods failed to identify major prey items known from morphological studies (e.g. no squid were identified in the scats of Adélie penguins, despite their known importance from traditional studies; Jarman et al. 2013).

Molecular dietary studies have also helped to expand the scope of marine trophic ecology, in particular due to their ability to consider different life stages, and finer temporal resolutions. For instance, for many seabirds, chick diet is much easier to observe than adult diet, and so the finding that chick and adult diets do not vary significantly in Atlantic puffins *Fratercula arctica* is valuable beyond studies of chick feeding (Bowser et al. 2013). The increased temporal resolution possible from more efficient molecular studies allows dietary composition to be monitored across the reproductive cycle (Cavallo et al. 2018), including in the non-breeding phase when dietary studies are much less common, particularly for migratory seabirds (e.g. Correia et al. 2019). Responses to prey availability can also be tracked – e.g. several seabirds shift to nutritionally-poor gelatinous prey when more energetically-valuable prey (e.g. krill, fish) are scarce (Jarman et al. 2013, McInness et al. 2017a, 2017b). Prey abundance is more important than energetic content to other predators too (e.g. Kleinschmidt et al. 2019), although some, such as Salish Sea orcas, seem to retain a preference for particular salmon species even when others are more abundant (Ford et al. 2016). Changes in diet can also be important in the context of environmental change and other human activities, with differing prey species composition in a predator's diet potentially indicating competition with fisheries or changing prey distributions (e.g. Jarman et al. 2013, McInness et al. 2017b). Better knowledge of predator diets can have implications for management both of the predators themselves

(e.g. the fact that semipalmated sandpiper diets are broader than previously thought implies both potential resilience to habitat loss but also the need for conservation management plans to consider a wider range of habitats; Gerwing et al. 2016), and of their prey (e.g. harbour seals consume species of low conservation concern in the autumn, but spring diets are dominated by species of high conservation concern; Thomas et al. 2017). More generally, when the diets of seabirds and other predators of conservation importance are heavily dependent on commercially important fish species, this reinforces the need for effective fisheries management of these stocks (Bowser et al. 2013).

As well as facilitating more detailed dietary analysis of previously well-studied groups such as seabirds and fish, molecular methods have enabled the first investigations into other taxa too, revealing essential information on basic life history of important marine groups. For instance, specialised dietary behaviours and distinct food preferences have been revealed by molecular analysis of benthic foraminifera (Chronopoulou et al. 2019), and the diet of the pelagic sea squirt *Dolioletta gegenbauri* has been shown to reflect the local marine eukaryotic microbial community, with selective feeding on larger prey items, which contradicts the passive feeding strategy described in traditional studies (Walters et al. 2019). Other novel trophic relationships can also be inferred from molecular studies. For instance, the presence of fungi detected in benthic foraminifera could imply a previously unknown parasitic relationship (Pangiota-Chronopoulou et al. 2019), while detection of fungal taxa in brown shrimp guts suggests symbiosis (Siegenthaler et al. 2019). In other situations, known parasites can be detected: the presence of Apicomplexa taxa in samples of pelagic sea squirts (Walters et al. 2019) and brown shrimp (Siegenthaler et al. 2019) has potential implications for transmission of these parasites in marine ecosystems. In some systems, it is possible to track the dynamics of known parasites with implications for commercial species. For instance, parasitic dinoflagellates of the genus *Hematodinium* infect the common shore crab *Carcinus maenas*, and molecular methods have revealed a high number of parasitised crabs with low parasite load in spring–summer, and a low number of parasitised crabs with high parasite load in autumn–winter (Davies et al. 2019). This is important, because shore crabs act as a parasite vector for the commercially important edible crab *C. pagurus*. This kind of information is important from an ecological and conservation management perspective too; for instance, the prevalence of known parasites (e.g. tapeworm DNA detected in penguin scats; Jarman et al. 2013) can provide valuable information on the health of predator populations.

A final example of how molecular methods can help to add clarity to marine trophic ecology concerns studies of carbon transfer from coastal ecosystems to offshore sediments. Molecular methods are more effective at tracking the origin and fate of blue carbon than traditional approaches (e.g. isotope analyses), and identifying the origin of carbon present in DNA in marine sediments can reveal the principal sources of blue carbon in a range of systems (Geraldi et al. 2019). Queirós et al. (2019) revealed a large seasonal flux of carbon from coastal macroalgae to deep waters, where it is incorporated into sediments and benthic food webs. Not only does this highlight the importance of coastal macroalgae as a food source for offshore benthic fauna, the net positive carbon flux into seabed plays a key role in carbon storage within seafloor habitats (Queirós et al. 2019). Seagrass meadows are another major blue carbon store, and molecular methods

have shown that seagrasses themselves are the primary contributor to this sediment blue carbon, and so carbon sequestration within seagrass meadows does not depend on connectivity with other habitats (Reef et al. 2017).

Together, these examples highlight the enormous potential of eDNA, metabarcoding, and more targeted techniques to increase the spatial and temporal resolution of trophic interactions that were largely known, to expand the known diet breadth of important marine species, to reveal hitherto unknown important food sources for well-known species, and to trace sources of carbon integral to seafloor functioning (Indicator C9). Beyond their role in quantifying food webs (Indicator C8) these dietary studies can also play a role in tracking the abundance and dynamics of commercially-important prey species (e.g. mackerel in seabird diets; Kleinschmidt et al. 2019) and the presence of non-native invasive species (e.g. the barnacle *Austrominius modestus* identified in the diet of *C. crangon* in two estuaries; Siegenthaler et al. 2019), both relevant to indicator D7. The DNA-based technologies used to assess trophic relationships overcome some of the limitations of traditional methods, e.g. their ability to identify prey items with no hard parts. However, they still require the availability of comprehensive reference libraries for prey items to be fully effective, particularly for predators that do not eat well-known or commercially important prey.

Reproduction and Population Dynamics

Reproductive biology and population dynamics are central to the sustainable management of fish and shellfish stocks (Indicator C8) and intersect considerably with conservation issues and biodiversity assessments. Using eDNA to profile reproduction and population dynamics is a relatively recent development, but already molecular assessments of this key component of marine ecosystem functioning can be grouped into three themes: spawning events and species outbreaks; juvenile dynamics; and population genetics and structure.

The release of billions of gametes into the ocean – a practice known as broadcast spawning – is the primary reproductive tactic for many marine species. This vast efflux of genetic material means, theoretically, that molecular technologies can be readily applied to monitor oceanic reproductive events. For example, Bayer et al. (2019) employed quantitative PCR to attempt real-time detection of sea scallop *Placopecten magellanicus* spawning events, by designing PCR primers specific to an Internal Transcribed Spacer (ITS) region that is unique to scallops. In a mesocosm experiment, ITS gene copy number strongly and positively correlated with scallop sperm cell count. In field applications, strong ITS signals coincided with known sea scallop reproductive periods, evidencing the potential of molecular techniques in detecting the reproductive events of a commercially-important broadcast spawning species. This quantitative assay (Bayer et al. 2019) represents a first step in estimating the size of larval output in natural settings, though size-fractionated samples are needed to help discriminate between sperm, eggs, embryos and larvae. Conducting mesocosm experiments to verify spawning events in natural settings has also illuminated the reproductive activity of Japanese eels *Anguilla japonica* – a commercially important species that covers vast distances across its lifecycle. Takeuchi

et al. (2019) demonstrated that, for captive eels, the volume of eDNA released generally increases across life history stages. This is attributed to associated body size increases and greater resilience at later stages (specifically, mature eels can shed and replace old or damaged cells – a substantial source of eDNA not relevant to more juvenile life stages that do not exfoliate). Given that eel eDNA in mesocosm water was up to 200-times greater post-spawning, accounting for developmental variation in eDNA production could allow not only for detection of eel spawning events in natural settings, but also for determining the relative abundance of different life stages.

The importance of monitoring spawning behaviours is not restricted to commercially-important marine species. Indeed, outbreaks of environmentally-damaging taxa – often fuelled by anthropogenic interference – pose a major challenge for marine management. The corallivorous sea-stars *Acanthaster spp.* are a considerable biotic threat to coral reefs. Declines in predatory tritons (family *Ranellidae*) due to over-harvesting have left *Acanthaster* outbreaks biotically unregulated, resulting in substantial predation of tropical corals. Across several mesocosm experiments, Uthicke et al. (2018) applied droplet digital PCR to demonstrate a positive linear relationship between *Acanthaster* biomass and eDNA. In natural settings, *Acanthaster* eDNA was detected in seawater from coral reefs in the midst of an outbreak – but not on pre-outbreak or post-outbreak reefs. Therefore, use of molecular techniques both to pre-empt and to retrospectively evidence *Acanthaster* outbreaks appears limited. Supplementing droplet digital PCR with a quantitative PCR approach did improve the range of eDNA detection by a factor of 45, however this was still inadequate in detecting early *Acanthaster* outbreaks. Similarly, in European waters, RNA signatures from the toxic raphidophyte *Heterosigma akashiwo* – a bloom-forming species that is a major agent of fish mortality and associated economic losses across the continent – strongly and positively correlated with manually-determined cell number (Blanco et al. 2013). Laboratory cultures of *H. akashiwo* allowed the effects of variable environmental conditions on bloom formation to be assessed, but results indicated that variable nutrient content in natural settings could undermine molecular detection of this problematic taxon.

Elsewhere, molecular methods are attempting to reveal population dynamics beyond reproductive events. Chum salmon *Oncorhynchus keta* are anadromous, migrating as juveniles downstream to the open-ocean. Accordingly, tracking the movements of this commercially-important species is challenging, yet represents an essential process for stock management. Across a series of eDNA assessments in both mesocosms and natural settings, Minegishi et al. (2019) showed that although eDNA detection rate was positively correlated with the number of salmon juveniles (which could help identify the primary juvenile habitats of chum salmon in the wild), there were numerous confounding factors, varying from the degree of aggressive interactions between juveniles (a correlate of fish density) to water temperature (with eDNA degrading faster in warmer waters). However, the high concentration of PCR-inhibiting substances in the sampled chum salmon habitats, sourced from coastal development and the remnant effects of a tsunami, precluded the application of molecular techniques for *in-situ* juvenile monitoring (Minegishi et al. 2019). While this may limit applications to monitoring some highly mobile species, molecular technologies have successfully been used to elucidate the genetic structure of populations of others such as the whale shark *Rhincodon typus*. Meekan et al. (2017)

acquired tissue samples from whale shark copepods, employing mtDNA sequencing to demonstrate that copepods have persistent and long-term associations with their host shark, and revealing the presence of two distinct whale shark populations: one in the Atlantic and one in the Indo-Pacific. Genetic material in seawater also provides an opportunity to non-invasively profile whale shark population structure. Again using mtDNA-targeted amplification, Sigsgaard et al. (2017) found a greater number of whale shark haplotypes in seawater samples than returned in traditional tissue analyses, with Arabian Gulf whale sharks shown to be significantly differentiated from the Atlantic, but not Indo-Pacific population. Moreover, concomitant sampling of mackerel tuna *Euthynnus affinis* eDNA confirmed reports that whale sharks aggregate in the Arabian Gulf in pursuit of mackerel tuna spawn – a revelation that can inform the conservation of this regional population, and could be of particular relevance in studies of other mobile taxa of conservation concern.

In coastal Arctic zooplankton communities, eDNA has been used to accurately profile juvenile demography of benthic taxa such as barnacles (*Cirripedia*). In Svalbard, Walczyńska et al. (2019) used Metazoa-specific barcoding to track fluxes in *Cirripedia* larval abundance in response to variable environmental factors. Larval abundance peaked in response to high chlorophyll *a* levels – indicating that mature barnacles reproduce in time for larvae to exploit the spring algal bloom. More generally, eDNA presents an opportunity to monitor distribution change in groups like barnacles which, owing to the long pelagic residence time of their larvae (1-2 months) coupled with the potential for ship and floating-plastic biofouling and ongoing environmental changes, are expected to shift polewards.

Anthropogenic Impacts and Conservation

Mitigating adverse human impacts on the marine realm is a prominent theme in the Outcome Indicator Framework, relevant to all three Indicators assessed here. The potential of eDNA technologies in both identifying and monitoring human impacts pertains to three particular areas: detecting invasive species, managing taxa of conservation concern, and determining the effects of anthropogenic practices and processes on local biota.

Ever-increasing maritime activity has transported non-native marine species over vast distances, often in ballast water or on the hulls of ships. Molecular methods are gradually showing their value in detecting and profiling invasive species - key to informing management and pre-empting eradication. The detection of harmfully-blooming algae (HAB) in Australian ports poses a major threat to the health of both humans and wildlife, with metabarcoding documenting the spread of HABs into waters of high conservation and human-use value (Shaw et al. 2019). One in three ballast tanks of vessels docked in Australian ports contained between one and eight HAB taxa - providing evidence to support calls to strengthen ballast water policies to mitigate further invasions. Employing such pre-emptive measures using eDNA was further justified by Miralles et al. (2019), who used molecular techniques to provide a higher resolution than traditional visual methods for detecting the invasive Atlantic slipper limpet *Crepidula fornicata* in the western

Cantabrian Sea. eDNA analysis showed the eastern Cantabrian Sea to be free from slipper limpets, providing impetus for measures to prevent an eastward spread of the species. Molecular methods also detected the non-native tubeworm *Ficopomatus enigmaticus* on colonised substrates before they were detected visually, demonstrating the potential to detect sessile invasives before settlement (Muñoz-Colmenero et al. 2018). Beyond detection, eDNA analyses have circumvented traditional methods in profiling the effects of invasives on native biodiversity. Dahl et al. (2017) metabarcoded the stomach contents of invasive Indo-Pacific red lionfish *Pterois volitans* in the Gulf of Mexico, which doubled the prey diversity of lionfish reported in traditional morphological studies. Contrary to morphological surveys, this analysis reported near-exclusive piscivory by adult lionfish, with commercially-important species (e.g. red snapper *Lutjanus campechanus*) and juvenile recruits (e.g. vermillion snapper *Rhomboplites aurorubens*) dominating the stomach contents.

Molecular approaches to invasive species detection have not been unanimously successful. For example, von Ammon et al. (2019) failed to detect the spread of the Mediterranean fanworm *Sabella spallanzanii* in New Zealand waters using eDNA, despite repeated visual detection. This may have been due to the eDNA secretion rate of *S. spallanzanii* being too low to detect with the method used, or microbial activity associated with biofilms in the sample area may have degraded *S. spallanzanii* eDNA before it could be detected. Equally, low taxonomic resolution of eDNA studies may preclude detection of some invasives (Holman et al. 2019), while the persistence of extracellular DNA from locally extinct organisms can mask the molecular detection of live invasives (Pochon et al. 2017). RNA-based sequencing - used to discriminate between extinct and extant genetic material - could rectify this, although artefacts specific to both RNA and DNA sequencing remain problematic (see Laroche et al. 2017). Certainly, eDNA holds promise for the early detection of invasives (Holman et al., 2019). Beyond detection, however, confounding environmental factors and molecular artefacts limit its wider use.

Environmental DNA has also been widely used to assess taxa of conservation concern. One major advantage of eDNA is the ability to detect low density taxa in relatively small seawater samples. For example, Hunter et al. (2018) combined droplet digital- and quantitative-PCR to sequence the eDNA of manatees (*Trichechus spp.*) from concentrations as low as three copies per microlitre of seawater. This demonstrates the extremely high detection power afforded when combining multiple assays, especially when compared to traditional aerial surveys, which are limited by the high water turbidity in manatee habitats. Elsewhere, Weltz et al. (2017) detected the endangered Maugean skate *Zearaja maugeana* in as little as one litre of seawater. Importantly, because *Z. maugeana* eDNA degrades faster in seawater of higher dissolved oxygen, detection will be dependent on environmental conditions, and the absence of eDNA identification may not rule out the presence of the species (Weltz et al. 2017). A similar message emerges from other studies. For instance, harbour porpoise *Phocoena phocoena* eDNA was only detected when present at densities much higher than typically observed in natural situations (Foote et al. 2012), although the cetacean-specific primer used in this study did detect a long-finned pilot whale *Globicephala melas* - a species rarely observed in the Baltic Sea,

suggesting that eDNA can be used to monitor transient marine mammals at extremely low densities (Foote et al. 2012).

In a conservation context, genetic analyses are perhaps most important in monitoring taxa that are not only rare, but keystone constituents of local ecosystems. River herring *Alosa spp*. are important nutrient transfer links between marine and freshwater ecosystems, and have declined due to overexploitation, most notably in Chesapeake Bay. Application of quantitative PCR on herring eDNA revealed high spatial segregation of two *Alosa* species (Plough et al., 2018), with alewife herring dominating in waters of high anthropogenic activity, while blueback herring was mainly detected in less-disturbed waters. These distribution patterns were not previously documented in traditional surveys but are essential for informing management of this important genus. The value of eDNA to monitor rare keystone taxa is further demonstrated in sharks in New Caledonia (Boussarie et al. 2018). Using eDNA sequencing with shark-specific primers, shark eDNA was detected in 91% of seawater samples – substantially greater than the detection rate of 15% and 54% in corresponding visual and Baited Remote Underwater Video (BRUV) censuses (two traditional shark monitoring methods), respectively (Boussarie et al. 2018). Indeed, only 9 of 26 species known to the area were detected in visual and BRUV surveys, compared to 13 using eDNA – reducing the 'dark diversity' of sharks in New Caledonia to 50%, with only a few hundred eDNA samples necessary to capture the entire known shark diversity of the region. This is far more efficient than increasing visual and BRUV effort. This could be particularly useful in resolving geographic ranges of poorly known species (e.g. those classified as Data Deficient on the IUCN Red List). Illuminating dark diversity in this way can help to set priorities for marine spatial management.

Environmental DNA is also highly suited to tracking diversity across gradients of human activities, including changes in benthic communities with proximity to oil platforms in the North Sea (Lanzén et al. 2016, Klunder et al. 2018), and increased diversity of sharks at sites with lowest anthropogenic impacts (Bakker et al. 2017). In another study, DNA metabarcoding of a bacteria-specific SSU rDNA gene region revealed changes in sediment bacteria community composition with increasing distances from salmon aquaculture cages, with communities under cages dominated by bacteria known to occupy organic-rich sediments (Stoeck et al. 2018). These changes in bacterial communities were highly congruent with macrofaunal indices constructed in previous studies, suggesting that rapid quantification of communities using eDNA could in some circumstances be used as an efficient indicator of human pressures. Application of eDNA technologies has also been useful in monitoring the ecological consequences of major pollution events, such as oil spills. Metabarcoding of sediments reveals the after-effects of such disasters as differences in sediment communities between contaminated and uncontaminated areas (Xie et al. 2018), with the presence of specific taxa also relevant to seafloor functioning, such as the relative abundances of sediment bacteria, protists and metazoans (which influences trophic interactions), and the presence of oil-degrading microbial taxa indicative of biodegradation of residual oil. Cumulative effects of multiple stressors can also be investigated, for instance combining oil contamination with increased acidification reduces the abundance of some functional groups (especially sulphate-reducers) which typically thrive in oil-contaminated sediments (Coelho et al. 2016). Thus, bio-recovery of oil-

contaminated sediments will be greatly impeded under realistic future acidification scenarios.

Complex interactions between different management objectives can also be revealed using eDNA. For example, fisheries discards are problematic economically but can provide an important source of food for seabirds. McInness et al. (2017b) used metabarcoding of scats of black-browed albatrosses *Thalassarche melanophris* to show that fisheries discards were found in anywhere between 0-60% of albatross scats, with a positive correlation observed between the proportion of samples containing discards and breeding success. This has important consequences for the anti-discard policies, as well as the design of by-catch reduction schemes, and can feed in to broader ecosystem-based fisheries management strategies.

Finally, DNA-based technologies have significant potential to contribute to issues of emerging conservation importance. A good example is the expected rapid increase of seafloor massive sulphide (SMS) mining at deep-sea hydrothermal systems (Boschen et al. 2016). Ensuring this is sustainable means taking measures to conserve the unique chemosynthetic communities found at active hydrothermal vents, as well as the diverse fauna which colonise inactive vents. Genetic tools can help to identify sites of equivalent diversity to be set-aside from mining activities, as well as quantifying connectivity between sites via larval dispersal (Boschen et al. 2016).

Direct Measures of Ecosystem Function

The above examples of the applications of DNA-based technologies to marine ecosystems typically mirror more traditional methods in that they measure proxies of ecosystem functions - e.g. testing for the presence, diversity, and/or abundance of key species. However there are also instances where more direct measures of ecosystem function may be possible. For instance, Queirós et al. (2019) show how the carbon storage potential of offshore sediments can in part be quantified by measuring the transport of coastal macroalgae offshore, using eDNA to identify macroalgal taxa present in sediment samples. Other approaches, such as high-throughput sequencing of ribosomal RNA, can directly reveal microbial activity within sediments. This approach has been used to show activity of distinct fungal communities which digest organic substrates and dead plant material that has been exported to the subseafloor (Orsi et al. 2013). The activity of specific functional genes associated with nitrogen cycling expressed by benthic microbial communities can also be measured using quantitative PCR, both *in situ* and in mesocosm experiments, which can directly test the functional consequences of environmental change including increased CO_2 and elevated temperature (Currie et al. 2017). These examples show the potential to directly measure ecosystem functions of high relevance in particular to Indicator C9, which could play a key role in developing metrics of marine ecosystem function.

Challenges for DNA-based methods

There is no doubt that developments in DNA analysis over the past ten years have revolutionised how we study biodiversity and ecosystem functioning. There are, however, challenges in both the field collection of samples and the technical implementation of DNA assays in order to generate robust results. Here we discuss those in relation to the marine environment and current best practice. In the following section we consider opportunities to meet these challenges that are likely to arise over the next 10 years as technology continues to develop.

Sampling challenges

Arguably the most important step in any eDNA survey, sampling effort needs to reflect the aims of the project and be designed so as to minimise the possibility of false positive and false negative results. Once DNA is released into the environment there are two processes that can influence its detection: degradation and dispersal.

The marine environment presents some unique challenges in terms of the distribution and spread of eDNA after it is released. Suspended DNA particles may be distributed horizontally via ocean currents or vertically in stratified layers of water. Vertical stratification can be caused by differences in temperature and salinity, and reduced by hydrodynamic mixing and inputs of freshwater inputs such as rivers and rainfall (Jeunen et al. 2020). These potential vertical differences are important to consider if sampling for specific taxa, although a study looking for pelagic fish off the south-west English coast trialled sampling at three depths and found no difference in the communities detected by eDNA at each sampling point (C. Brodie, pers. comm., UK DNA conference 2020). However, this should be verified according to the aims of any particular study to maximise the chance of capturing the eDNA of interest.

Variation in particle size of eDNA will also affect its dispersal and distribution. However, eDNA transport follows similar dynamics to that of fine particulate organic matter – a useful standard to predict eDNA movement in aquatic systems (Harrison et al. 2019). The source of shed DNA will also influence where it is detectable. For example, fewer than 6% of eukaryotic families were detected in common between eDNA from surface water, sediment, settlement plates and plankton tows (Koziol et al 2019), again highlighting the need to design a suitable sampling strategy according to the aims of the study.

DNA in the environment can be intracellular within tissues or whole cells or extracellular, which can affect the likelihood of it being captured and analysed. For example, Ramirez et al. (2018) were interested in assessing prokaryote communities in sediments and found that, although extracellular DNA makes up the majority of DNA in marine sediment, it did not interfere with sediment community profiling due to its fast rate of degradation. The large quantities of extracellular DNA in sediments could be problematic for surveying these communities, as the sediments in question may not be the source of the molecules, or may not be accessible if adsorbed to substrate, and therefore lead to false positive results. It has been proposed that eRNA is a better marker to profile sediment communities due to

its high turnover rate, and hence increased likelihood to represent local communities, and direct link to metabolic activity (Torti et al. 2015).

DNA degradation can be affected by a variety of biotic and abiotic processes such as decay due to bacterial action, temperature, exposure to UV light, and particularly of importance in the marine environment, salinity, all of which can affect detectability (Harrison et al. 2019). It is therefore recommended to collect a suite of environmental data alongside eDNA samples in order to examine environmental variation and compare samples and studies in this context (Harrison et al. 2019). In the marine environment DNA degradation also varies according to location, being 1.6 times faster in inshore versus offshore waters (Collins et al. 2018).

A further aspiration for eDNA research is to infer the population size or biomass of the species present, in addition to their presence or absence. As shown above, there has been considerable recent progress towards this aim, however it remains ambitious given the challenges of DNA dispersal and degradation. An alternative to estimating population size based on eDNA concentration is to use information on genetic variability, such as mitochondrial haplotype frequencies (e.g. Sigsgaard et al. 2016). These approaches, however, are restricted to providing minimum estimates of population size and the challenge remains to relate this to age structure to infer further information about population size and genetic variation, such as further proof of concept studies estimating effective population size from mitochondrial haplotypes, or using genetic variation from nuclear DNA regions.

Although the rate of DNA *degradation* between different life stages of fish appears to be the same, the rate of DNA *release* can differ between juveniles and adults. In bluegill sunfish the much larger adults release DNA at 12 times the rate of juveniles, although juveniles release DNA at four times the rate of adults per unit of wet mass, suggesting that biomass of populations dominated by juveniles could be overestimated from eDNA (Maruyama et al. 2014). Stress can also increase DNA release, with 100-fold increase in tissue shedding under some conditions (Harrison et al. 2019, Sassoubre et al. 2016). Knowledge of DNA release rate or starting concentration and decay rates is needed if relating eDNA detection to species biomass (Sassoubre et al. 2016).

Quantification of prey biomass through dietary analysis can also be confounded by DNA degradation, as well as differential digestibility. Analysing little penguin diet in a feeding trial, Deagle et al. (2010) found that the main food items dominated the sequencing data, whereas sequence number from items fed in lower proportions did not reflect those amounts, a pattern which may be driven by differential degradation of prey tissue. Prey detection can be done by analysing either stomach contents or scats. For the latter, the age of the scat and substrate on which it is sampled can influence the success of DNA amplification. McInnes et al. (2017) found that dry scats amplified more successfully than fresher or more recent ones, however dry scats contained a higher proportion of non-food derived DNA (e.g. from fungal taxa), indicating that scats should be collected when as fresh as possible.

Technical challenges

How samples are processed after collection is also crucial to successful implementation of eDNA monitoring. From sample collection through to data analysis there can be variation in methodology, and it is important to know how choices made over each step can influence the outcome of any monitoring effort. Developing standard practices for eDNA metabarcoding assays that share the same aim will be an important process in developing monitoring protocols that allow the generation of both long-term and comparative data sets to be generated (see Goodwin et al. 2017 for further discussion).

Many marine eDNA studies will be based upon filtered water samples, and choice of filter material and pore size, and how the DNA is subsequently isolated, can all influence the amount and type of DNA retained. A study comparing filters and extraction methods found that cellulose-nitrate filters filtered larger volumes of water before becoming clogged, and that a pore size of 0.2 µm combined with Qiagen DNA extraction kits captured the highest yield of DNA (Jeunen et al. 2019), providing a useful starting guide for future projects.

Due to eDNA often being degraded and in low concentration, it is susceptible to contamination, both from other sources of higher molecular weight DNA and among the eDNA samples themselves. Good laboratory practice and inclusion of negative controls and PCRs are therefore crucial to ensuring robust results. As contamination can occur at any stage of the sampling and data generation, Thomsen & Willerslev (2015) advise incorporating blank negative samples at each of the sampling/filtering, DNA extraction and PCR stages.

Metabarcoding relies on the use of ‘universal’ barcoding primers that will amplify a broad range of taxa for a specific genetic region. In reality, primers can be biased towards preferentially binding to and amplifying DNA from certain taxa above others, and the choice of which primers to use is therefore an important step in study design. The priority will be to amplify the target taxa with minimal bias, and potentially exclude certain taxonomic groups. For example, studies targeting eukaryote diversity would ideally avoid amplification of prokaryotic DNA, so as to maximise sequence information for species of interest. Likewise diet studies can be designed to avoid amplifying the DNA of the predator (e.g. Peters et al. 2015). Positive control or mock community mixtures of DNA can be used to test primer suitability (Goodwin et al. 2017).

Data analysis pipelines and the choice of software are other potential sources of variability when analysing sequence data. Studies have shown that parameter choice can make very little difference to ecological inferences from metabarcoding data (e.g. Clare et al. 2016), although a good way to assess pipeline performance is through the use of simulated or test datasets (Goodwin et al. 2017). The availability of reference DNA sequences is critical in order to gain an accurate measure of biodiversity from eDNA. The absence of species in databases can lead to an underestimation of species richness, or lead to erroneous conclusions of species presence if eDNA sequences are incorrectly assigned to the next closest match. A recent gap analysis of reference libraries for aquatic organisms found that although fish were well covered, there were fewer records for marine molluscs and ascidians (Weigand et al. 2019). As another example of data gaps and taxonomic biases,

only around 18% of described marine species currently exist in the Barcode of Life Database, and nearly a third of these are bony fish which constitute only 7% of all marine species (TW, unpublished analysis). Filling in these gaps needs to be a key priority for current and future projects.

Despite these challenges, the use of eDNA is providing many useful insights into marine functioning and biodiversity, often with the potential to be more efficient and cheaper than other established survey techniques. eDNA can, indeed, often go beyond what is possible with morphological taxonomic assessments, such as identification of otherwise cryptic species (e.g. copepods, Jeunen et al. 2020) and microbial assemblages (Goodwin et al. 2017).

Horizon Scan of Opportunities

The last decade has seen significant advances in the development and implementation of DNA-based technology for environmental monitoring. This has been due to a combination of developments in sequencing technology, the generation of novel information such as reference sequence databases, and funding of projects to develop and test new methodologies. With a critical mass of researchers with appropriate skills and knowledge building, and sequencing technology continuing to advance in capacity and reduce in cost, opportunities for new assays for the assessment of ecological functioning in marine environments can be expected to follow.

Capacity building for eDNA knowledge in the UK has been facilitated by the establishment of the UK DNA Working Group, a network of research academics, end-users and other stakeholders who have met at annual conferences since 2014. The group was instrumental in securing funding opportunities by emphasising the potential of eDNA methodology to NERC's Science Committee, resulting in eDNA being among the first 'highlight topics' funded in 2015. With these projects now coming to an end, further targeted funding in order to develop research projects and translate them into monitoring tools will be crucial. Likewise, it will be important to continue with high-quality scientific training in both wet-lab techniques and bioinformatics. Research-council funded capabilities, such as the NERC Biomolecular Analysis Facility (NBAF), can provide access to cutting-edge technology, cleanroom facilities, high performance computing infrastructure and training in the latest practices for the UK research community; the Sheffield and Liverpool nodes of NBAF specialise in metabarcoding and microbial metagenomics, respectively. NERC is committed to further funding of state-of-the-art facilities accessible to the UK research community through the commissioning of its Environmental Omics Facility later this year. This, along with the recently established Defra DNA Centre of Excellence, will facilitate the development and implementation of eDNA monitoring tools in the UK over the coming years.

The commonest technique currently employed for environmental monitoring using eDNA is metabarcoding, combined with sequencing on an Illumina platform. Amplicons of up to ~500 bp in length are obtained and sequenced to a depth of tens to hundreds of thousands of reads per sample. Alternatives to Illumina technology are the long-read sequencers from Pacific Biosciences and Oxford Nanopore Technology, which can generate sequence reads in the order of 10–100 kb, or more, in length. Longer amplicons could in many cases increase the taxonomic resolution of metabarcoding datasets (e.g. Tedersoo et al. 2018), although the lower number of DNA fragments that can be sequenced per run currently results in a higher per-sample cost; these methods are at present more suitable for surveying less taxonomically diverse ecosystems where lower sequencing depth per sample is not prohibitive.

Long-read sequencing technologies are likely to have an increasing impact on the availability of reference sequences for taxonomic assignment from metabarcoding. The longer read lengths and increased accuracy of the PacBio platform over recent years makes it suitable for high-throughput generation of established DNA barcode regions from

identified specimens (e.g. the ~658 bp COI gene, Hebert et al. 2018), meaning that gaps in reference databases can be filled more efficiently when sample material is available. These technologies will also be instrumental in the Darwin Tree of Life Project (https://www.darwintreeoflife.org/), which aims to sequence all 60,000 eukaryote species in the UK over the next ten years. This endeavour will generate a comprehensive database of barcode genes against which metabarcoding data can be screened and identified. However, the initial focus will be on the more easily attainable common terrestrial vertebrates and invertebrates, so it will be towards the end of this project when we see the full benefit for eDNA surveys of marine life.

Metagenomics approaches that do not require the use of PCR, and hence avoid the limitations of this approach, are also likely to be of benefit for future monitoring applications, not only for genomic signatures of metabolic function but also for taxonomic profiling and quantification of population size. This requires a significantly higher sequencing depth per sample in order to generate enough coverage of the taxonomically-informative genome regions, although the increased cost of sequencing can be offset against the time and consumables saving of eliminating the PCR amplification step. Analysis pipelines are being developed to identify and quantify both prokaryotic and eukaryotic species assemblages (Greenfield et al. 2019; Ji et al. 2019). For eukaryotes, however, species present in low abundance are difficult to detect and the approach is currently more suited to bulk sampling of individuals (e.g. Ji et al. 2019) rather the eDNA, although this is an active area of research.

Opportunities are increasing for the autonomous collection of water samples and eDNA, alongside associated environmental metadata. The Plymouth Marine Laboratory employ autonomous data collection buoys in the western English Channel (https://www.pml.ac.uk/Facilities/Data_buoys_en) along with manually-collected weekly water and monthly sediment samples. This allows the analysis of, for example, seasonal peaks in echinoderm spawning and how this is connected to the sediment community (K. Tait, pers. comm., UK DNA conference 2020). Water samples can be collected using remotely operated vehicles (e.g. Everett et al. 2018), or even using environmental sample processors that can be programmed to filter and preserve water samples (e.g. Yamahara et al. 2019). Such systems are also being commercially developed in the UK; Applied Genomics offer a system that can filter up to 50 litres of water over pre-set time periods. DNA sequencing is also becoming more portable with sequencing devices and USB-powered sample preparation (https://nanoporetech.com/products/voltrax) available from Oxford Nanopore Technologies. Real-time sequencing and hence bio-monitoring is therefore now achievable in the field (Krehenwinkel et al. 2019), and feasibly could become autonomous in the near future.

Sample storage is another area to prioritise in the near future. Samples of eDNA collected now will provide a valuable benchmark against which to assess future change, and should also be stored as an archive in order to take advantage of future technological developments. The UK's natural history museums could take the lead on this, with initiatives such as CryoArks expanding to incorporate eDNA (https://www.cryoarks.org/).

With the increasing publication of studies of eDNA in the marine environment there is the need to establish at which point any particular method is at the stage where it can be applied for routine monitoring. A validation scale has been proposed for eDNA studies that employ single-species qPCR assays in order to inform potential end users on the reliability of the approach (https://edna-validation.com/). We propose that a similar scheme indicating technology readiness levels for metabarcoding approaches should be implemented to increase the understanding of the progression of applications from basic principles through to proof of concept and pilot studies to full implementation, and highlight those expected to be most easily put into practice.

These developments in eDNA technology can lead to new assessments of ecological functioning, such as faster evaluations of species presence–absence in marine ecosystems, rapid and comprehensive gut content analysis allowing more comprehensive quantification of food webs with finer spatial and temporal resolution, plus an increased ability to estimate biomass and abundance. Over the coming period there will be the opportunity to implement new environmental monitoring legislation and methodology as the UK leaves the EU. We have the required technology and methodology, and now need to work with the Defra DNA Centre of Excellence and other stakeholders to identify monitoring priorities.

Recommendations for Future Focus

The above sections explore a range of challenges and opportunities for the widespread adoption of DNA-based methods for monitoring marine ecosystem function. From these, the following specific recommendations should be priorities for future research and development in this area:

- Develop commonalities or standardised approaches to bioinformatics pipelines to ensure reliability of implementing DNA methodology to marine ecosystem monitoring.
- Develop sampling technology, such as autonomous sample collection, to overcome the operational challenges of implementing large-scale monitoring.
- Develop DNA sequence reference databases to allow robust inferences to be made from eDNA data.
- Develop a validation scale for marine eDNA monitoring metrics to easily permit assessment of the readiness of methods to be routinely applied.
- Work with the biodiversity informatics community to develop clear pipelines to ensure that new DNA-derived databases are fully interoperable with other classes of biodiversity data.

References

BAKER, C. S., Steel, D., Nieukirk, S., & Klinck, H. (2018). Environmental DNA (eDNA) from the wake of the whales: Droplet digital PCR for detection and species identification. *Frontiers in Marine Science*, *5*(APR), 1–11. https://doi.org/10.3389/fmars.2018.00133

BAKKER, J., Wangensteen, O. S., Chapman, D. D., Boussarie, G., Buddo, D., Guttridge, T. L., Hertler, H., Mouillot, D., Vigliola, L., & Mariani, S. (2017). Environmental DNA reveals tropical shark diversity in contrasting levels of anthropogenic impact. *Scientific Reports*, *7*(1), 1–11. https://doi.org/10.1038/s41598-017-17150-2

BAYER, S. R., Countway, P. D., & Wahle, R. A. (2019). Developing an eDNA toolkit to quantify broadcast spawning events of the sea scallop Placopecten magellanicus: Moving beyond fertilization assays. *Marine Ecology Progress Series*, *621*, 127–141. https://doi.org/10.3354/meps12991

BEAUMONT, N. J., Austen, M. C., Mangi, S. C., & Townsend, M. (2008). Economic valuation for the conservation of marine biodiversity. *Marine Pollution Bulletin*. https://doi.org/10.1016/j.marpolbul.2007.11.013

BERRY, T. E., Saunders, B. J., Coghlan, M. L., Stat, M., Jarman, S., Richardson, A. J., Davies, C. H., Berry, O., Harvey, E. S., & Bunce, M. (2019). Marine environmental DNA biomonitoring reveals seasonal patterns in biodiversity and identifies ecosystem responses to anomalous climatic events. *PLoS Genetics*, *15*(2), 1–19. https://doi.org/10.1371/journal.pgen.1007943

BLANCO, E. P., Hagström, J., Salomon, P. S., & Granéli, E. (2013). Detection of Heterosigma akashiwo (Hada) using specific RNA probes: Variability of RNA content with environmental conditions. *Harmful Algae*, *24*, 80–88. https://doi.org/10.1016/j.hal.2013.02.001

BOSCHEN, R. E., Collins, P. C., Tunnicliffe, V., Carlsson, J., Gardner, J. P. A., Lowe, J., McCrone, A., Metaxas, A., Sinniger, F., & Swaddling, A. (2016). A primer for use of genetic tools in selecting and testing the suitability of set-aside sites protected from deep-sea seafloor massive sulfide mining activities. *Ocean and Coastal Management*, *122*(March), 37–48. https://doi.org/10.1016/j.ocecoaman.2016.01.007

BOUSSARIE, G., Bakker, J., Wangensteen, O. S., Mariani, S., Bonnin, L., Juhel, J. B., Kiszka, J. J., Kulbicki, M., Manel, S., Robbins, W. D., Vigliola, L., & Mouillot, D. (2018). Environmental DNA illuminates the dark diversity of sharks. *Science Advances*, *4*(5). https://doi.org/10.1126/sciadv.aap9661

BOWSER, A. K., Diamond, A. W., & Addison, J. A. (2013). From puffins to plankton: A DNA-based analysis of a seabird food chain in the Northern Gulf of Maine. *PLoS One*, *8*(12), 1–16. https://doi.org/10.1371/journal.pone.0083152

BRYANT, J. A., Aylward, F. O., Eppley, J. M., Karl, D. M., Church, M. J., & DeLong, E. F. (2016). Wind and sunlight shape microbial diversity in surface waters of the North Pacific Subtropical Gyre. *ISME Journal*, *10*(6), 1308–1322. https://doi.org/10.1038/ismej.2015.221

CARDINALE, B. J., Srivastava, D. S., Duffy, J. E., Wright, J. P., Downing, A. L., Sankaran, M., & Jouseau, C. (2006). Effects of biodiversity on the functioning of trophic groups and ecosystems. *Nature*. https://doi.org/10.1038/nature05202

CAVALLO, C., Chiaradia, A., Deagle, B. E., McInnes, J. C., Sánchez, S., Hays, G. C., & Reina, R. D. (2018). Molecular analysis of predator scats reveals role of salps in temperate inshore food webs. *Frontiers in Marine Science*, *5*(OCT), 1–14. https://doi.org/10.3389/fmars.2018.00381

CLARE EL, Chain FJJ, Littlefair JE, Cristescu ME (2016) The effects of parameter choice on defining molecular operational taxonomic units and resulting ecological analyses of metabarcoding data. *Genome*, 59, 981–990.

COELHO, F. J. R. C., Cleary, D. F. R., Costa, R., Ferreira, M., Polónia, A. R. M., Silva, A. M. S., Simões, M. M. Q., Oliveira, V., & Gomes, N. C. M. (2016). Multitaxon activity profiling reveals differential microbial response to reduced seawater pH and oil pollution. *Molecular Ecology*, *25*(18), 4645–4659. https://doi.org/10.1111/mec.13779

COLLINS RA, Wangensteen OS, O'Gorman EJ *et al.* (2018) Persistence of environmental DNA in marine systems. *Communications Biology*, 1, 185.

CORREIA, E., Granadeiro, J. P., Mata, V. A., Regalla, A., & Catry, P. (2019). Trophic interactions between migratory seabirds, predatory fishes and small pelagics in coastal West Africa. *Marine Ecology Progress Series*. https://doi.org/10.3354/meps13022

CURRIE, A. R., Tait, K., Parry, H., de Francisco-Mora, B., Hicks, N., Mark Osborn, A., Widdicombe, S., & Stahl, H. (2017). Marine microbial gene abundance and community composition in response to ocean acidification and elevated temperature in two contrasting coastal marine sediments. *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2017.01599

DAHL, K. A., Patterson, W. F., Robertson, A., & Ortmann, A. C. (2017). DNA barcoding significantly improves resolution of invasive lionfish diet in the Northern Gulf of Mexico. *Biological Invasions*, *19*(6), 1917–1933. https://doi.org/10.1007/s10530-017-1407-3

DAVIES, C. E., Batista, F. M., Malkin, S. H., Thomas, J. E., Bryan, C. C., Crocombe, P., Coates, C. J., & Rowley, A. F. (2019). Spatial and temporal disease dynamics of the parasite Hematodinium sp. In shore crabs, Carcinus maenas. *Parasites and Vectors*,*12*(1), 1–15. https://doi.org/10.1186/s13071-019-3727-x

DEAGLE, B. E., Chiaradia, A., McInnes, J., & Jarman, S. N. (2010). Pyrosequencing faecal DNA to determine diet of little penguins: Is what goes in what comes out? *Conservation Genetics*, *11*(5), 2039–2048. https://doi.org/10.1007/s10592-010-0096-6

DEAGLE, B. E., Kirkwood, R., & Jarman, S. N. (2009). Analysis of Australian fur seal diet by pyrosequencing prey DNA in faeces. *Molecular Ecology*, *18*(9), 2022–2038. https://doi.org/10.1111/j.1365-294X.2009.04158.x

DJURHUUS, A., Pitz, K., Sawaya, N. A., Rojas-Márquez, J., Michaud, B., Montes, E., Muller-Karger, F., & Breitbart, M. (2018). Evaluation of marine zooplankton community structure through environmental DNA metabarcoding. *Limnology and Oceanography: Methods*,*16*(4), 209–221. https://doi.org/10.1002/lom3.10237

EVERETT, M. V., & Park, L. K. (2018). Exploring deep-water coral communities using environmental DNA. *Deep-Sea Research Part II: Topical Studies in Oceanography*, *150*, 229–241. https://doi.org/10.1016/j.dsr2.2017.09.008

FOOTE, A. D., Thomsen, P. F., Sveegaard, S., Wahlberg, M., Kielgast, J., Kyhn, L. A., Salling, A. B., Galatius, A., Orlando, L., & Gilbert, M. T. P. (2012). Investigating the Potential Use of Environmental DNA (eDNA) for Genetic Monitoring of Marine Mammals. *PLoS One*, *7*(8), 2–7. https://doi.org/10.1371/journal.pone.0041781

FORD, M. J., Hempelmann, J., Hanson, M. B., Ayres, K. L., Baird, R. W., Emmons, C. K., Lundin, J. I., Schorr, G. S., Wasser, S. K., & Park, L. K. (2016). Estimation of a killer whale (Orcinus orca) population's diet using sequencing analysis of DNA from feces. *PLoS One*, *11*(1), 1–14. https://doi.org/10.1371/journal.pone.0144956

GERALDI, N. R., Ortega, A., Serrano, O., Macreadie, P. I., Lovelock, C. E., Krause-Jensen, D., Kennedy, H., Lavery, P. S., Pace, M. L., Kaal, J., & Duarte, C. M. (2019). Fingerprinting Blue Carbon: Rationale and Tools to Determine the Source of Organic Carbon in Marine Depositional Environments. *Frontiers in Marine Science*, *6*(May), 1–9. https://doi.org/10.3389/fmars.2019.00263

GERWING, T. G., Kim, J.-H., Hamilton, D. J., Barbeau, M. A., & Addison, J. A. (2016). Diet reconstruction using next-generation sequencing increases the known ecosystem usage by a shorebird. *The Auk*, *133*(2), 168–177. https://doi.org/10.1642/auk-15-176.1

GOODWIN, K. D., Thompson, L. R., Duarte, B., Kahlke, T., Thompson, A. R., Marques, J. C., & Caçador, I. (2017). DNA sequencing as a tool to monitor marine ecological status. *Frontiers in Marine Science*, *4*(MAY), 1–14. https://doi.org/10.3389/fmars.2017.00107

GREENFIELD, P., Tran-Dinh, N., & Midgley, D. (2019). Kelpie: Generating full-length "amplicons" from whole-metagenome datasets. *PeerJ*. https://doi.org/10.7717/peerj.6174

GUARDIOLA, M., Wangensteen, O. S., Taberlet, P., Coissac, E., Uriz, M. J., & Turon, X. (2016). Spatio-temporal monitoring of deep-sea communities using metabarcoding of sediment DNA and RNA. *PeerJ*, *2016*(12), 1–31. https://doi.org/10.7717/peerj.2807

HARRISON, J. B., Sunday, J. M., & Rogers, S. M. (2019). Predicting the fate of eDNA in the environment and implications for studying biodiversity. *Proceedings of the Royal Society B. Biological Sciences*,*286*(1915), 20191409. https://doi.org/10.1098/rspb.2019.1409

HEBERT, P. D. N., Braukmann, T. W. A., Prosser, S. W. J., Ratnasingham, S., deWaard, J. R., Ivanova, N. V., Janzen, D. H., Hallwachs, W., Naik, S., Sones, J. E., & Zakharov, E. V. (2018). A Sequel to Sanger: Amplicon sequencing that scales. *BMC Genomics*. https://doi.org/10.1186/s12864-018-4611-3

HOLMAN, L. E., de Bruyn, M., Creer, S., Carvalho, G., Robidart, J., & Rius, M. (2019). Detection of introduced and resident marine species using environmental DNA metabarcoding of sediment and water. *Scientific Reports*, *9*(1), 1–10. https://doi.org/10.1038/s41598-019-47899-7

HUNTER, M. E., Meigs-Friend, G., Ferrante, J. A., Kamla, A. T., Dorazio, R. M., Diagne, L. K., Luna, F., Lanyon, J. M., & Reid, J. P. (2018). Surveys of environmental DNA (eDNA): A new approach to estimate occurrence in Vulnerable manatee populations. *Endangered Species Research*, *35*, 101–111. https://doi.org/10.3354/esr00880

JAKUBAVIČIUTE, E., Bergström, U., Eklöf, J. S., Haenel, Q., & Bourlat, S. J. (2017). DNA metabarcoding reveals diverse diet of the three-spined stickleback in a coastal ecosystem. *PLoS One*, *12*(10), 1–16. https://doi.org/10.1371/journal.pone.0186929

JARMAN, S. N., McInnes, J. C., Faux, C., Polanowski, A. M., Marthick, J., Deagle, B. E., Southwell, C., & Emmerson, L. (2013). Adélie penguin population diet monitoring by analysis of food DNA in scats. *PLoS One*, *8*(12). https://doi.org/10.1371/journal.pone.0082227

JEUNEN G-J, Knapp M, Spencer HG *et al.* (2019) Species-level biodiversity assessment using marine environmental DNA metabarcoding requires protocol optimization and standardization. *Ecology and Evolution*, 9, 1323–1335.

JEUNEN G, Lamare MD, Knapp M *et al.* (2020) Water stratification in the marine biome restricts vertical environmental DNA (eDNA) signal dispersal. *Environmental DNA*, 2, 99–111.

JI, Y., Huotari, T., Roslin, T., Schmidt, N. M., Wang, J., Yu, D., & Ovaskainen, O. (2019). SPIKEPIPE: A metagenomic pipeline for the accurate quantification of eukaryotic species ccurrences and intraspecific abundance change using DNA barcodes or mitogenomes. *BioRxiv*. https://doi.org/10.1101/533737

KELLY, R. P., Port, J. A., Yamahara, K. M., & Crowder, L. B. (2014). Using environmental DNA to census marine fishes in a large mesocosm. *PLoS One*, *9*(1). https://doi.org/10.1371/journal.pone.0086175

KLEINSCHMIDT, B., Burger, C., Dorsch, M., Nehls, G., Heinänen, S., Morkūnas, J., Žydelis, R., Moorhouse-Gann, R. J., Hipperson, H., Symondson, W. O. C., & Quillfeldt, P. (2019). The diet of red-throated divers (Gavia stellata) overwintering in the German Bight (North Sea) analysed using molecular diagnostics. *Marine Biology*, *166*(6). https://doi.org/10.1007/s00227-019-3523-3

KLUNDER, L., Lavaleye, M. S. S., Filippidi, A., van Bleijswijk, J. D. L., Reichart, G.-J., van der Veer, H. W., Duineveld, G. C. A., & Mienis, F. (2018). Impact of an artificial structure on the benthic community composition in the southern North Sea: assessed by a morphological and molecular approach. *ICES Journal of Marine Science*. https://doi.org/10.1093/icesjms/fsy114

KNUDSEN, S. W., Ebert, R. B., Hesselsøe, M., Kuntke, F., Hassingboe, J., Mortensen, P. B., Thomsen, P. F., Sigsgaard, E. E., Hansen, B. K., Nielsen, E. E., & Møller, P. R. (2019). Species-specific detection and quantification of environmental DNA from marine fishes in the Baltic Sea. *Journal of Experimental Marine Biology and Ecology*,*510*(September), 31–45. https://doi.org/10.1016/j.jembe.2018.09.004

KOZIOL, A., Stat, M., Simpson, T., Jarman, S., DiBattista, J. D., Harvey, E. S., Marnane, M., McDonald, J., & Bunce, M. (2019). Environmental DNA metabarcoding studies are critically affected by substrate selection. *Molecular Ecology Resources*, *19*(2), 366–376. https://doi.org/10.1111/1755-0998.12971

KREHENWINKEL, H., Pomerantz, A., & Prost, S. (2019). Genetic biomonitoring and biodiversity assessment using portable sequencing technologies: Current uses and future directions. In *Genes*. https://doi.org/10.3390/genes10110858

LAMB, P. D., Hunter, E., Pinnegar, J. K., Creer, S., Davies, R. G., & Taylor, M. I. (2017). Jellyfish on the menu: MtDNA assay reveals scyphozoan predation in the Irish sea. *Royal Society Open Science*, *4*(11). https://doi.org/10.1098/rsos.171421

LANZÉN, A., Lekang, K., Jonassen, I., Thompson, E. M., & Troedsson, C. (2016). High-throughput metabarcoding of eukaryotic diversity for environmental monitoring of offshore oil-drilling activities. *Molecular Ecology*, *25*(17), 4392–4406. https://doi.org/10.1111/mec.13761

LAROCHE, O., Wood, S. A., Tremblay, L. A., Lear, G., Ellis, J. I., & Pochon, X. (2017). Metabarcoding monitoring analysis: The pros and cons of using co-extracted environmental DNA and RNA data to assess offshore oil production impacts on benthic communities. *PeerJ*. https://doi.org/10.7717/peerj.3347

LIU, Y., Wikfors, G. H., Rose, J. M., McBride, R. S., Milke, L. M., & Mercaldo-Allen, R. (2019). Application of Environmental DNA Metabarcoding to Spatiotemporal Finfish Community Assessment in a Temperate Embayment. *Frontiers in Marine Science*,*6*(November), 1–12. https://doi.org/10.3389/fmars.2019.00674

MARUYAMA, A., Nakamura, K., Yamanaka, H., Kondoh, M., & Minamoto, T. (2014). The release rate of environmental DNA from juvenile and adult fish. *PLoS One*, *9*(12), 1–13. https://doi.org/10.1371/journal.pone.0114639

McINNES, J. C., Alderman, R., Deagle, B. E., Lea, M. A., Raymond, B., & Jarman, S. N. (2017). Optimised scat collection protocols for dietary DNA metabarcoding in vertebrates. *Methods in Ecology and Evolution*, *8*(2), 192–202. https://doi.org/10.1111/2041-210X.12677

McINNES, J. C., Alderman, R., Lea, M. A., Raymond, B., Deagle, B. E., Phillips, R. A., Stanworth, A., Thompson, D. R., Catry, P., Weimerskirch, H., Suazo, C. G., Gras, M., & Jarman, S. N. (2017). High occurrence of jellyfish predation by black-browed and Campbell albatross identified by DNA metabarcoding. *Molecular Ecology*. https://doi.org/10.1111/mec.14245

McINNES, J. C., Jarman, S. N., Lea, M. A., Raymond, B., Deagle, B. E., Phillips, R. A., Catry, P., Stanworth, A., Weimerskirch, H., Kusch, A., Gras, M., Cherel, Y., Maschette, D., & Alderman, R. (2017). DNA metabarcoding as a marine conservation and management tool: A circumpolar examination of fishery discards in the diet of threatened albatrosses. *Frontiers in Marine Science*, *4*(AUG), 1–22. https://doi.org/10.3389/fmars.2017.00277

MEEKAN, M., Austin, C. M., Tan, M. H., Wei, N. W. V., Miller, A., Pierce, S. J., Rowat, D., Stevens, G., Davies, T. K., Ponzo, A., & Gan, H. M. (2017). iDNA at sea: Recovery of whale shark (Rhincodon typus) mitochondrial DNA sequences from the whale shark copepod (Pandarus rhincodonicus) confirms global population structure. *Frontiers in Marine Science*, *4*(DEC), 1–8. https://doi.org/10.3389/fmars.2017.00420

MINEGISHI, Y., Wong, M. K. S., Kanbe, T., Araki, H., Kashiwabara, T., Ijichi, M., Kogure, K., & Hyodo, S. (2019). Spatiotemporal distribution of juvenile chum salmon in Otsuchi Bay, Iwate, Japan, inferred from environmental DNA. *PLoS One*, *14*(9), 1–22. https://doi.org/10.1371/journal.pone.0222052

MIRALLES, L., Parrondo, M., Hernández de Rojas, A., Garcia-Vazquez, E., & Borrell, Y. J. (2019). Development and validation of eDNA markers for the detection of Crepidula fornicata in environmental samples. *Marine Pollution Bulletin*, *146*(June), 827–830. https://doi.org/10.1016/j.marpolbul.2019.07.050

MIYA, M., Sato, Y., Fukunaga, T., Sado, T., Poulsen, J. Y., Sato, K., Minamoto, T., Yamamoto, S., Yamanaka, H., Araki, H., Kondoh, M., & Iwasaki, W. (2015). MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: Detection of more than 230 subtropical marine species. *Royal Society Open Science*, *2*(7). https://doi.org/10.1098/rsos.150088

MOUILLOT, D., Bellwood, D. R., Baraloto, C., Chave, J., Galzin, R., Harmelin-Vivien, M., Kulbicki, M., Lavergne, S., Lavorel, S., Mouquet, N., Paine, C. E. T., Renaud, J., & Thuiller, W. (2013). Rare Species Support Vulnerable Functions in High-Diversity Ecosystems. *PLoS Biology*. https://doi.org/10.1371/journal.pbio.1001569

MUÑOZ-COLMENERO, M., Ardura, A., Clusa, L., Miralles, L., Gower, F., Zaiko, A., & Garcia-Vazquez, E. (2018). New specific molecular marker detects Ficopomatus enigmaticus from water eDNA before positive results of conventional sampling. *Journal for Nature Conservation*, *43*(March), 173–178. https://doi.org/10.1016/j.jnc.2017.12.004

ORSI, W., Biddle, J. F., & Edgcomb, V. (2013). Deep Sequencing of Subseafloor Eukaryotic rRNA Reveals Active Fungi across Marine Subsurface Provinces. *PLoS One*, *8*(2). https://doi.org/10.1371/journal.pone.0056335

PANAGIOTA-CHRONOPOULOU, M., Salonen, I., Bird, C., Reichart, G. J., & Koho, K. A. (2019). Metabarcoding insights into the trophic behavior and identity of intertidal benthic foraminifera. *Frontiers in Microbiology*, *10*(MAY), 1–16. https://doi.org/10.3389/fmicb.2019.01169

PATERSON, D.M., E.C. Defew, J. Jabour (2012) Ecosystem function and co-evolution of terminology in marine science and management. In: Solan, M., R.J. Aspen, D.M. Paterson (Eds) Marine Biodiversity and Ecosystem Functioning: Frameworks, Methodologies, and Integration. Oxford University Press.

PAWLOWSKI, J., Christen, R., Lecroq, B., Bachar, D., Shahbazkia, H. R., Amaral-Zettler, L., & Guillou, L. (2011). Eukaryotic richness in the abyss: Insights from pyrotag sequencing. *PLoS One*, *6*(4). https://doi.org/10.1371/journal.pone.0018169

PETERS KJ, Ophelkeller K, Bott NJ *et al.* (2015) Fine-scale diet of the Australian sea lion (*Neophoca cinerea*) using DNA-based analysis of faeces. *Marine Ecology*, 36, 347–367.

PINNEGAR, J. K., Goñi, N., Trenkel, V. M., Arrizabalaga, H., Melle, W., Keating, J., & Óskarsson, G. (2014). A new compilation of stomach content data for commercially-important pelagic fish species in the Northeast Atlantic. *Earth System Science Data Discussions*. https://doi.org/10.5194/essdd-7-197-2014

PLOUGH, L. V., Ogburn, M. B., Fitzgerald, C. L., Geranio, R., Marafino, G. A., & Richie, K. D. (2018). Environmental DNA analysis of river herring in Chesapeake Bay: A powerful tool for monitoring threatened keystone species. *PLoS One*, *13*(11), 1–25. https://doi.org/10.1371/journal.pone.0205578

POCHON, X., Zaiko, A., Fletcher, L. M., Laroche, O., & Wood, S. A. (2017). Wanted dead or alive? Using metabarcoding of environmental DNA and RNA to distinguish living assemblages for biosecurity applications. *PLoS One*, *12*(11), 1–19. https://doi.org/10.1371/journal.pone.0187636

PORT, J. A., O'Donnell, J. L., Romero-Maraccini, O. C., Leary, P. R., Litvin, S. Y., Nickols, K. J., Yamahara, K. M., & Kelly, R. P. (2016). Assessing vertebrate biodiversity in a kelp forest ecosystem using environmental DNA. *Molecular Ecology*, *25*(2), 527–541. https://doi.org/10.1111/mec.13481

QUEIRÓS, A. M., Stephens, N., Widdicombe, S., Tait, K., McCoy, S. J., Ingels, J., Rühl, S., Airs, R., Beesley, A., Carnovale, G., Cazenave, P., Dashfield, S., Hua, E., Jones, M., Lindeque, P., McNeill, C. L., Nunes, J., Parry, H., Pascoe, C., … Somerfield, P. J. (2019). Connected macroalgal-sediment systems: blue carbon and food webs in the deep coastal ocean. *Ecological Monographs*, *89*(3), 1–21. https://doi.org/10.1002/ecm.1366

RAMÍREZ, G. A., Jørgensen, S. L., Zhao, R., & D'Hondt, S. (2018). Minimal Influence of Extracellular DNA on Molecular Surveys of Marine Sedimentary Communities. *Frontiers in Microbiology*, *9*(December), 1–12. https://doi.org/10.3389/fmicb.2018.02969

REEF, R., Atwood, T. B., Samper-Villarreal, J., Adame, M. F., Sampayo, E. M., & Lovelock, C. E. (2017). Using eDNA to determine the source of organic carbon in seagrass meadows. *Limnology and Oceanography*, *62*(3), 1254–1265. https://doi.org/10.1002/lno.10499

ROUX, S., Brum, J. R., Dutilh, B. E., Sunagawa, S., Duhaime, M. B., Loy, A., Poulos, B. T., Solonenko, N., Lara, E., Poulain, J., Pesant, S., Kandels-Lewis, S., Dimier, C., Picheral, M., Searson, S., Cruaud, C., Alberti, A., Duarte, C. M., Gasol, J. M., … Sullivan, M. B. (2016). Ecogenomics and potential biogeochemical impacts of globally abundant ocean viruses. *Nature*, *537*(7622), 689–693. https://doi.org/10.1038/nature19366

SALTER, I., Joensen, M., Kristiansen, R. et al. Environmental DNA concentrations are correlated with regional biomass of Atlantic cod in oceanic waters. Commun Biol 2, 461 (2019). https://doi.org/10.1038/s42003-019-0696-8

SASSOUBRE, L. M., Yamahara, K. M., Gardner, L. D., Block, B. A., & Boehm, A. B. (2016). Quantification of Environmental DNA (eDNA) Shedding and Decay Rates for Three Marine Fish. *Environmental Science and Technology*, *50*(19), 10456–10464. https://doi.org/10.1021/acs.est.6b03114

SHAW, J. L. A., Weyrich, L. S., Hallegraeff, G., & Cooper, A. (2019). Retrospective eDNA assessment of potentially harmful algae in historical ship ballast tank and marine port sediments. *Molecular Ecology*, *28*(10), 2476–2485. https://doi.org/10.1111/mec.15055

SIEGENTHALER, A., Wangensteen, O. S., Benvenuto, C., Campos, J., & Mariani, S. (2019). DNA metabarcoding unveils multiscale trophic variation in a widespread coastal opportunist. *Molecular Ecology*, *28*(2), 232–249. https://doi.org/10.1111/mec.14886

SIGSGAARD, E. E., Nielsen, I. B., Bach, S. S., Lorenzen, E. D., Robinson, D. P., Knudsen, S. W., Pedersen, M. W., Jaidah, M. Al, Orlando, L., Willerslev, E., Møller, P. R., & Thomsen, P. F. (2017). Population characteristics of a large whale shark aggregation inferred from seawater environmental DNA. *Nature Ecology & Evolution*,*1*(1), 7–11. https://doi.org/10.1038/s41559-016-0004

SIGSGAARD, E. E, Torquato, F., Frøslev, T. G., Moore, A. B. M., Mølgård Sørensen, J., Range, P., Ben Hamadou, R., Sanvig Bach, S., Rask Møller, P., & Thomsen, P. F. (2019). Using vertebrate environmental DNA from seawater in biomonitoring of marine habitats. *Conservation Biology*, cobi.13437. https://doi.org/10.1111/cobi.13437

SINNIGER, F., Pawlowski, J., Harii, S., Gooday, A. J., Yamamoto, H., Chevaldonné, P., Cedhagen, T., Carvalho, G., & Creer, S. (2016). Worldwide analysis of sedimentary DNA reveals major gaps in taxonomic knowledge of deep-sea benthos. *Frontiers in Marine Science*, *3*(JUN), 1–14. https://doi.org/10.3389/fmars.2016.00092

SOLAN, M., Cardinale, B. J., Downing, A. L., Engelhardt, K. A. M., Ruesink, J. L., & Srivastava, D. S. (2004). Extinction and ecosystem function in the marine benthos. *Science*. https://doi.org/10.1126/science.1103960

STAT, M., Huggett, M. J., Bernasconi, R., Dibattista, J. D., Berry, T. E., Newman, S. J., Harvey, E. S., & Bunce, M. (2017). Ecosystem biomonitoring with eDNA: Metabarcoding across the tree of life in a tropical marine environment. *Scientific Reports*, *7*(1), 1–11. https://doi.org/10.1038/s41598-017-12501-5

STOECK, T., Frühe, L., Forster, D., Cordier, T., Martins, C. I. M., & Pawlowski, J. (2018). Environmental DNA metabarcoding of benthic bacterial communities indicates the benthic footprint of salmon aquaculture. *Marine Pollution Bulletin*, *127*(October), 139–149. https://doi.org/10.1016/j.marpolbul.2017.11.065

TAKEUCHI, A., Iijima, T., Kakuzen, W., Watanabe, S., Yamada, Y., Okamura, A., Horie, N., Mikawa, N., Miller, M. J., Kojima, T., & Tsukamoto, K. (2019). Release of eDNA by different life history stages and during spawning activities of laboratory-reared Japanese eels for interpretation of oceanic survey data. *Scientific Reports*, *9*(1), 1–9. https://doi.org/10.1038/s41598-019-42641-9

TEDERSOO, L., Tooming-Klunderud, A., & Anslan, S. (2018). PacBio metabarcoding of Fungi and other eukaryotes: errors, biases and perspectives. *New Phytologist*. https://doi.org/10.1111/nph.14776

THOMAS, A. C., Nelson, B. W., Lance, M. M., Deagle, B. E., & Trites, A. W. (2017). Harbour seals target juvenile salmon of conservation concern. *Canadian Journal of Fisheries and Aquatic Sciences*, *74*(6), 907–921. https://doi.org/10.1139/cjfas-2015-0558

THOMSEN, P. F., Kielgast, J., Iversen, L. L., Møller, P. R., Rasmussen, M., & Willerslev, E. (2012). Detection of a Diverse Marine Fish Fauna Using Environmental DNA from Seawater Samples. *PLoS One*, *7*(8), 1–9. https://doi.org/10.1371/journal.pone.0041732

THOMSEN PF, Willerslev E (2015) Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*, 183, 4–18.

THURMAN, L. L., Barner, A. K., Garcia, T. S., & Chestnut, T. (2019). Testing the link between species interactions and species co-occurrence in a trophic network. *Ecography*. https://doi.org/10.1111/ecog.04360

TORTI, A., Lever, M. A., & Jørgensen, B. B. (2015). Origin, dynamics, and implications of extracellular DNA pools in marine sediments. *Marine Genomics*, *24*, 185–196. https://doi.org/10.1016/j.margen.2015.08.007

UTHICKE, S., Lamare, M., & Doyle, J. R. (2018). eDNA detection of corallivorous seastar (Acanthaster cf. solaris) outbreaks on the Great Barrier Reef using digital droplet PCR. *Coral Reefs*, *37*(4), 1229–1239. https://doi.org/10.1007/s00338-018-1734-6

VAUSE, B. J., Morley, S. A., Fonseca, V. G., Jazdzewska, A., Ashton, G. V., Barnes, D. K. A., Giebner, H., Clark, M. S., & Peck, L. S. (2019). Spatial and temporal dynamics of Antarctic shallow soft-bottom benthic communities: Ecological drivers under climate change. *BMC Ecology*, *19*(1), 1–14. https://doi.org/10.1186/s12898-019-0244-x

VON AMMON, U., Wood, S. A., Laroche, O., Zaiko, A., Lavery, S. D., Inglis, G. J., & Pochon, X. (2019). Linking Environmental DNA and RNA for Improved Detection of the Marine Invasive Fanworm Sabella spallanzanii. *Frontiers in Marine Science*, *6*(October), 1–13. https://doi.org/10.3389/fmars.2019.00621

VUILLEMIN, A., Magritsch, T., Vargas, S., Pockalny, R., Smith, D. C., Hondt, S. D., & Orsi, W. D. (2018). Microbial diversity , abundance and metabolism in oxic and anoxic abyssal clays from the North Atlantic Ocean *Proceedings of the 20th EGU General Assembly, p*2937.

WALCZYŃSKA, K. S., Søreide, J. E., Weydmann-Zwolicka, A., Ronowicz, M., & Gabrielsen, T. M. (2019). DNA barcoding of Cirripedia larvae reveals new knowledge on their biology in Arctic coastal ecosystems. *Hydrobiologia*, *837*(1), 149–159. https://doi.org/10.1007/s10750-019-3967-y

WALTERS, T. L., Lamboley, L. M., López-Figueroa, N. B., Rodríguez-Santiago, Á. E., Gibson, D. M., & Frischer, M. E. (2019). Diet and trophic interactions of a circumglobally significant gelatinous marine zooplankter, Dolioletta gegenbauri (Uljanin, 1884). *Molecular Ecology*, *28*(2), 176–189. https://doi.org/10.1111/mec.14926

WANG, X., Singh, P., Gao, Z., Zhang, X., Johnson, Z. I., & Wang, G. (2014). Distribution and diversity of planktonic fungi in the west pacific warm pool. *PLoS One*, *9*(7), 1–7. https://doi.org/10.1371/journal.pone.0101523

WEIGAND H, Beermann AJ, Čiampor F *et al.* (2019) DNA barcode reference libraries for the monitoring of aquatic biota in Europe: Gap-analysis and recommendations for future work. *Science of the Total Environment*, 678, 499–524.

WELTZ, K., Lyle, J. M., Ovenden, J., Morgan, J. A. T., Moreno, D. A., & Semmens, J. M. (2017). Application of environmental DNA to detect an endangered marine skate species in the wild. *PLoS One*, *12*(6), 1–16. https://doi.org/10.1371/journal.pone.0178124

XIE, Y., Zhang, X., Yang, J., Kim, S., Hong, S., Giesy, J. P., Yim, U. H., Shim, W. J., Yu, H., & Khim, J. S. (2018). eDNA-based bioassessment of coastal sediments impacted by an oil spill. *Environmental Pollution*, *238*, 739–748. https://doi.org/10.1016/j.envpol.2018.02.081

YAMAHARA, K. M., Preston, C. M., Birch, J., Walz, K., Marin, R., Jensen, S., Pargett, D., Roman, B., Ussler, W., Zhang, Y., Ryan, J., Hobson, B., Kieft, B., Raanan, B., Goodwin, K. D., Chavez, F. P., & Scholin, C. (2019). In situ autonomous acquisition and preservation of marine environmental dna using an autonomous underwater vehicle. *Frontiers in Marine Science*, *6*(JUL), 1–14. https://doi.org/10.3389/fmars.2019.00373

YAMAMOTO, S., Masuda, R., Sato, Y., Sado, T., Araki, H., Kondoh, M., Minamoto, T., & Miya, M. (2017). Environmental DNA metabarcoding reveals local fish communities in a species-rich coastal sea. *Scientific Reports*, *7*(December 2016), 1–12. https://doi.org/10.1038/srep40368

ZINGER, L., Amaral-Zettler, L. A., Fuhrman, J. A., Horner-Devine, M. C., Huse, S. M., Welch, D. B. M., Martiny, J. B. H., Sogin, M., Boetius, A., & Ramette, A. (2011). Global patterns of bacterial beta-diversity in seafloor and seawater ecosystems. *PLoS One*, *6*(9), 1–11. https://doi.org/10.1371/journal.pone.0024570

Think-Piece 3: DNA biomonitoring in the terrestrial biome

Elizabeth Clare

School of Biological and Chemical Sciences, Queen Mary University of London

Contents

Figures

Summary

Lay Summary

Molecular (primarily DNA) based approaches are becoming extensive and pervasive and produce a sometimes dizzying array of options to incorporate into our methods of biomonitoring. These methods can be largely divided into species specific approaches and ecosystem level approaches. Species approaches seek to detect and identify key species of interest such as natives of special concern, or non-natives that represent an invasive threat. Ecosystem level approaches consider issues of process and structure, rather than individual species identities. Both approaches can provide vital insight into the status and resilience of ecosystems and their services and it is necessary to strike a balance between directives to act as custodians for life (e.g. Natural England) and the necessity of foreplaning for the necessary exploitation of natural capital to support society. The objective of this think piece was to consider the terrestrial landscape, explore the applications of existing and emerging technologies and to speculate on the metrics that are needed to best fit future societal needs. In the following pages, I have attempted to do that, while also strongly advocating for an integration across ecosystems and stronger partnerships between regulatory bodies and academic research, and a transnational approach.

In general, there are three message I hope to convey here.

1. It is important to validate new technologies to permit continuity with current methodologies, but the emphasis needs to change from one of solving every potential source of error to one of acknowledging error and learning how to incorporate multiple error profiles in monitoring.
2. We need to consider both species level and ecosystem level process approaches to monitoring and develop metrics which can be applied across traditional ecological boundaries and at scale so that we can manage local landscapes, while balancing this against future global use.
3. We need to increase the transnational approaches to ecosystem monitoring through regulatory and academic collaboration so that the latest technological advances can be more readily incorporated at a faster pace.

If these three can become standard operating procedure, then biomonitoring for the next generation will become a process of adopting new technologies as they emerge with the net benefit of rapid advances in the core goals of species and ecosystem surveillance.

Executive Summary

The purpose of this document is to summarise the case for genetic approaches to be integrated into terrestrial biosurveys (targeted and more general monitoring). I provide an overview of current and emerging technologies, speculate on appropriate targets for “next generation biomonitoring”, propose metrics for development, and discuss challenges

moving forward and appropriate solutions. In this case with a particular focus on the terrestrial domain e.g. soils.

The most prominent and emerging technologies include DNA barcoding, which allows DNA to be used to identify taxa in a nearly automated system and which can be expanded to DNA metabarcoding where complex mixed samples (soil cores, leaf litter) can be analysed as a slurry for taxonomic composition. Beyond taxonomic identity, metagenomic technologies can be used to reconstruct all genomes in a sample and to assay for function directly in a process called functional genomics. In functional genomics, genes known to be responsible for a particular function (e.g. nitrogen fixation) are the measurement of interest, without concern for underlying taxonomic diversity. Such processes are listed as the known current pressures on soils in the Defra 25 Year Environmental Plan. Scaling up metatranscriptomic assays for expressed components of DNA (e.g. RNA), provide the opportunity to look at active ecological functions (e.g. cycling of a nutrient) rather than potential functions (presence of a species that *might* provide that task) and can be expanded to address some demographic questions (e.g. presence of age classes). All of these will be augmented by the emergence of mobile labs which can be deployed in remote areas and coupled to artificial intelligence (AI) and machine learning to provide the opportunity for automation.

While much effort has concentrated on monitoring critically endangered species, potential targets for biomonitoring of ecosystem functions should deemphasise single species approaches. This should be done in favour of communities such as fungal, microbial, macro-invertebrate (such as nematode) and plant communities which are not traditionally monitored but carry out some of the most fundamental global ecosystem processes. They are also known to promote ecosystem resilience and may not follow patterns of global species distribution associated with better studied groups.

Metrics for development should reduce the focus on specific species and increase focus on non-taxonomic assays for specific functions such as carbon cycling, nitrogen fixation etc. Metrics should measure demographic trends such as age structures and recruitment to the next generation and species interactions which consider how interactions may respond to a-biotic environmental conditions independently of the underlying community, thus separating “function” from the presence of a specific species. To this we must also consider how we manage resources for use today vs. preservation for potential future functional requirements. Finally, some measure of ecosystem “fitness” which considers natural resistance and resilience of a system, needs to be validated. All must take into account technological integration with other non-molecular next generation biomonitoring systems which are already being deployed.

To implement this, we must address methods of quantification, technological integration and resilience to technological change, global access to information and regulatory agencies, policy makers and end users must engage with academic research at all levels. Finally, we must abandon a compartmental view of isolated ecosystems with a renewed focus on the interfaces between systems. With this we must increase the spatial and temporal scale of monitoring from regional and national levels to continental and integrated multi-ecosystem perspectives led by global consortia providing a more

biologically meaningful scope and resilience to local changes in funding and government priorities.

Introduction

The foremost challenge of our time is to accurately and rapidly measure biodiversity, assess the effects of environmental change on the current and future global ecosystems and evaluate ecosystem level capacity to respond to global fluctuations in climate, habitat, and resources. The biodiversity crisis coupled with climate change is producing more rapid environmental alterations than at any previous point in history and cascading environmental transformation threatens every aspect of life for every organism on the planet.

Novel technological and analytical approaches are required to establish new baselines and monitor biodiversity in real time, identify species interactions, and assess ecosystem level responses so that effective predictions can be made about future events. We need the capacity to scale our measurements from individual organisms to whole communities and landscapes. This must be multifaceted, including estimates of biodiversity, characterizing their relationships and the flow of energy between systems, as well as direct measurements of function. In an age of global change, this represents the most pressing challenge: to understand ecosystem function sufficiently to predict and plan for the future.

In order to achieve this, our approach to biomonitoring must shift from one focussed on flagship species and those with clear taxonomic designations and specific metrics to describe them, to a broader approach. One that encompasses genes to ecosystem level assays with a focus on *function*, or the capacity of a system to provide a property or process. Such an approach can encompass both well-known and lesser known groups (e.g. fungi) and will necessitate new types of questions such as:

Are the genes for function X present in this ecosystem?

Does this ecosystem have the capacity to provide service X for the next 100 years if we remove or alter resources?

The journal Frontiers in Environmental Sciences, recently released a collection of nine reviews and articles under the heading of “A Next-Generation of Biomonitoring to Detect Global Ecosystem Change” (https://www.frontiersin.org/research-topics/8325/a-next-generation-of-biomonitoring-to-detect-global-ecosystem-change). Collectively they address the problem of how to think on a much bigger scale for current and future biomonitoring. In particular, in their paper “Key Questions for Next-Generation Biomonitoring” Makiola et al. (2020) recognise two categories of issues which are important to summarise here. While their excellent review is not specific to DNA approaches, molecular techniques feature broadly in their discussion and I specifically highlight their 10 point argument, because they are well aligned with the arguments I will make throughout this “think piece” and are reminiscent of the considerations reported as guiding the development of the Defra 25 year Environmental Plan. The concordance in arguments suggests a consensus exists among developers and practitioners on the way forward.

In the category of “current” topics, Makiola et al. (2020) argue:

1. That the benefits of next-generation biomonitoring need to be better communicated to society, scientists and policymakers (the slow uptake by decision makers often resulting from miscommunication).
2. The appropriate spatial-temporal scale for biomonitoring need to be vastly increased, despite socio-economic and political pressures that keep us short sighted (the next election or funding cycle etc.). One solution suggested is to limit the scope of biomonitoring to vast, mostly automated, scales to detect change, and only then focus on explanation and prediction by team specialists thus reducing the resources needed while increasing the scale of monitoring. To this I add that it would require a complete removal of the “national approach” where most countries take a totally independent unintegrated approach, or worse yet, management is organised at a sub-national, municipal or regional scale. While there is value in local ecosystem benefits such as flood prevention and the clear societal benefits of contact with nature, specific trans-continental approaches have demonstrated potential ways forward at scale (e.g. EU water framework directive, DNAqua-Net, Earth Microbiome Project, Autonomous Reef Monitoring Structure, to name a few).
3. We must find a balance between specific and generic methodologies, and that next generation methodologies are becoming more generic as they develop, making them more applicable to diverse scenarios. The net result will be a reduction in the piece-meal approach we currently use to a more standardised global approach.
4. The development of new metrics (which I expand upon later) is mandatory.
5. We require the integration of machine learning with next generation monitoring, particularly for the assay of species interactions when scales of measurement are larger than current statistical approaches can accommodate. The authors make one interesting argument; that much of the data needed exists behind publication barriers preventing the immediate use of these statistical approaches, but a relaxation of copywrite law might jump start this process.
6. We must address the key technical challenges of next generation biomonitoring including such problems as false positive and false negative errors and quantifying uncertainty (and many others I expand upon later, particularly the issue of quantification).
7. We must apply biomonitoring to risk management, particularly the development of whole ecosystem predictive models rather than just descriptive models - almost certainly requiring artificial intelligence/machine learning approaches.

In the category of “outlook” topics Makiola et al. (2020) argue:

1. That advances in genomics tools, such as whole community genomics, will permit community metaphylogenomics, high resolution analysis of individual variation and population genetics and enhanced functional genetics (discussed later).
2. Future advances in computing and bioinformatics will need to address the scale of data generation and change in our approach to data accessibility and standards.
3. Advancements in both technology and modelling will permit a more integrated approach to both traditional and new metrics.

In this broader context, DNA-based technologies to measure changes in biodiversity, species interactions and ecosystem services at multiple scales from individuals to landscapes, must be integrated with artificial intelligence, remote sensing and global modelling, moving away from the theoretical constructs to actual measurements of systems and processes. The ability to access trace materials like eDNA, coupled with current and emerging technologies such as metabarcoding, metagenomics, metatranscriptomics and machine learning, are on the verge of becoming standard operational parameters in both research and regulatory approaches. Further developments in “mobile lab” technologies will extend our capabilities to include rapid responses in remote field locations, an approach which promises assessments of ecosystems at a scale previously impossible. But these must be integrated into other “Next-Generation Biomonitoring” approaches.

In the following pages I will address current and emerging technological advances, appropriate targets for new monitoring scales, a set of new metrics that need to be developed, challenges in integrating biomonitoring approaches and case studies which provide examples of new pathways forward.

Existing and emerging DNA technologies and their applications

For most applications and metrics currently employed in terrestrial biomonitoring, alpha diversity, a measure of how many of each type of species exist in a system, is a fundamental requirement. To this we normally need to add some degree of knowledge of home range, habitat requirement, niche, abundance, distribution and other diversity metrics etc. for added value. In addition to visual surveys, a wide variety of techniques are employed from camera trapping (Burton et al. 2015), to trace material collections such as hairs (Berezowska-Cnota et al. 2017), chewed forage (real or artificial) (Howe et al. 2009), audio recordings of songs (e.g. birds and bats) (Adams et al. 2012), PIT (Passive Integrated Transponder) tagging (Gibbons et al. 2004), radio tracking (Gottwald et al. 2019) and GPS (Global Positioning System) monitoring (Kays et al. 2015) using both manual and remote sensing methods. To this suite of tools molecular methods have been well integrated across many taxonomic groups now for decades and have included immunological methods, protein assays and, in the last 20 years, DNA-based approaches (Symondson 2002, Pompanon et al. 2012). A DNA-based biomonitoring approach was adopted for comparisons of bacterial communities long before it was adapted to eukaryotic life (Floyd et al. 2002). For the most part, Eukaryotic mitochondrial genes have been favoured, (particularly for animals) but with different choices for different taxonomic groups (e.g. ND2 in Birds, CytB for mammals).

DNA-based biomonitoring gained particular traction in the early 2000s with the term “molecular barcodes” being coined as a metaphor (Floyd et al. 2002) for the use of 18S molecular operational taxonomic units (MOTU) to address the diversity and taxonomic confusion of soil nematodes. It has become most famous now under the increasingly broad term of “DNA barcoding” which, in a strict definition, refers to the use of the cytochrome c oxidase subunit 1 (COI) region for animal identification and discrimination (Hebert et al. 2003) but in a more relaxed definition includes the use of internal transcribed spacer (ITS) for many fungi (Seifert 2009), 12S, 16S, 18S (e.g. Floyd et al. 2002) for more difficult taxonomic groups and a suit of markers used in different combinations for plants (CBOL 2009). Regardless of the marker, the approach is the same with the expectation that a single species will be characterised by one or a small number of very closely related haplotypes at the target marker and that intraspecific divergence is much lower than interspecific divergence permitting a robust and repeatable identification by anyone anywhere on any fragment of biological material (Hebert et al. 2003). For the most part this works very well with the two main advantages being, the lack of specific taxonomic expertise required and the ability to work with even the most fragmentary degraded materials.

With the advent of high-throughput sequencing platforms and their rapid development in capacity and quality, most environmental monitoring by DNA is now encompassed by the field of “metabarcoding” (Taberlet et al. 2012). While the principle is the same as DNA barcoding, the genetic regions targeted are smaller and the capacity for identifications, millions of times larger. The skill and training, particularly in bioinformatics has dramatically

increased, though the explosion of modern platforms for analysis are reversing this last trend to some extent (e.g. Ji et al. 2019) though “off the shelf” presents its own challenges.

Metabarcoding targets intracellular DNA or more non-standard sources of DNA such as environmental “eDNA” (Bohmann et al. 2014), shed into an organism’s environment either while it is alive or after its death, or the more recently dubbed invertebrate derived “iDNA”(Schnell et al. 2012, 2015) which relates to any DNA carried in the gut of an invertebrate. Lesser used ancient “aDNA” and other terms are also sometimes encountered. Given this linguistic confusion I will thus only use the terms DNA or eDNA from this point on, but recognise this suite of subtleties. The metabarcoding approach really first took off in ecology as a method of diet analysis following the groundwork in immunological, Sanger sequencing targeted and microsatellite approaches (Symondson 2002) and became available on a widespread basis less than a decade ago. The fast uptake of the technology can be seen by its description as a speculative and prohibitively expensive technology as recently as the early 2000s (Symondson et al. 2003) but adopted on a widespread basis and well established less than a decade later (Pompanon et al. 2012).

Species biomonitoring (Figure 2) appears to have been a bit slower but was also widely adopted along the same timelines with many very excellent reviews on technique and application available (e.g. Bohmann et al. 2014, Thomsen and Wilerslev 2015, Ruppert et al. 2019).

While complicated by the multitude of factors which will degrade DNA in often unpredictable or unexpected ways, eDNA-based biomonitoring has the same advantage as DNA barcoding in that it potentially addresses the problem of identifying species with a minimum of taxonomic expertise and minimal disturbance to the taxa themselves (Ruppert et al. 2019). It is now possible to measure an entire community, including rare and elusive taxa and accelerate the identification of novel taxa. It is still extremely limited in determining any form of population size or age structure senses, but there are speculative approaches which may determine age, sex and condition structures e.g. targeting genes only expressed during some life stages or specific biomarkers of age structure such as patterns of DNA methylation, though this has not been widely explored (De Paoli-Iseppi 2017). It is extremely useful in detecting new invasive species (Xu et al. 2017) or monitoring critically endangered taxa where capture is not an option (Holmes et al. 2009). All of which fit with the thematic indicators listed and several headline points (e.g. D2, Headline 3-8, 12, 15, H1, H2) in the Defra 25 Year Environmental Plan.

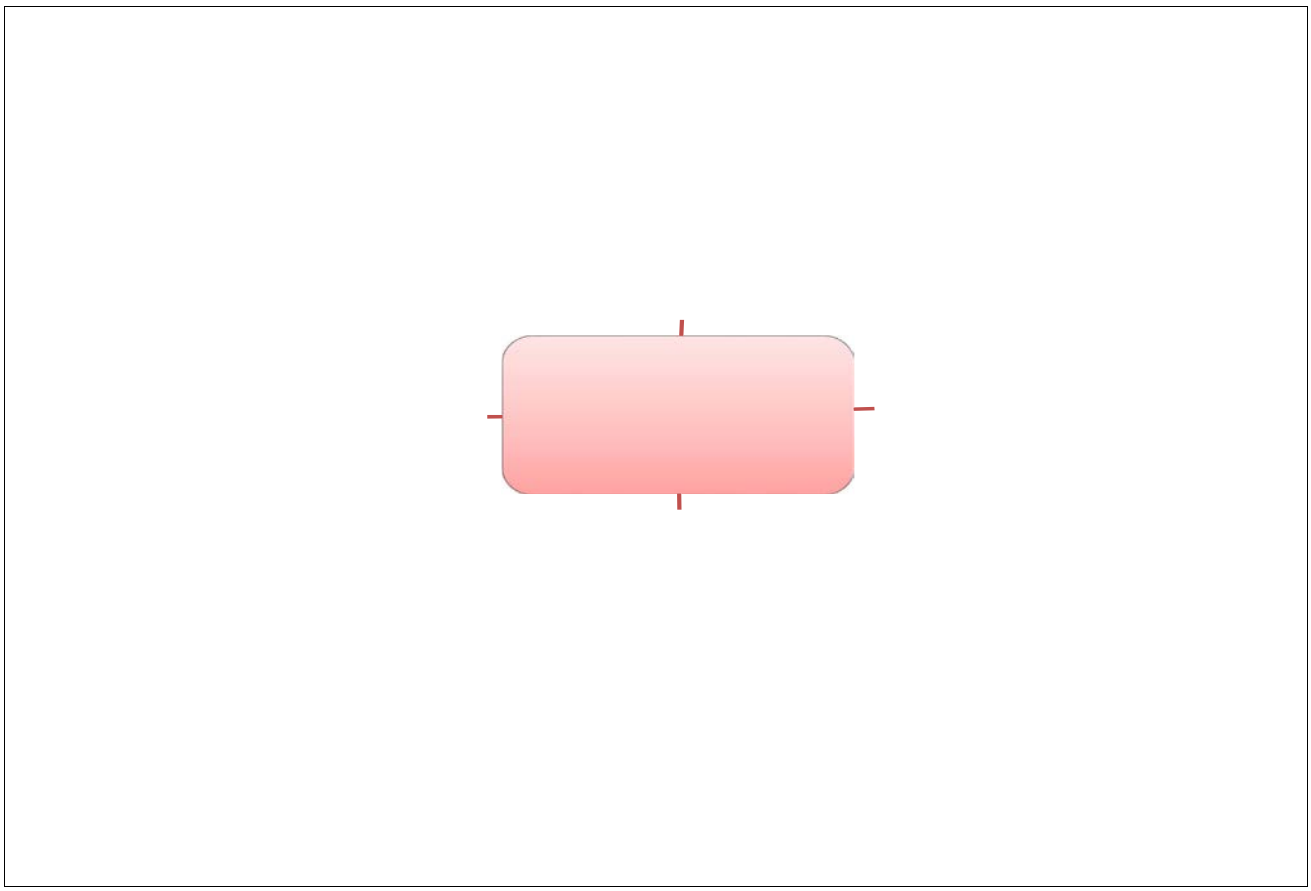

Figure 2 Targets for global biomonitoring using metabarcoding. Adapted with permission from Ruppert et al. 2019

Terrestrial biomonitoring using molecular tools is most commonly applied to soils. It was first used to diagnose fungal communities (Buee et al. 2009) but has also been applied to describe both above and below ground communities e.g. plants (Fahner et al. 2016), vertebrates (Drummon et al. 2015) or mixed communities (e.g. Epp et al. 2012 examined multiple communities from fungi and bryophytes to birds). Other good targets for molecular approaches in terrestrial biomonitoring and community composition are root biomass, leaf litter (e.g. Yang et al. 2014), and even air samples (Johnson et al. 2019a) such as bulk sampling of pollen (Bell et al. 2019) or Malaise traps (Marquina et al. 2019). One of the more charismatic approaches is to use species interactions to monitor a target population. For example, blowflies (Lee et al. 2016) and leeches (Schnell et al. 2012, 2015, 2018, Drinkwater et al. 2019) have been used to monitor local mammal populations. In a comparison with camera trap data, Leempoel et al. (2020), found high concordance between species detected in camera traps and through eDNA derived from soil samples with the added benefit of eDNA finding numerous small mammals not recorded on cameras (Figure 3). A similar comparison of mammal DNA carried by flies and camera trapping found low concordance, with camera traps finding mostly larger mammals while flies carried DNA from primarily smaller bodied species (Gogarten et al. 2020). The variety of outcomes suggests a role for complementary technologies. In many contexts DNA is a new, rather than a replacement tool for biomonitoring, offering different costs and benefits in analysis, implementation and continuity with long-standing monitoring programmes.

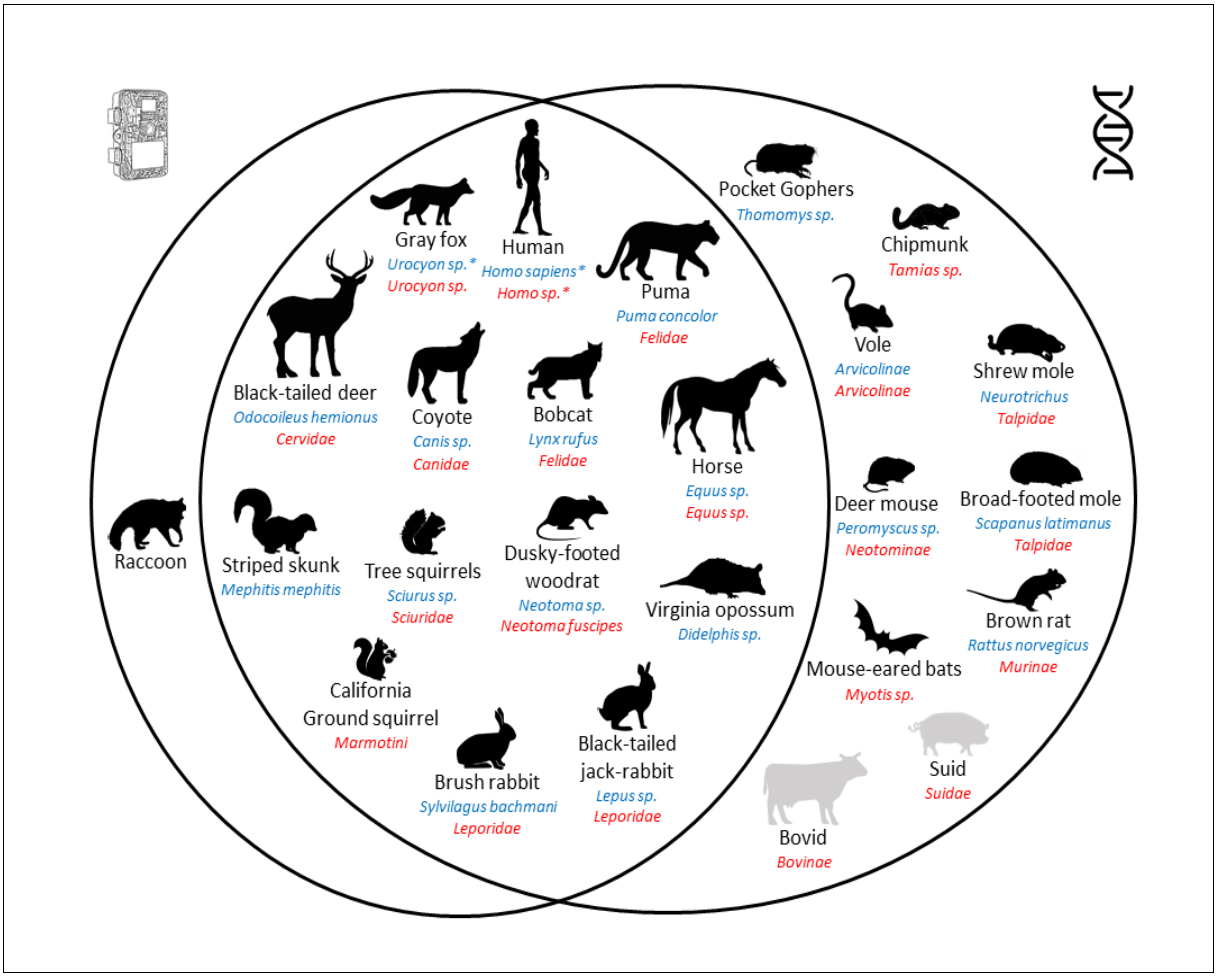

Figure 3 ven diagram of mammals detected from camera trapping vs. eDNA metabarcoding. Adapted with permission from Leempoel et al. 2020

One of the problems with most metabarcoding approaches is the issue of primer amplification biases. Some taxa will simply be amplified, sequenced and thus detected at a greater rate because of various biases in how PCR works. Approaches to counter this have included the use of multiple primers to at least vary the biases and better primer design and testing.

Similar to metabarcoding, metagenomics seeks to describe the biodiversity in a sample of mixed origin. While metabarcoding targets only a small homologous fragment in all taxa in the sample, metagenomics targets the entire genome of every taxa. This imposes a very clear trade off. Entire genomes will always provide extensive information on the taxa, however, because of the sequencing depth required to generate a full genome, the method is unlikely to recover the full complement of a diverse sample. Metabarcoding is more likely to recover the full biodiversity, but with much more limited information about any one taxon. Metagenomic approaches are known by a variety of different names and often target the assembly of a particular part of the genome, usually an organelle (plastid, mitochondria) which is present in much higher copy number than the nuclear genome. The technique often has no PCR and thus should produce a more representative outcome than PCR-based approaches.

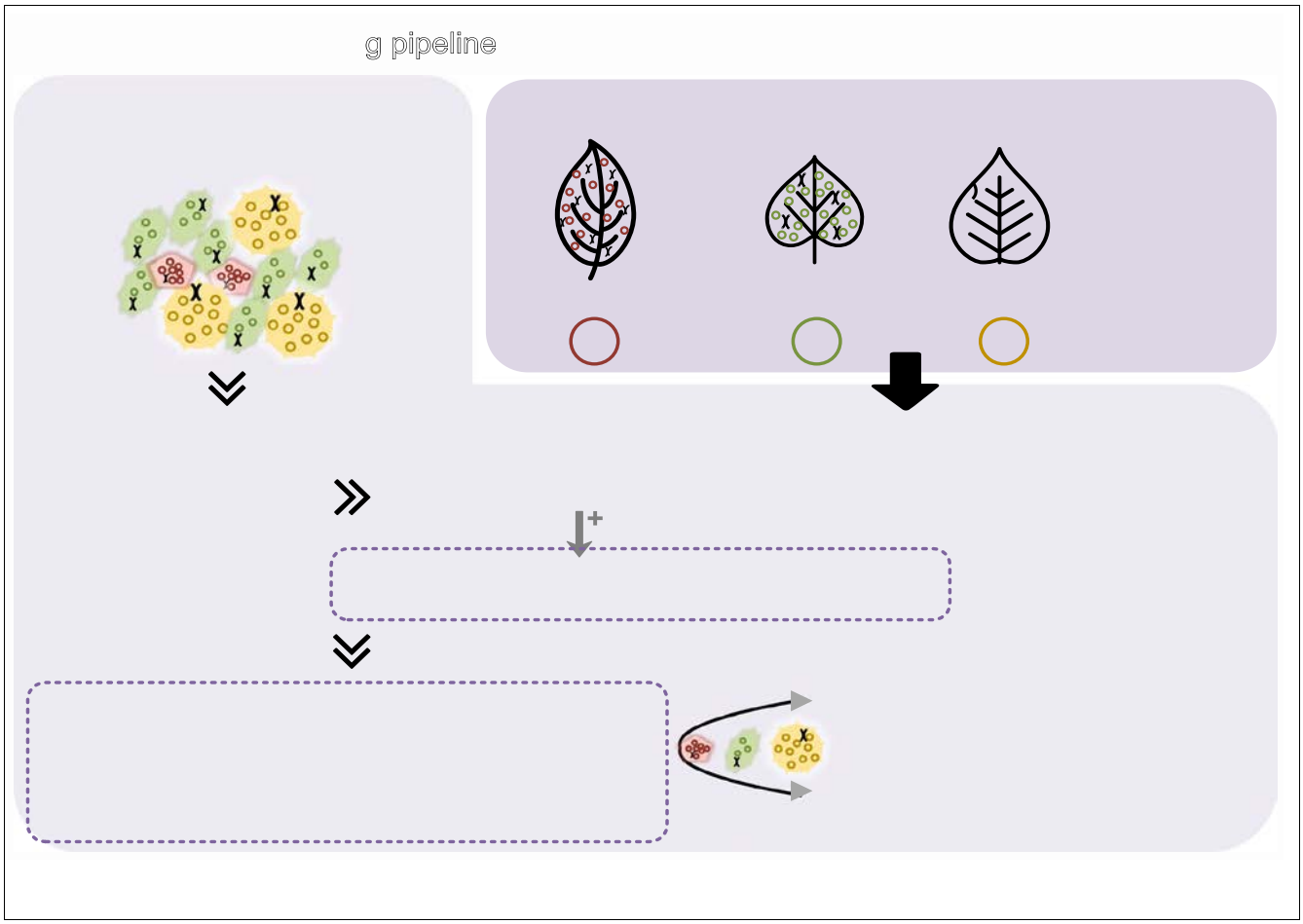

Figure 4 Pollen genome skimming used to identify plants in a known pollen mix were able to identify all expected plants and provide good estimates of pollen numbers compared to traditional pollen counts. Adapted with permission from Lang et al. 2019

Crampton-Platt et al. (2016) reviewed the use of “mitochondrial metagenomics” to map low coverage sequencing of mitochondrial genomes from a bulk collection of invertebrates to existing libraries of mitochondrial genomes and described the potential for monitoring community turn over and community phylogenetics. Lang et al. (2019) used “genome skimming” of known pollen mixes (mock communities) to bulk sequence the contents (Figure 4) and were able to identify all species and found that the pollen frequencies estimated from sequencing were highly correlated to traditional pollen counts. In a comparative experiment Srivathsan et al. (2015) used metabarcoding and metagenomics to analyse the diet of a leaf feeding monkey. They targeted *trnL* and full chloroplast genomes to identify the plants and observed that full chloroplast genomes produced better identifications to expected plants but missed rare dietary items. In contrast *trnL* sequences recovered a wider variety of items but with lower resolution on the identifications. This trade-off will be more exaggerated with full genomes compared to this organellar genome approach. While the future of these analyses does likely rest with a metagenomic method, this will really reach an operational capacity with increases in sequencer capacity and reductions in cost, and more accessibility of good rapid bioinformatics approaches.

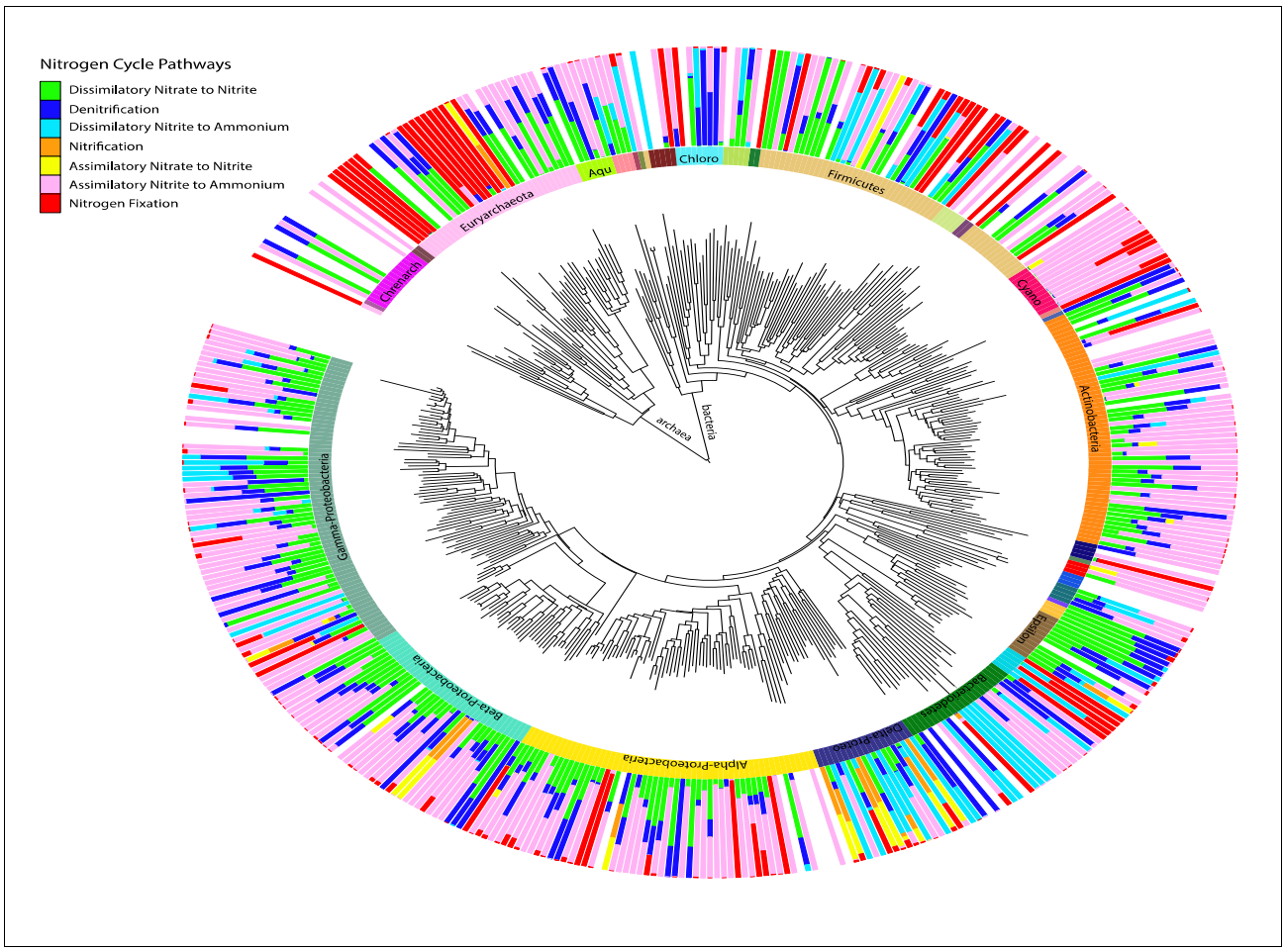

Figure 5 Phylogenetic reconstruction of nitrogen cycling pathways from soil metagenomes. Outer circle is the proportion of N-cycling reads assigned to each pathway while the inner circle indicates the major classes and phyla. Adapted with permission from Nelson

Even more enticing, emerging technologies include arrays and microarrays (Nilsson et al. 2019), single-celled genomic approaches where entire genomes might be constructed from a single cell and metatranscriptomics where expressed genes are assayed (Creer et al. 2016). These methods might allow for much more targeted assessments of biodiversity with the potential to assess age structures, reproductive condition etc. of communities (De Paoli-Iseppi 2017). However, the next most likely technological step is to move out of a lab-based environment and into the field. Various mobile lab technologies are now available and, while most lack the efficiency and scale associated with lab-based methods, they are extremely promising. Collection and transport of biological samples in an increasingly complex regulatory landscape has become one of the most serious factors limiting DNA-based field studies. In the short term we need to decide whether we can adapt mobile technology to meet regulatory standards and whether we can improve the quality of the analysis to meet with research needs. In the long term, this technology is on the cusp of becoming a major tool in environmental management, and different analytical systems (BENTO www.bento.bio, miniPCR www.minipcr.com etc.) are on the market. In some cases these technologies are already of extremely high quality and have gained some media attention (e.g. the “genes in space” programme https://www. genesinspace.org/) and are improving rapidly. In a recent experiment we took a combination of FTA

cards (filter paper), mini PCR and the Oxford Nanopour MinION into a remote Belize forest and confirmed mammal field identifications on the spot (unpublished data).

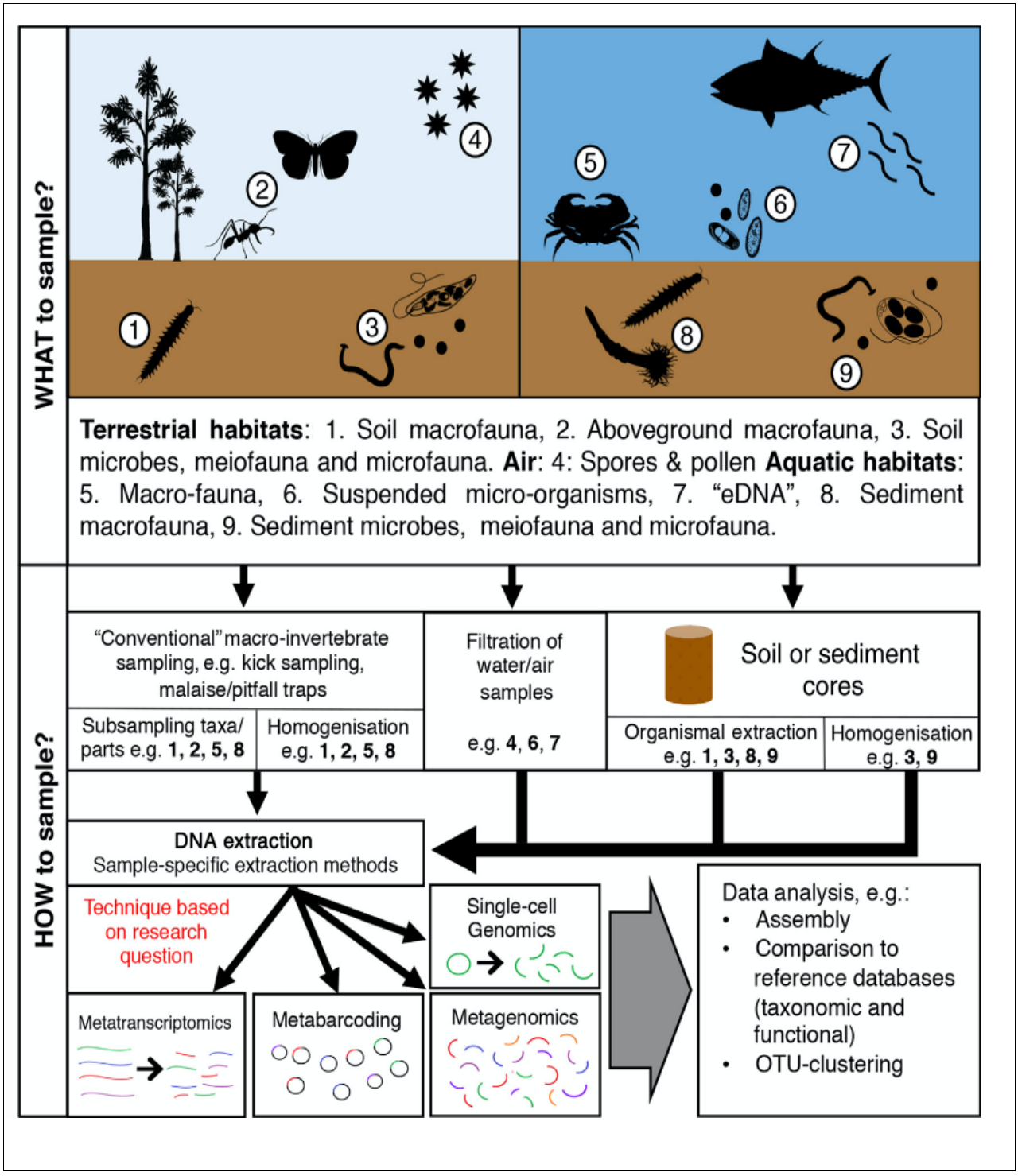

Figure 6 An often-bewildering set of methods are available and determining the correct approach is complex. Adapted with permission from Creer et al. 2016

In the immediate future, the biggest challenge will be to decide which approach is best for a given problem; which gene target will be best to identify or analyse the problem at hand and which analysis method is most likely to provide an outcome (Figure 6). Even more complex can be the issue of which validation technique will be best to address reliability. This is not as simple as asking advice, because opinions on best practice vary widely.

However, some excellent reviews are now available including Creer et al. 2016, Alberdi et al. 2018, Bohmann et al. 2014. Ruppert et al. 2019 to name just a few.

What terrestrial components should we target?

With the diversity of approaches comes an equally complex diversity of terrestrial targets for biomonitoring. The short answer is that we should be monitoring everything. This is not such an outlandish target in the not so distant future with the continually decreasing cost of DNA sequencing and increasing resources for analysis. The Earth BioGenome Project is a global effort to generate a dataset of 1.5 million genomes from species of eukaryotes worldwide as part of an even loftier goal to sequence the genome of everything (https://www.earthbiogenome.org/) and the related "Darwin Tree of Life" (https://www.darwintreeoflife.org/) specifically targeting UK genomes. If such targets are not the stuff of fiction but a real scientific programme, then why a modified metabarcoding or metagenomic approach to monitor all terrestrial biodiversity should be so loftier a goal. The sequencing power and technical expertise are less demanding. So it is perhaps a realistic target to say that whatever we do, that should be the ultimate goal. In the more immediate future what sub targets could lead us to this? Here the discussion becomes more one of informed speculation but all of the following would meet the requirement of indicator D7 Species supporting ecosystem function and E7 healthy soils of the Defra 25 year plan and should be strongly considered as new targets where DNA approaches can be implemented.

A hierarchical mixed approach is perhaps an optimum. In a proposed plan, we could target specific representative "flagship species" of each biome for monitoring. These species would provide continuity with current practices and include taxa like bees and birds where extensive records already exist. This continuity is vital for long term monitoring. To this we must consider the addition of new targets such as non-traditional pollinators (e.g. hoverflies, mammals, birds) which carry out the same functions and may provide redundancy for ecosystem services. It will be important to focus on major taxonomic clades and communities which are key to ecosystem functions but where taxonomic based or flagship species monitoring is not an efficient practice. Assemblages of species have conservation value in their own right and monitoring entire communities may prevent declines and extinctions of species of interest (e.g. D5 D6 in the Defra 25 year plan), while simultaneously conserving a wide variety of ecosystem functions. All will require DNA-based approaches and probably a combination of metabarcoding, metagenomics and functional genomics.

Fungal communities

There are an estimated 2-4 million fungal species (Hawkswroth et al. 2017) which carry out an unbelievable variety of ecosystem processes and mostly exist as saprotophs fundamental in processes such as decay and soil formation. In mutualistic and parasitic relationships they play a central role in nutrient cycling and movement of energy through terrestrial food webs (Nilsson et al. 2019). Among their most familiar ecosystem functions

is likely their mycorrhizal associations with roots, but they are also fundamental in aquatic processes (e.g. Grossart et al. 2019) and most of their taxonomic associations are poorly delineated. Soil fungal diversity is strongly associated with climate and chemistry (pH, temperature, carbon cycling) but may not follow standard models of diversity distribution (Větrovský et al. 2019) and may promote adaptation or resilience to environmental change. For example, arbuscular mycorrhizal fungi associated with agriculture, promote drought and pathogen tolerance and nutrient capture in plants (Davison et al. 2015). They have a diversity peak in grasslands that appears decoupled from latitudinal gradients with very low levels of endemism (Davison et al. 2015). Because of their highly varied functions they are clearly a priority target.

Microbial communities

Microbial communities are key players in terrestrial ecosystem functioning as part of nitrogen (Kowalchuk and Stephen 2001) and carbon cycling (Hogberg et al. 2001), primary production, decomposition and in symbiotic and pathogenic relationships with every other species on the planet. They flourish in even the most extreme environments and, as a consequence, they pay a vital role both directly and indirectly in regulating the climate and have both direct and indirect effects on plant productivity, promoting diversity and in symbiotic relationships (van der Heijden et al. 2008). Pathogenic bacteria also contribute to patterns of plant community turnover and plant-soil feedback and through a complex interplay of suppressing some species allowing others to dominate (van der Heijden et al. 2008). Soil microbial diversity has been positively associated with multiple ecosystem functions on a global scale (Delgado-Baqueizo et al. 2016). Of particular research interest is how fungal and bacterial communities individually and jointly influence systems. For example, ecosystems with enhanced rates of nutrient availability are typically dominated by bacteria and may be associated with faster growing plant species, higher leaf litter quality and disturbed habitats, while systems dominated by fungi are characterised by slower nutrient cycling and are associated with more well developed soils, richer organic matter and less disturbed habitats (van der Heijden et al. 2008). Along with fungi, and particularly because the methods of DNA-based assessment are so well-established, microbial communities must also be a key target for ecosystem monitoring.

Soil nematode communities

Estimates suggest nematodes are the most abundant animals on earth dominating soil communities and accounting for up to 80% of animals on land playing vital roles in soil food webs as bacteriovores, fungivores, herbivores, omnivores and predators, processing organic nutrients and microorganism populations (van den Hoogen et al. 2019). Similarly to fungal diversity, they seem to not follow standard models of distribution (Figure 7) with abundance peaks in high latitudes with larger soil carbon resources (van den Hoogen et al. 2019). Some of the earliest DNA-based eukaryotic biomonitoring approaches were applied to nematode diversity and indeed both the terms “barcoding” and “molecular operational taxonomic units” (MOTU) were introduced to apply to soil nematodes (Floyd et al. 2002). The most recent analyses of nematode diversity and distribution hint at the

magnitude of their role in global carbon cycling and organic matter turn over and the unusual spatial patters of this processing and also provide a quantitative model for assessing functions on a regional and global scale (van den Hoogen et al. 2019).

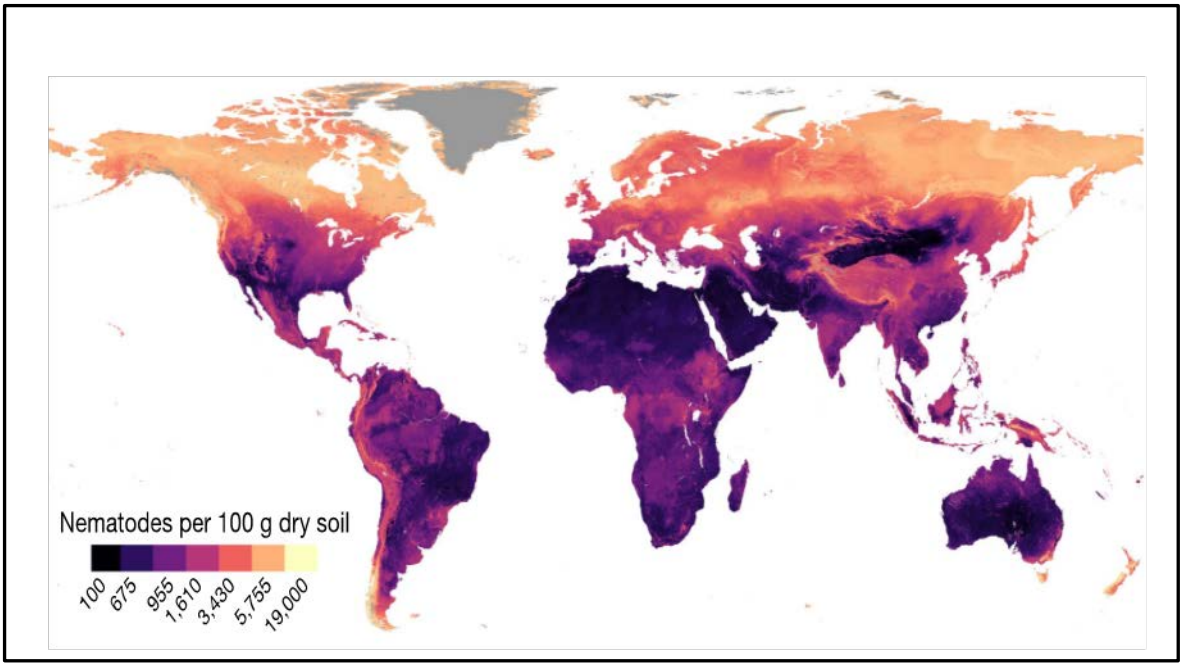

Figure 7 Global distribution of soil nematode abundance may follow carbon deposits rather than standard patterns of tropical abundance. Adapted with permission from van den Hoogen et al. 2019

Plant communities

Pollen in substrate samples has long been used as a record of past climate and ecosystem monitoring (e.g. Opel et al. 2018) and pollen profiling is already a well-developed discipline in the climate sciences, partly because of the long-term preservation of pollen grains into the fossil record. The addition of DNA-based monitoring of both airborne and soil/substrate pollens provides a good potential way to monitor primary productivity across a large scale. Some of the earliest soil metabarcoding approaches were pollen targeting (e.g. Yoccoz et al. 2012) and similarly the emerging air sampling techniques have mostly targeted plant DNA from pollen or non-pollen (Johnson et al. 2019a/b) sources. While non-pollen DNA will be nearly impossible to separate from pollen sources the potential to monitor the lowest trophic levels both above and below soil level is an exceptionally important target in assessing ecosystem fitness from the bottom up and as a basic measure of energy flowing into the terrestrial system.

In the above four major groups, a combination of metabarcoding, metagenomic and functional genomic approaches can work in combination to target assessments. They all have at least a partial focus on soils, where many of the primary ecosystem functions take place including primary productivity, nutrient sequestration, cycling and turnover. However, the same approach can be used for other targets. For example, bulk invertebrate collections, surveys of leaf litter and compost detritus for the action of invertebrate

detritivores can be assayed in the same way and more unusual sources for the above (air samples, tree cores, plant surfaces etc.) should be considered.

Future Metrics of Ecosystem Function

1. Most ecological approaches to ecological function rely on quantifying the presence or absence of a set of taxa that are known or thought to perform the function of interest. Their presence, absence or abundance infers the status of the function. One problem with the flagship species approach is that it cannot scale to any global measure, as almost no species is panmictic in distribution. One exciting alternative approach is to ignore taxonomic identity entirely and measure ecosystem functions directly. Functional genomic techniques can be used to survey for key functional genes to characterise a habitat and are mostly applied in microbial work. For example, in aquatic systems detecting the active ammonia monooxygenase and nitrite reductase genes can identify nitrifying and denitrifying microbial functions, key to global nitrogen cycling. Moving forward it would be useful to develop and establish a functional genomics approach to ecosystem assessment in the terrestrial biomes and this would be compatible with the Indicator D7 Species supporting ecosystem function of the Defra 25 Year Environmental Plan. This is the ideal use of environmental samples and mobile technology since it can allow us to detect the active ecosystem function on the spot (rather than just the species involved) but application of these methods is currently limited. The practicality of this approach will explode in the next decade as the Earth BioGenome Project begins with a global effort to generate a dataset of 1.5 million genomes from species of eukaryotes worldwide and related UK initiatives such as the Darwin Tree of Life project target UK species. As these genomes become available, the targeting of functional genes underpinning key ecosystem processes will become a reality. I think it is key for regulatory agencies and academic research to engage in this programme going forward as an outgrowth of work on eDNA and mobile DNA lab technologies.
2. A great many ecological processes are not currently measured directly, but are inferred from some other, easier to measure, observation of process. Demographic processes often fall into this category. A priority should be to develop metrics that address these targets directly. For example, we measure pollination by visitation and/or pollen transfer, and we can quantify seed set following this process. However, what we really want to measure is the recruitment from seeds that come from the pollinator’s visitation. Similarly, for seed dispersal we most frequently measure seeds in droppings, or seeds germinating under a roost when we want to measure recruitment to the next generation and contributions of dispersal to plant population genetics and population spread and sustainability over time. Some aspects of these processes are currently augmented by DNA-based technologies. For example, we can measure seed dispersal when the disperser cannot consume the seed but only the fruit pulp refining our understanding of the mutualistic network. However, understanding the ultimate recruitment and population turnover effect on plants is inefficient, at best, impossible in most cases. Emerging genomic

methods may address a variety of demographic measures and metrics. DNA methylation patterns may indicate population age structure (Paoli-Iseppi et al. 2017) and assaying for genes expressed only during specific life stages may give an indication of juveniles, reproductive capacity etc. Current and past effective population size can be measured using a variety of genetic and genomic approaches from measurements of diversity to coalescent models (e.g. Hill and Baele 2019). Individual dispersal and fecundity can be measured directly or indirectly using a variety of DNA-based approaches. Developing an accurate set of demographic metrics for populations which consider age structure and contribution to future populations should be prioritised.

3. How many species are there and how do they interact? Fundamental to our understanding of ecosystem function, is knowing what is present, and how it interacts with both the biotic and abiotic environment. While these are not metrics *per se*, or not new ones, knowing both is fundamental to understanding function and miserably difficult to ascertain. For example, a species inventory is a measure of community composition, but until a measure of interaction is generated (e.g. a food web) and monitored over time, then no function or dysfunction can be measured. Currently both composition and interactions are impossible to measure completely. All food webs and biodiversity inventories are incomplete and thus actual functions only inferred. Major target groups (e.g. fungi, microbes) responsible for some of the most important terrestrial functions (primary nutrient cycling, carbon sequestration, augmenting plants grown in mycorrhizal associations) are rarely included in food webs and interaction networks or even in biological inventories. DNA-based techniques to generate species inventories is augmenting our measures of ecological structure such as food webs (e.g. Wirta et al. 2014). From these, we can better measure specific aspects of biological function such as resilience and energy transfer. Until inventories and networks are “complete” these remain proxies. However, when complete, they should allow us to use functional ecological principles to predict aspects of network structure, expected species interactions and in turn actually predict or identify missing species from inventories (Figure 8).

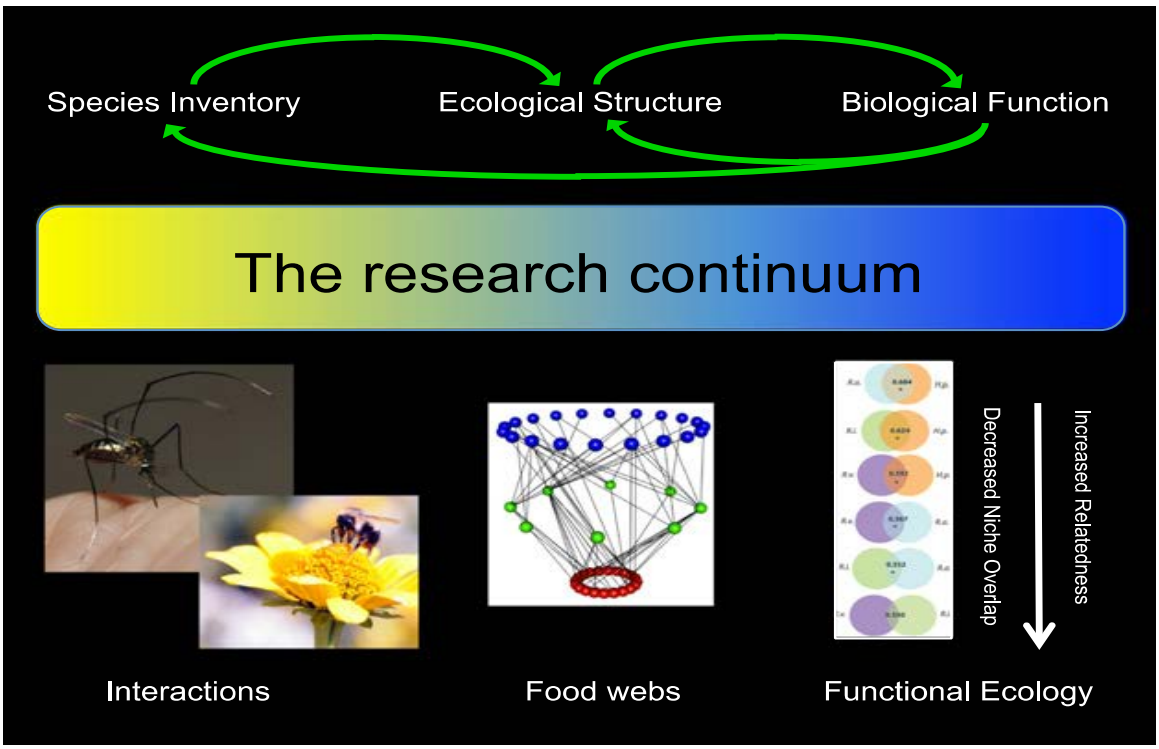

Figure 8 Structural diagram of integrating biomonitoring, network generation and functional ecological inference in a feedback system which would theoretically allow for knowledge of degrading functions to be used to predict the source biotic problems. Adapted with permission from Clare et al. 2014

Complicating matters is the problem that species interactions themselves react to environmental filters (Poisot et al. 2017) and this is crucially important to understanding the maintenance of ecosystem processes and functions. The presence of a species does not always predict the presence of a particular interaction or ecosystem function and vice a versa. This growing understanding that interactions may respond to environmental gradients independently of species composition (Poisot et al. 2017) raises questions about why such trophic behaviour should change and whether we should prioritize the conservation of species, functions or interactions which are three subtly, but distinctly different targets for biomonitoring.

4. Along with this potential for a metric of ecological function from complete inventories and networks we need to tease apart how we should manage systems for structure vs. function. Are we more interested in preserving ecological structure or managing for the ecosystem function directly? However, “function” is a very difficult thing to actually measure, and there will be a trade-off where managing for tomorrow may mean compromising current ability to exploit the functions of the system. Here we must draw a very clear line between management for function for current use and management for biodiversity which may preserve options for the future. Understanding that trade-off is a priority for ecosystem sustainability and fits the three main categories of consideration regarding natural capital “reducing pressures, improving condition or maximising benefits” and headline 8 in the Defra 25 Year Environmental Plan. Rossberg at al. (2017) argue that chief among the targets for sustainability of functional resources must be a change from high-level

qualitative language in policy documents “good environmental status” to more quantitative languages and approaches and to acknowledge that potential uses of future generations may not be the same uses we have today. Rossberg et al. (2017) argue that a framework for sustainability of ecosystem function must include a measure of recovery time of a function within a defined time to a pre-pressure state while acknowledging that since indicators of function respond slowly, pressures exerted on them must be measured along with the function itself to safeguard future sustainability.

5. While managing ecosystem function is normally the target of metric measurement, a continual problem is that these metrics are proxies for actual ecosystem “fitness”. Fitness is a loaded term in biological research. In evolutionary biology a structure leads to a function and we assess the fitness of that structure-function with some measure of population turnover or recruitment. In ecosystem ecology, we don’t appear to have an actual metric of what ecosystem “fitness” in the evolutionary sense might be and this may not be possible since “fitness” is normally associated with an individual rather than a population or system directly. In this context, trying to assay function is actually probably impossible in an evolutionary sense. We need first to define what ecosystem fitness might be and then work backwards to understand function. Whitford et al. (1999) observed that metrics of resistance and resilience of grasses to a gradient of grazing intensity varied along with drought and time to recovery to a pre-stress state and also varied with ecosystem stress. They argued that measurements of known natural resistance and resilience characteristics of a system could be used as a test of an ecosystem’s fitness and that such fitness tests should be used as a warning system for ecosystem functional degeneration. Some similar measurements at scales of landscape and whole ecosystems permit a more detailed measure of function.

6. Technological integration for global metrics of ecosystem function must also be a target. While I have left it to last, and the focus of this document is DNA-based approaches, the most important metric for development is one which integrates a wide variety of high level “next generation” methodologies. For example, a variety of reviews, opinion pieces and research papers have argued for the widespread integration of eDNA and genomic approaches with food web ecology along with approaches in artificial intelligence (Clare et al. 2019, Evans et al., 2016, Roslin & Majaneva 2016, González-Varo et al. 2014, Bohan et al. 2017). What is really needed is global integration of a much wider variety of information driven systems. Integration of things like Google Earth Engine, a cloud based planetary geospatial analysis system (Gorelick et al. 2017), Landsat satellite remote sensing (https://www.sciencedirect.com/topics/earth-and-planetary-sciences/landsat), LIDAR high resolution mapping and more functional biological measures such as remote eDNA surveys and AI driven integration into dynamic food web modelling, to name just a few. Independently all these systems are being deployed to measure earth functions in an explosion of applications, but very little integration has been proposed or applied. An example of where such combined approaches can be implemented could include augmented monitoring of area change and function in broadleaved and conifer woodland (D3 Defra 25 Year Environment Plan). Metrics which take multiple sources such as these into account will be uniquely valuable to solve the problem of measuring and monitoring ecosystem health, function and fitness and to manage sustainability moving forward.

Grand challenges of integrating DNA into biomonitoring programmes

Before complete integration of DNA into global biomonitoring a number of challenges need to be addressed. These are not insurmountable, but they are controversial, and efforts need to be put into determining the best practice to address these.

Abundance, biomass and the quantification problem

One of the most controversial challenges in the use of DNA assays in ecological study is the problem of estimating biomass or abundance of the target (D4, D6 headline 7 Defra 25 Year Environment Plan). In microbial ecology, it has become common to use the relative abundance of sequencing reads associated to a taxa from metabarcoding as a proxy for abundance (e.g. Amend et al. 2010). This can be further refined using some version of the amplicon sequence variants (ASV) approach which attempts to control potential sequencing errors so that retained haplotypes are “real” (Callahan et al. 2017). The main argument against this approach comes from biases in how primers differentially amplify targets and other evidence that everything from collection and extraction protocols and bioinformatics steps impact on the relative representation of the sequence reads (Deagle et al. 2013) and thus any estimate of abundance or biomass. This is particularly true of Eukaryotes where frequent targets like mitochondria or plastid DNA will vary substantially between tissue type (Barazzoni et al. 2000) or reproductive condition (Cotterill et al. 2013). In some analyses, relative abundance has proven reliable (Deagle et al. 2019) but it remains controversial to impose any such measure without extensive testing and even then, only in relatively simple systems, particularly if PCR is involved (Deagle et al. 2019). Promising solutions include PCR free metagenomic approaches, for example Lang et al. (2019)’s genome skimming of known pollen mixes found that the pollen frequencies estimated from sequencing were highly correlated to traditional pollen counts. However many, like the Lang et al. (2019) example work with highly controlled mixes (mock communities) rather than natural samples. Clearly this type of work should be the focus of additional validation.

Adjusting to changes in technology and achieving continuity

It is interesting that so much focus has been placed on the potential error rates of DNA-based technologies. There are thousands of publications devoted to discussion of these issues. This often leads to the regulatory response that we can’t or should not use these technologies until all problems are solved. This is a demonstrably silly position to take. All forms of monitoring are error prone. Acoustic monitoring for bats is limited by the sensitivity of various receivers, direction of sampling, and unable to quantify individuals (e.g. Adams et al. 2012) but is still widely used to monitor activity and species composition because netting is too labour intensive and biased against high flying species. In aquatic systems there is evidence that AC and DC based electrofishing produce biases in species catch and in the captures per unit of effort (Porreca et al. 2013) and similarly different types of netting are biased by size, net avoidance behaviour, weather and baiting etc.

(Jellyman and Graynoth 2005). New biases in current accepted methods are discovered all the time. The expectation that DNA approaches should somehow be held to a higher standard is a major limitation on the uptake of these new technologies. A problem for changing to an eDNA-based approach, or even incorporating this into existing practices is achieving continuity with previous work in the face of continually changing practices. A comparison of high-throughput sequencing platforms by Glenn (2011) demonstrated a confusing array of options and how their names have changed as companies bought and sold platforms. In only a few years since that publication, most of the technologies he reviews no longer exist and for those that do (e.g. Illumina, PacBIO) the methods and chemistry are very different now. This turnover makes continuity of monitoring extremely difficult if we are trying to compare data produced in different ways, and if sampling becomes unrepeatable in a key technological approach. There are several solutions to this. One involves extensive continual validation using mock communities as positive controls. The second is to archive samples to provide comparative data and the third involves the use of assays like rarefaction curves to look at sampling completeness. But some flexibility is needed, which can be hard to integrate into a regulatory and legal framework.

Competing trade-offs between length and depth goals of sequencing

One example of a trade off in technological development is a changing focus from platforms that achieve long sequencing length (e.g. Roche 454 produced 1000p reads, IonTorrent up to 450bp reads), which gave way to shorter length but greater depth platforms (e.g. Illumina MiSeq and HiSeq) and now systems which again target length (PacBio SEQUEL). Length will provide better taxonomic identification; depth will provide greater coverage of rarer taxa. Such trade-offs need to be taken into account as part of validation. One reason for this is the dual use of sequencing platforms for assembling genomes against a scaffold of a known reference genome, where depth is more important than individual read length, and taxonomic identification where length may be more important than depth. Again, the emergence of metagenomics and the newest approaches like SEQUEL will start to address this, though the cost of some technologies still make them untenable for most long term or widespread biomonitoring approaches.

Completeness and clarity of databases

For any metabarcoding or metagenomic approach to work a good reference database is needed. A number of very large references now exist. NCBI's GenBank is the largest repository of molecular data but is not specific to any particular target. NCBI's organelle resources contain records of full mitochondrial (currently n=10,561) and plastid (currently n=820) genomes. BOLD (www.barcodinglife.org) contains the largest homologous reference collection for COI data, a frequent target for Eukaryotic metabarcoding. It also contains smaller collections of ITS, rbcL and matK references that are searchable (7.8 million sequences at time of writing). ITS is the most common marker for fungal metabarcoding and the largest reference collection is in the UNITE database (https://unite.ut.ee/). For microbial metabarcoding 16S or other ribosomal data is normally

targeted, and the SILVA database (https://www.arb-silva.de/) contains curated collections of small and large subunit ribosomal rRNA sequences. The same problems impact all databases. First, as taxonomic designations change, data is rarely updated so that one species or taxa may become represented by multiple names. Second, an estimated 90% of taxa have no current Linnaean species designation and have been described as “dark taxa” (Page, 2016) so that even a perfect match to a reference in a database will fail to generate a name.

The main solution to this problem is a combination of generating private dedicated databases or dispensing with taxonomic designations as the primary means to track an operational taxonomic unit through time and space. The BOLD database has already implemented an OTU based system called the Barcode Index Number (BIN) which provides interim designations that allows for comparisons across samples even when Linnaean designations are incomplete or in flux (Ratnasingham and Hebert 2013). Similar OTU based systems exist for other databases and operationalise them for analytical systems. While this is common practice in other scientific disciplines, it has been long resisted in biology, but is the only way forward to deal with the Linnaean bottleneck. One less recognised problem is serial identification leading to inflated error rates. For example, Unknown sequence A is 99% similar to species 1 so is designated species 1 and deposited in a database. Unknown sequence B is 98% similar to Unknown A which now has a name. However Unknown B is really only 96% similar to the original reference sequence. This can get compounded over time as the “cloud” of sequences gets larger. The net impact of this is unknown at this stage but should be considered.

Operational constraints

Some of the main practical limitations include the costs associated with both conversion to new technologies where new lab-based investment will be needed, and ongoing investments required in consumables and sequencing. However, these costs are falling quickly, in particular sequencing costs have declined dramatically over the last decade, having previously followed Moore’s law on declining costs but dramatically dropped with the advent of high throughput platforms. (Figure 9).

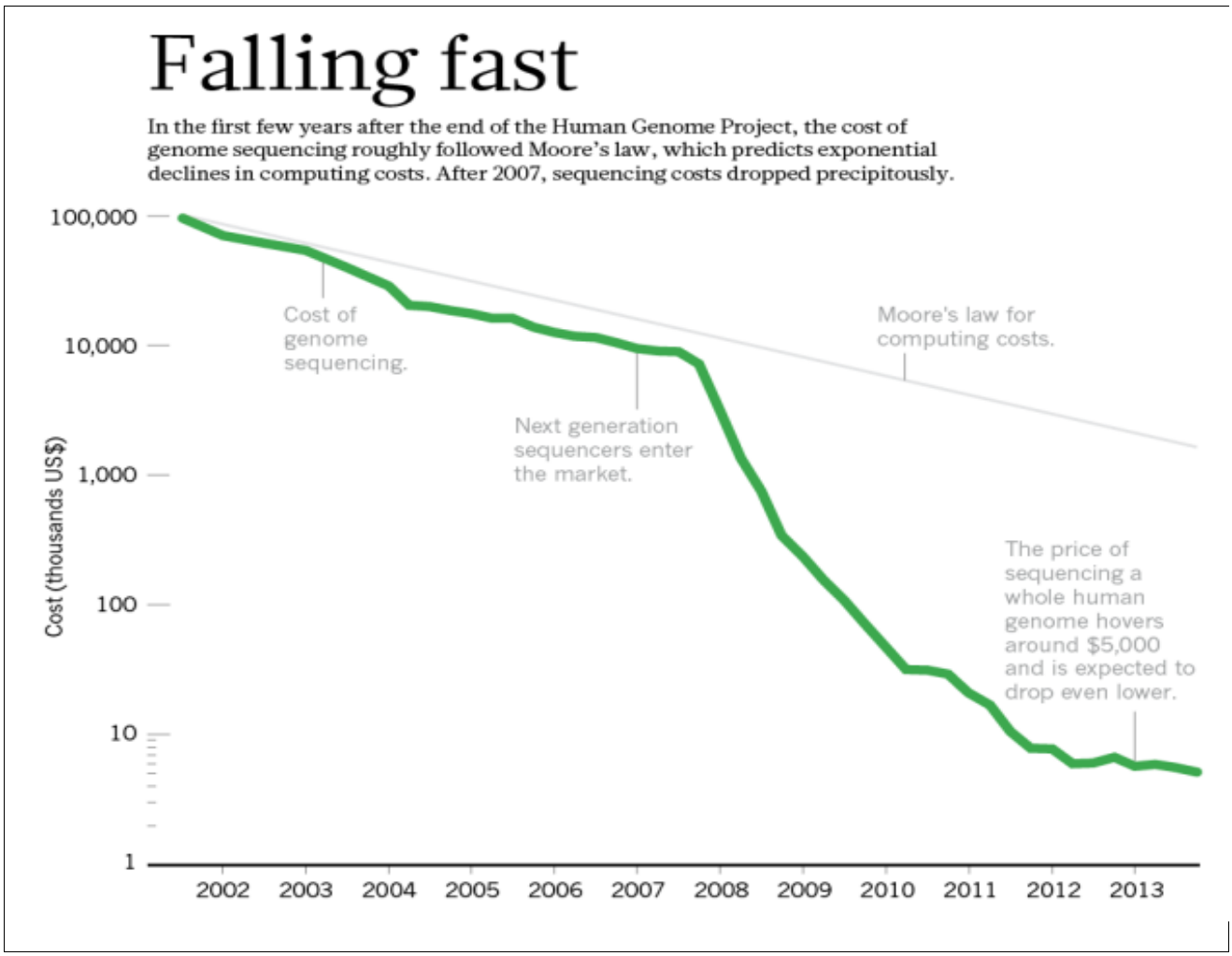

Figure 9 Famously sequencing costs declined following Moore's law but this changed with the advent of high-throughput sequencing and the falling costs have made these technologies accessible to most research labs and regulators. Adapted with permission from Hayden 2014

More complex is the problem of comparability with existing data and appropriate experimental design to achieve an objective. There are operational issues in sample collection, preservation and transportation, choices around the appropriate replication (technical vs. biological), choices of positive and negative controls and validation tests both before and after analysis. The approach which is likely to produce highest success is a phased introduction of new methods with an emphasis on backwards compatibility with established methods.

However, the acceptable practice needs to not focus on replication of a particular method "use chemical brand X" but with an emphasis on objectives and diverse methods which can independently address those objectives. Higher success will be met with combined approaches than a focus on only one approach. For example, to generate the best estimate of fish species richness in a river, using electrofishing, fish counters, acoustic scanners, sein netting etc. in combination will always generate a better outcome than any one technique on its own. DNA approaches should not be held to any different standard.

The approach should be to use multiple techniques (e.g. metabarcoding and functional genomics) in combination to meet the objectives.

Along with this is the need for technical training and computational resources. Initially these were some of the main limitations for most users however, the wide variety of platforms now available both online and by local command line is exploding and making the analyses accessible. This has advantages, widening the user pool, but also raises an issue of data quality where many steps in the analysis are not explained or, worse, concealed from the end users, making the analysis almost ‘too easy’ with serious analytical implications that are not obvious. For example, some analyses will automatically use sequence read numbers as a measure of abundance and skip to ecological models and outcomes, leaving the user without an option to use or not use read numbers as appropriate. The solution to this is wider testing, more standardisation and more interactive platforms that preserve the user-friendly interfaces, while incorporating more user choice so that the implication of choices can be assessed for each new analysis.

Case study 1: Biomonitoring soils and detritus

Soil metabarcoding and metagenomics is one of the most well explored terrestrial bulk sampling DNA-based techniques, with thousands of publications in just the last couple of years. The topics range from comparisons of soil type impacted by pollution (e.g. Sun and Badgley 2019) and agricultural practices (e.g. Treonis et al. 2018), forest soils (e.g. Müller et al. 2019) deserts and paleoclimate (Díaz et al. 2019), targeting microbes (e.g. George et al. 2019), nematodes (e.g. Treonis et al. 2018), fungi (e.g. George et al. 2019), insects, plants (e.g. Zinger et al. 2019) and vertebrates (e.g. Leempoel et al. 2020) to name only a limited selection. As a consequence, there is a vast literature on which to base a forward-looking bio-monitoring campaign and for the development of novel metrics of function.

In an interesting multi-taxa metabarcoding study of tropical soil community assembly, Zinger et al. (2019) studied community assembly in a 12ha tropical forest plot. They analysed 19 taxonomic groups from microbes to fungi, invertebrates, plants and other mesofauna and found primarily stochastic assembly with aluminium, topography and plant species weekly predicting bacteria, protists and fungi (Zinger et al. 2019). Interestingly they also found that body size predicted assembly with larger taxa showing more stochastic assembly than smaller microbes (Zinger et al. 2019). This type of community level analysis is an emerging discipline but extremely promising for understanding changes in total community composition rather than focussing on flagship taxa.

In an investigation of soil rehabilitation following mining activity, Sun and Badgley (2019) used soil functional metagenomics to investigate the functional microbial community in a 31-year time series of soils collected after reforestation. They found that while some changes in microbial functional genes suggested increasing metabolic activity related to the carbon cycling and ammonia and nitrite oxidizing bacteria during restoration, there was minimal change in key N-cycling functional genes and those related to methane consumption (Sun and Badgley 2019). For example, key methanotrophs and methane monooxygenase genes decreased in relative abundance at all former mine sites over the time series but not at control sites suggesting that these soils have become a methane source rather than a sink (Sun and Badgley 2019). This combined taxonomic and functional investigation is extremely interesting as a way to assay ecosystem functional change and suggests a clear path forward for monitoring restoration ecology of soils.

These two different examples, one of a taxonomically focussed study of community assembly and one which focuses on how genomic approaches can be used to monitor and quantify whether key ecosystem functions have been restored, provide two clear analytical options and pathways forward for terrestrial ecosystem monitoring. The advantage in both is the global view of “function”. For example, in the restoration of mined soils, assaying for a flagship taxon (e.g. related to carbon cycling) would have suggested good recovery, while considering both the community of taxa and of functional genes suggests a much more nuanced outcome after three decades of attempted restoration. This more complex, but more informative method is surely the way forward in ecosystem functional monitoring.

Case study 2: Biomonitoring terrestrial life with air

One of the often-cited advantages of the DNA approach, particularly eDNA, is that it can be a non-invasive method of conducting biomonitoring. Among the most exciting applications in this area is the use of metabarcoding to analyse the content of air samples. This has a dual potential of assessing air quality (Headline 1 of the Defra 25 Year Environmental Plan) along with biomonitoring. Nicolaisen et al. (2017) collected 152 air samples from rooftops in urban settings and over oil seed rape fields in the USA and analysed fungal content using a combination of ITS1 metabarcoding and qPCR for targeted detection of plant pathogens. While they observed that Basidiomycota were in higher abundance in the autumn and spring, they found little difference in the near surface air fungal community, though agricultural areas had a lower diversity of fungal species detected. Plant pathogens were equally likely in both urban and agricultural areas (Figure 10).

Air sampling is also a good source for anemophilous (wind-pollenated) plant species detection. Sherwood et al. (2017) used a metabarcoding approach to determine that windward and leeward airborne algal diversity differed dramatically across the Ko'Olau mountain range in Hawaii. Similarly, Korpelainen and Pietilainen (2017) collected air samples in buildings to analyse pollen exposure and found a wide variety of both indoor and outdoor plants and were able to detect a spike in potential allergens in some summer collected samples. Johnson et al. (2019a) have also explored the detection rates for non-anemophilous species by sampling air using dust traps and comparing the collection of the wind pollinated genus *Bouteloua* and insect pollinated (*Prosopis glandulosa*) which was not flowering at the time. Despite the lack of pollen, they were able to detect both *Bouteloua* and *Prosopis* indicating that non-pollen DNA was present in the air and suggesting a more complex ecology of airborne eDNA. In a review of the topic, Johnson et al. (2019b) expand the definition of airborne eDNA to include leaf and flower fragments along with pollen and present analytical comparisons which demonstrate that collection, extraction and PCR approaches all influence plant detection.

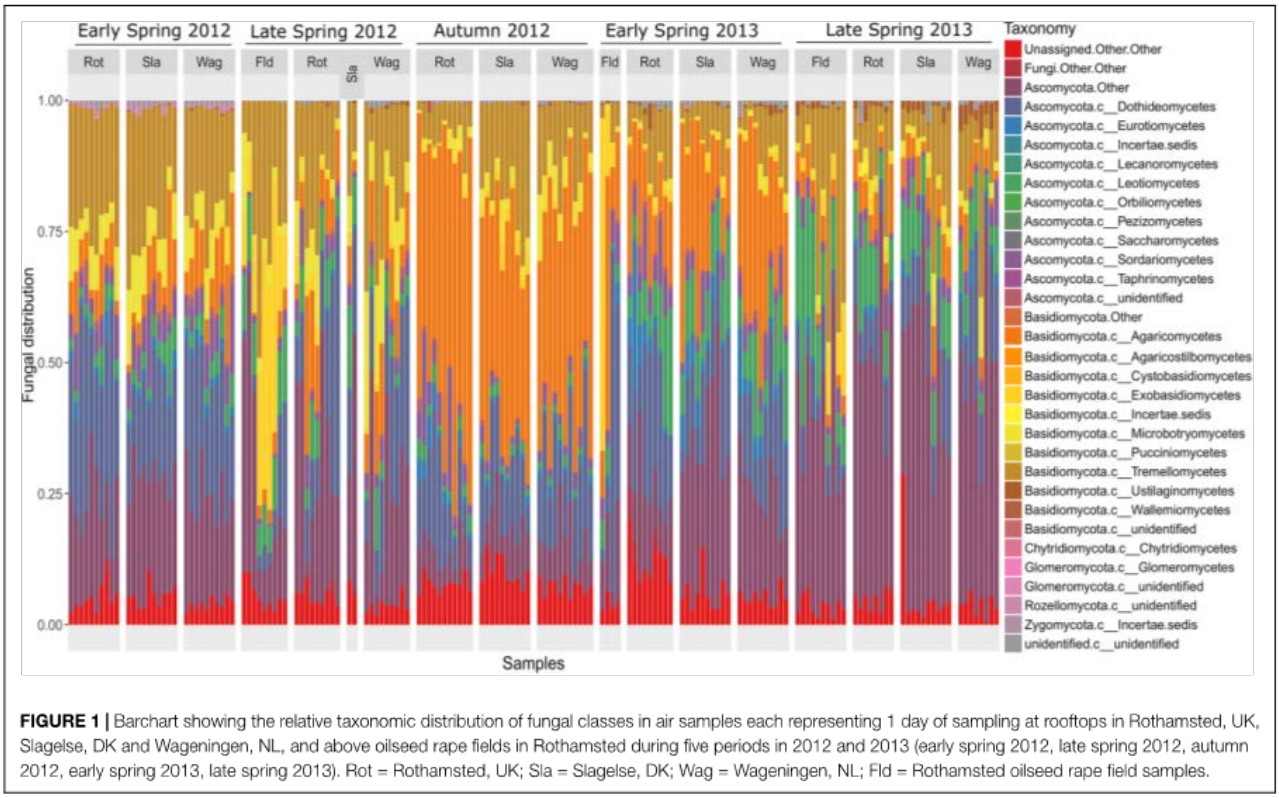

FIGURE 1 | Barchart showing the relative taxonomic distribution of fungal classes in air samples each representing 1 day of sampling at rooftops in Rothamsted, UK, Slagelse, DK and Wageningen, NL, and above oilseed rape fields in Rothamsted during five periods in 2012 and 2013 (early spring 2012, late spring 2012, autumn 2012, early spring 2013, late spring 2013). Rot = Rothamsted, UK; Sla = Slagelse, DK; Wag = Wageningen, NL; Fld = Rothamsted oilseed rape field samples.

Figure 10 Relative taxonomic distribution of fungal classes detected in air sample across seasons. Adapted with permission from Nicolaisen et al. 2017

To date, I can find no published case where animal DNA has been targeted in air, however, it was the subject of a high school science fair project where Yuma Okamoto and So Tsukamoto, of Shizuoka Prefectural Kakegawa-Nishi High School in Kanegawa, Japan constructed a bird detector based on airborne DNA and traced the spatial temporal patters of DNA degradation (https://www.sciencenewsforstudents.org/blog/eureka-lab/isef-2019-two-teens-pull-dna-birds-out-air). While to date, most studies are narrow in scope, it is an exciting option for biomonitoring given the ubiquity of air filtering devices available and commonly deployed to measure air quality already. Filters in these devices could be excellent sources of eDNA for species biomonitoring immediately.

Case study 3: Assessing ecological interactions and food webs

While DNA-based biomonitoring is a vital method of assessing ecosystems, truly understanding functions and changes in functions requires us to measure interactions, resilience, stability etc. to understand the existing structure and its capacity to recover from disturbance. Pollination, parasitism, nutrient cycling, energy transport etc. all result from species interactions and to measure their dynamics and response to fluctuations requires much more than knowledge of who is present in a system.

Molecular approaches to detecting species interactions started long before DNA-based approaches and generally targeted predation. Immunoassays using targeted antibody techniques were used for decades (reviewed in Boreham and Ohiagu 1978) and ELISA with both polyclonal and monoclonal antibodies were used in predation studies (Sunderland 1988). Protein electrophoresis as a technique for the analysis of gut contents was reviewed two decades ago by Murray et al. (1989) where staining for enzyme activity was compared to known prey banding patterns. However, these were largely replaced by DNA-based techniques towards the end of the 90s and start of the 2000s (Symondson 2002), due to the tremendous reduction in cost and laboratory equipment needed. Targeting of mitochondrial DNA started with feeding trials by Chen et al. (2000) and two main approaches quickly developed, one which focussed on targeted methods using specific primers (reviewed by King et al. 2008) vs. a diversity approach where highly general primers target the breadth of potential prey. Within a decade the rise of high-throughput sequencers has again transformed this (reviewed by Pompanon et al. 2012) by scaling up and expanding the applications to entire food webs and interaction networks.

The main place where DNA will push the boundaries of network modelling is in node resolution. In a dramatic demonstration of this Wirta et al. (2014) used complimentary DNA barcoding to augment high arctic parasitoid networks and found dramatic alterations in perceived network structure. They observed that for some measures, the addition of DNA-based node resolution caused these metrics to vary by as much as 5x more than the average variability between networks from around the world. Their conclusion was that combinations of approaches are vital to accurately depict networks structure. Gonzalez-Varo et al. (2014) applied a barcode approach to track avian seed dispersal by sequencing avian DNA from the surface of seeds allowing the dispersal pattern to be tracked with no observation needed at all.

While these examples employed the simplest of all molecular approaches, others have attempted to incorporate metabarcoding data into networks (e.g. Evans et al. 2016, Bell et al. 2017, Clare et al. 2019) though this has more methodological challenges (Figure 11). Metabarcoding has particularly been applied to pollination networks. Pornon et al. (2016) found 2.5x more plant species in plant-pollinator interactions when metabarcoding was used to identify pollen. And Pornon et al. (2017) found many “hidden interactions” in pollination networks and suggests that most pollinators are much more generalistic in their approach than previously thought. Similarly, hoverflies were found to be more generalist in their flower visitation than previously described when metabarcoding was applied to their

pollen loads (Lucas et al. 2018a) and that this behaviour increased towards late summer (Lucas et al. 2018b). Compared to traditional light microscopy, metabarcoding detected pollen transported by more individual moths and between more moths and plants than previously known, suggesting an unexpectedly complex nocturnal pollination network (Macgregor et al. 2019).

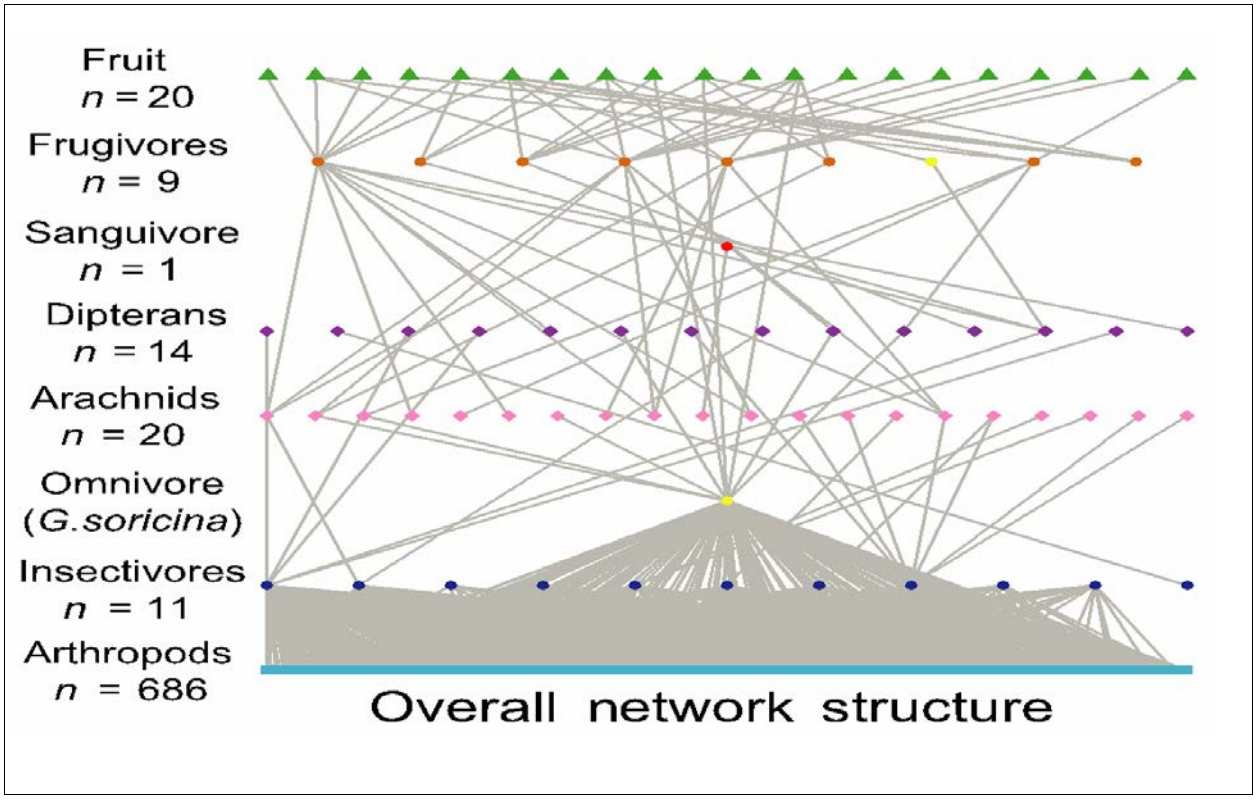

Figure 11 Network of networks generated entirely using DNA-based approaches employed both DNA barcoding and DNA metabarcoding to determine the structure of a neotropcial bat community. Adapted from Clare et al. 2019

As a general observation, metabarcoding finds more interactions between more species than any previous method of constructing networks and this increases specific network properties such as generality and connectance. It will also have a likely knock on effect of increasing measures of network robustness through increased functional redundancy, but may reduce estimates of network compartmentalisation which has implications for our understanding of how perturbations can move through ecosystems.

Why the biome-based method is counter productive

The vast majority of our traditional approaches to ecology and biomonitoring are based on targeting a key species, a discrete biome or a trophic category. We are used to talking about “frugivores” or terrestrial biomonitoring. Even the title and scope of this document was to discuss the terrestrial biome, but I have found it impossible not to mention other systems and this is a reflection of the reality of ecosystems. While we have acknowledged for decades that the interaction between biomes, particularly in transition zones, is a fundamental characteristic of these systems (Schindler and Smits 2017) and the Defra 25 Year Environmental Plan lists things like “connectivity of habitats” as a headline indicator (D1, Headline 7), there is a broad gap between those studying aquatic systems and those studying terrestrial systems (Soininen et al. 2015) and few attempting to bridge this gap. However, such barriers are both artificial and harmful to our ability to understand ecosystem functions.

There are many one-off analyses which address this problem of trying to categorise species and systems and make specific observations about the very grey area between these traditional roles. For example, we investigated the diet and behaviour of the neotropical bat *Glossophaga soricina* classified as a “nectarivore” but sometimes reported to consume insects. We first demonstrated that it commonly consumes insects and that it is not an opportunistic behaviour while also feeding at flowers but the result of a novel echolocation system and hunting strategy (Clare et al. 2014a). Second, we suggested using food webs that, rather than a specialist feeder, this species could act as a keystone species in recovering neotropical systems (Clare et al. 2019). It is highly likely that most nectarivores and frugivores are actually omnivores (we have now observed insect material in guano from a great many bats traditionally classified as frugivores) and it has been demonstrated that in places like Madagascar, fruits are actually too protein deficient to support primates (Ganzhorn et al. 2009) and some degree of omnivorous supplementation is certain to be happening on a very wide scale. This break down of traditional trophic categories can be mirrored in many systems and should not be shrugged off when trying to understand trophic interactions and ecosystem services such as nectarivory and seed dispersal. Similarly, the movement of energy between biomes is extremely common and examples are easy to find. In our analysis of the insectivorous bat *Myotis lucifugus* we were able to actually accurately classify local aquatic habitats just by what we found in the diet of the terrestrial predator (Clare et al. 2011, 2014b) because they primarily forage over water and were consuming species known to be freshwater environmental indicators. If they were foraging over the water, they were also almost certainly depositing guano and urine back into this system making a direct energy transfer from aquatic to terrestrial and back to aquatic in one set of interactions.

It should be absolutely impossible to ignore the ways that terrestrial and aquatic systems, or marine and freshwater systems interact (D1 Defra 25 Year Environment Plan), particularly in the UK where we have the massive Thames river system where marine and freshwater species are interacting constantly. But in a very real and measurable way all biomes interact all the time. For example, aquatic and terrestrial food webs are tightly linked and an integrative approach should be incorporated into research campaigns

(Soininen et al. 2015) and, indeed, metaecosystem dynamics is now a theoretical framework and major funding schemes are starting to recognise this as an important research direction. But these interactions should be obvious. For example, agricultural run-off of both wastes and fertilisers leads to eutrophication, which also leads to structural changes in aquatic ecosystems which interacts with warming trends (Binzer et al. 2016). In a meta-analysis of previous work, Bartels et al. (2012) reported that the interaction between terrestrial and aquatic systems is asymmetric with aquatic systems receiving higher subsidies (e.g. the movement of nutrients appears to be a net of terrestrial to aquatic) and that both top-down and bottom-up ecosystem effects are impacted by these allochthonous movements of resources. This asymmetry is likely driven by the movement of water (Schindler and Smits 2017). While the concept of cross-system subsidies is gaining in focus, the extended impact of changes in one biome on another is less well known. For example, Schindler and Smits (2017) argue for more focus on the impact of anthropogenic changes to both aquatic and terrestrial landscapes and that these spatial-temporal changes in subsidy delivery will have consequences for functions in both ecosystems. To do this, they argue for more parallel studies of biotic and abiotic mechanisms of resource subsidy and how these processes impact productivity of both biomes.

Clearly, the focus going forward for all proposed methods, metrics and approaches, must take a larger systems-based approach where we discard the traditional boundaries of ecological study and ecosystem biomonitoring and shift to a more integrated approach.

An end to regional and national approaches: One significant issue in establishing next generation biomonitoring is selecting an appropriate spatial-temporal scale for monitoring. The flagship “indicator” species approach is, by its nature, spatially limited to a range or distribution and feeds into a local approach where programmes are built and maintained by national governments, or even local municipalities. There is value in a granular approach and the old adage, “think globally, act locally” is a good sentiment but it does not make for an advanced next generation biomonitoring system and this transnational view is echoed in headline 16, and indicators K1, K2, K2 and K4 of the Defra 25 Year Environmental Plan. Barriers need to be removed as fast as possible. The current regional and national approach must be expanded. Major international policy already exists in this area (e.g. Convention on Biological Diversity) and several networks have breached these traditional barriers: DNAqua-Net (https://dnaqua.net/) is developing pan-european DNA-based monitoring; IBOL (https://ibol.org/) has tried to unify approaches and standards for biodiversity assays; the ARMS programme (https://www.oceanarms.org/) uses passive collectors to sample understudied marine fauna and the Earth Microbiome Project (http://www.earthmicrobiome.org/) was established to characterise global microbial diversity at the taxonomic and functional level. This global (and often autonomous) thinking has three immediate advantages: 1) it removes political boundaries which make no biological sense, 2) it provides the potential of long term continuity when short term turnover of political systems and funding keep us otherwise short sighted and 3) it decouples “experts” from location, meaning each country need not develop specialities in every method but can rely on autonomous data collection and the activation of global response teams when a problem is detected. Some disciplines have whole heartedly

adopted global consortia, and the success of CERN, the international space station and astronomical arrays with multi-national teams and funding are a clear model for global approaches to big questions. Biological disciplines have not traditionally taken this route but this must be embraced to address long term ecosystems survival.

Conclusions and recommendations

How shall we move from general suggestions to operationalisation and on what timeline? I make the following practical suggestions:

- Regulatory bodies must urgently engage the scientific research community at all levels. Rather than waiting for technologies to mature, they should be part of their development. This will include meaningful collaboration and investment in joint ventures and joint advisory expert panels which include a diverse set of approaches. This will allow regulatory agencies to be first off the ground in using new technologies and allow researchers and developers to gain insight into the requirements and applications of their developments.
- The current lack of engagement has caused one very serious problem in that it is unclear if any of the case studies or technologies outlined above meet the regulatory requirements of current UK or international biomonitoring policy. Because of the lack of engagement between these two, we must now engage in retroactive validation, and this is not high priority for most scientists already using these approaches (and is very hard to fund). Such engagement needs to happen far earlier and include international partner agencies so that a more global approach can be actioned, and validation should be part of development, not post-development usage.
- To remedy this, we need an urgent joint initiative – the establishment of a diverse expert working group with the explicit goal of identifying key technologies and appropriate controlled validation experiments, and with a budget to implement those plans for the UK on a tight timeline, for example ready for the “National Ecosystem Assessment” suggested for 2022 in the Defra 25 year Environmental Plan (Figure 12). This will almost certainly require extremely large and carefully manipulated experimentation on national scale, but such approaches are the only way to operationalise these approaches and should be implemented immediately. There is no timeline to success in this. The time for planning was a decade ago. The time for action is now.

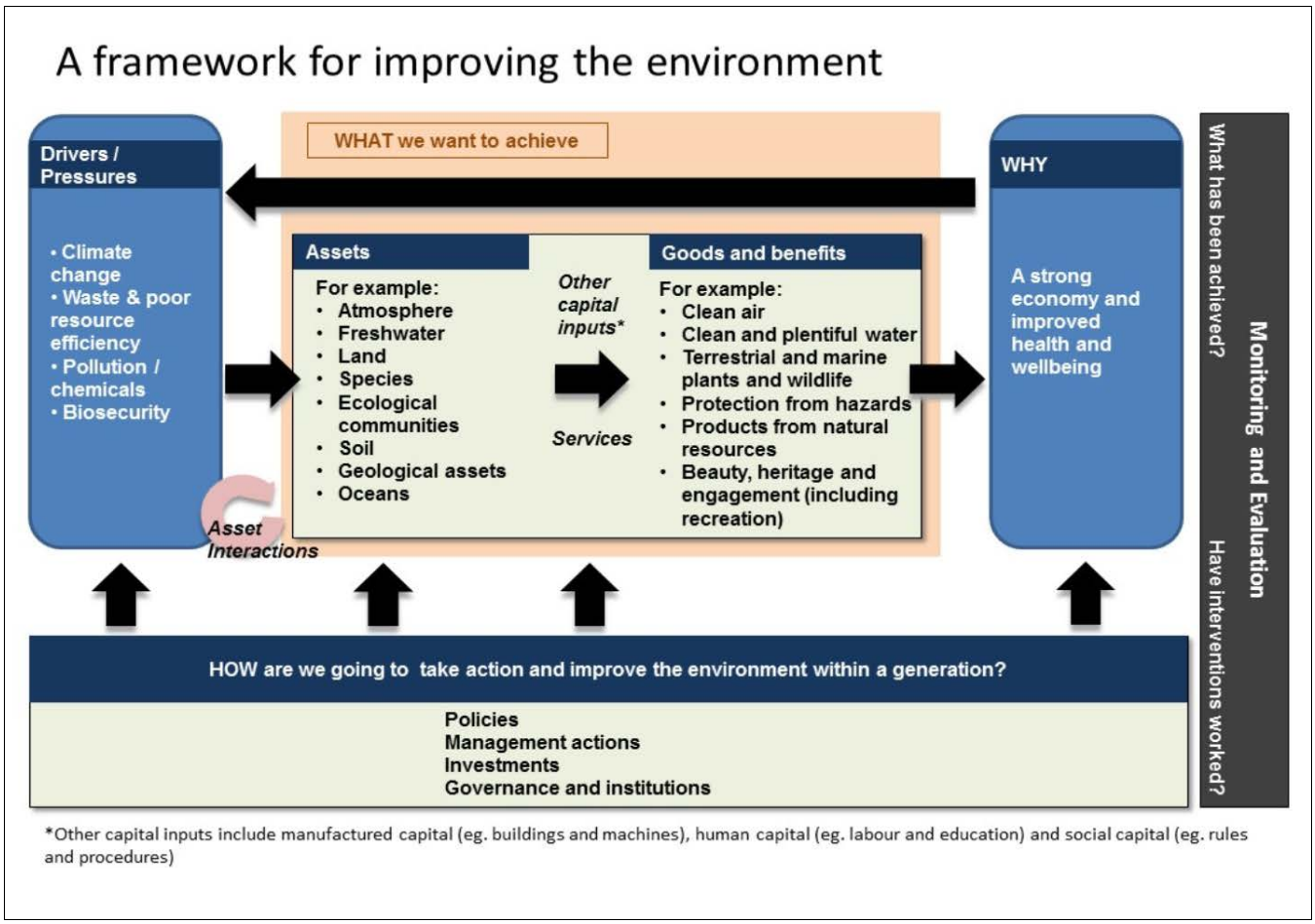

Figure 12 The conceptual framework for improving the environment from which the Defra 25 Year Environment Plan will use to evaluate progress. Adapted with permission from Defra

References

ADAMS AM, Jantzen MK, Hamilton RM, Fenton MB. 2012. Do you hear what I hear? Implications of detector selection for acoustic monitoring of bats. *Methods in Ecology and Evolution*. 3(6):992-998

ALBERDI A, Aizpurua O, Gilbert MTP, Bohmann K. 2018. Scrutinizing key steps for reliable metabarcoding of environmental samples. *Methods in Ecology and Evolution*. 9(1):134-147

AMEND AS, Seifert KA, Bruns TD. 2010. Quantifying microbial communities with 454 pyrosequencing: does read abundance count? *Molecular Ecology*. 19(24):5555-5565

BARTELS P, Cucherousset J, Steger K, Eklöv P, Tranvik LJ, Hillebrand H. 2012. Reciprocal subsidies between freshwater and terrestrial ecosystems structure consumer resource dynamics. *Ecology*. 93(5):1173-1182

BELL KL, Burgess KS, Botsch JC, Dobbs EK, Read TD, Brosi BJ. 2019. Quantitative and qualitative assessment of pollen DNA metabarcoding using constructed species mixtures. *Molecular Ecology*. 28(2):431-435

BELL KL, Fowler J, Burgess KS, Dobbs EK, Gruenwald D, Lawley B, Morozumi C, Brosi BJ. 2017. Applying pollen DNA metabarcoding to the study of plant-pollinator interactions. *Applications In Plant Sciences*. 5(6):1600124

BEREZOWSKA-CNOTA T, Luque-Márquez I, Elguero-Claramunt I, Bojarska K, Okarma H, Selva N. 2017. Effectiveness of different types of hair traps for brown bear research and monitoring. *PLoS One* 12(10): e0186605

BINZER A, Guill C, Rall BC, Brose U. 2016 Interactive effects of warming, eutrophication and size structure: impacts on biodiversity and food-web structure. *Global Change Biology*. 22(1): 220-227

BOHMANN K, Evans A, Gilbert MTP, Carvalho GR, Creer S, Knapp M, Yu DW, de Bruyn M. 2014. *Environmental DNA for wildlife biology and biodiversity monitoring*. 29(6):358-367

BOHAN DA, Vacher C, Tamaddoni-Nezhad A, Raybould A, Dumbrell AJ, Woodward G. 2017. Next-generation global biomonitoring: Large-scale, automated reconstruction of ecological networks. *Trends in Ecology & Evolution*. 32(7): 477–487

BOREHAM PFL, Ohiagu CE. 1978. The use of serology in evaluating invertebrate prey-predator relationships: a review. *Bulletin of Entomological Research*. 68(2): 171–194

BARAZZONI RK, Short KR, Nair KS. 2000. Effects of aging on mitochondrial DNA copy number and cytochrome c oxidase gene expression in rat skeletal muscle, liver, and heart. *Journal of Biological Chemistry*. 275(5):3343–3347

BUÉE M, Reich M, Murat C, Morin E, Nilsson RH, Uroz S, Martin F. 2009. 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. *New Phytologist*. 184:449-456

BURTON AC, Neilson E, Moreira D, Ladle A, Steenweg R, Fisher JT, Bayne E, Boutin S. 2015. Wildlife camera trapping: a review and recommendations for linking surveys to ecological processes. *Journal of Applied Ecology*. Monitoring Wildlife: 675-685

CALLAHAN BJ, McMurdie PJ, Holmes SP. 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *The ISME Journal.* 11:2639-2643

CBOL Plant Working Group. A DNA barcode for land plants. *Proceedings of the National Academy of Sciences*. 106(31):12794-12797

CHEN Y, Giles KL, Payton ME, Greenstone MH. 2000. Identifying key cereal aphid predators by molecular gut analysis. *Molecular Ecology*. 9(11):1887–1898

CLARE EL, Barber BR, Sweeney BW, Hebert PDN, Fenton MB. 2011. Eating local: influences of habitat on the diet of little brown bats (*Myotis lucifugus*). *Molecular Ecology*. 20(8): 1772-1780

CLARE EL, Goerlitz HR, Drapeau VA, Holderied MW, Adams AM, Nagel J, Dumont ER, Hebert PDN, Fenton MB. 2014a. Trophic niche flexibility in *Glossophaga soricina*: how nectar seeker sneaks an insect snack. *Functional Ecology*. 28(3):632-641

CLARE EL, Symondson WO, Broders H, Fabianek F, Fraser EE, MacKenzie A, Boughen A, Hamilton R, Willis CKR, Martinez-Nuñez F, Menzies AK, Norquay KJO, Brigham M, Poissant J, Rintoul J, Barclay RM, Reimer JP. 2014b. The diet of *Myotis lucifugus* across Canada: assessing foraging quality and diet variability. *Molecular Ecology*. 23(15):3618-3632

CLARE EL, Fazekas AJ, Ivanova NV, Floyd RM, Hebert PDN, Adams AM, Nagel J, Girton R, Newmaster SG, Fenton MB. 2019. Approaches to integrating genetic data into ecological networks. *Molecular Ecology*. 28(2):503-519

COTTERILL M, Harris SE, Collado Fernandez E, Lu J, Huntriss JD, Campbell BK, Picton HM. 2013. The activity and copy number of mitochondrial DNA in ovine oocytes throughout oogenesis in vivo and during oocyte maturation in vitro. *Molecular Human Reproduction*. 19(7):444–450

CRAMPTON-PLATT A, Yu DW, Zhou X, Vogler AP. 2016. Mitochondrial metagenomics: letting the genes out of the bottle. *GigaScience*. 5(15). DOI 10.1186/s13742-016-0120-y

CREER S, Deiner K, Frey S, Porazinska D, Taberlet P, Thomas WK, Potter C, Bik HM. 2016. The ecologist's field guide to sequence-based identification of biodiversity. *Methods in Ecology and Evolution*. 7(9): 1008-1018

DAVISON J, Moora M, Öpik M, Adholeya A, Ainsaar L, Bâ A, Burla S, Diedhiou AG, Hiiesalu I, Jairus T, Johnson NC, Kane A, Koorem K, Kochar M, Ndiaye C, Pärtel M, Reier Ü, Saks Ü, Singh R, Vasar M, Zobel M. 2015. Global assessment of arbuscular mycorrhizal fungus diversity reveals very low endemism. *Science*. 349(6251):970-973

DE PAOLI-ISEPPI R, Deagle BE, McMahon CR, Hindell MA, Dickinson JL, Jarman SN. 2017. Measuring animal age with DNA methylation: from humans to wild animals. *Frontiers in Genetics*. 8:106

DEAGLE BE, Thomas AC, Shaffer AK, Trites AW, Jarman SN. 2013. Quantifying sequence proportions in a DNA-based diet study using Ion Torrent amplicon sequencing: which counts count? *Molecular Ecology Resources*. 13(4):620-633

DEAGLE BE, Thomas AC, McInnes JC, Clark LJ, VesterinenEJ, Clare EL, Kartzinel TR, Evenson JP. 2019. Counting with DNA in metabarcoding studies: How should we convert sequence reads to dietary data? *Molecular Ecology*. 28(2):391-406

DELGADO-BAQUERIZO M, Maestre FT, Reich PB, Jeffries TC, Gaitan JJ, Encinar D, Berdugo M, Campbell CD, Singh BK. 2016. Microbial diversity drives multifunctionality in terrestrial ecosystems. *Nature Communications*. 7:10541

DÍAZ FP, Latorre C, Carrasco-Puga G, Wood JR, Wilmshurst JM, Soto DC, Cole TL, Gutiérrez RA. Multiscale climate change impacts on plant diversity in the Atacama Desert. *Global Change Biology*. 25(5):1733-1745

DRINKWATER R, Schnell IB, Bohmann K, Bernard H, Veron G, Clare E, Gilbert MTP, Rossiter SJ. 2018. Using metabarcoding to compare the suitability of two blood-feeding leech species for sampling mammalian diversity in North Borneo. *Molecular Ecology Resources*. 19(1):105-117

DRUMMOND AJ, Newcomb RD, Buckley TR, Xie D, Dopheide A, Potter BCM, Heled J, Ross HA, Tooman L, Grosser S, Park D, Demetras NJ, Stevens MI, Russell JC, Anderson SH, Carter A, Nelson N. 2015. Evaluating a multigene environmental DNA approach for biodiversity assessment. *GigaScience*. 4(1): s13742–015–0086–1, https://doi.org/10.1186/s13742-015-0086-1

EPP LS, Boessenkool S, Bellemain EP, Haile J, Esposito A, Riaz T, Erséus C, Gusarov VI, Edwards ME, Johnsen A, Stenøien HK, Hassel K, Kauserud H, Yoccoz NG, Bråthen KA, Willerslev E, Taberlet P, Coissac E, Brochmann C. 2012. New environmental metabarcodes for analysing soil DNA: potential for studying past and present ecosystems. *Molecular Ecology*. 21(8):1821–1833

EVANS DM, Kitson JJN, Lunt DH, Straw NA, Pocock MJO. 2016. Merging DNA metabarcoding and ecological network analysis to understand and build resilient terrestrial ecosystems. *Functional Ecology*. 30(12):1904-1916

FAHNER NA, Shokralla S, Baird DJ, Hajibabaei M. 2016. Large-scale monitoring of plants through environmentzal DNA metabarcoding of soil: recovery, resolution, and annotation of four DNA markers. *PLoS One*. 11(6):e0157505

FLOYD R, Abebe E, Papert A, Blaxter M. 2002. Molecular barcodes for soil nematode identification. *Molecular Ecology*. 11(4): 839-850

GANZHORN JU, Arrigo-Nelson S, Boinski S, Bollen A, Carrai V, Derby A, Donati G, Koenig A, Kowalewski M, Lahann P, Norscia I, Polowinsky SY, Schwitzer C, Stevenson PR, Talebi MG, Tan C, Vogel ER, Wright PC. 2009. Possible fruit protein effects on primate communities in Madagascar and the Neotropics. *PLoS One* 4(12):e8253. doi:10.1371/journal.pone.0008253

GEORGE PBL, Lallias D, Creer S, Seaton FM, Kenny JG, Eccles RM, Griffiths RI, Lebron I, Emmett BA, Robinson DA, Jones DL. Divergent national scale trends of microbial and animal biodiversity revealed across diverse temperate soil ecosystems. *Nature Communications*. 10: 1107

GIBBONS WJ, Andrews KM. 2004. PIT Tagging: simple technology at its best. *BioScience*. 54(5): 447–454

GOGARTEN JF, Hoffmann C, Arandjelovic M, Sachse A, Merkel K, Dieguez P, Agbor A, Angedakin S, Brazzola G, Jones S, Langergraber KE, Lee K, Marrocoli S, Murai M, Sommer V, Kühl H, Leendertz FH, Calvignac-Spencer S. 2020 Fly-derived DNA and camera traps are complementary tools for assessing mammalian biodiversity. *Environmental DNA*. 2(1):63-76

GORELICK N, Hancher M, Dixon M. Ilyushchenko S, Thau D, Moore R. 2017. Google earth image: Planetary-scale geospatial analysis for everyone. *Remote Sensing of Environment*. 202:18-27

GLENN TC. 2011. Field guide to next-generation DNA sequencers. *Molecular Ecology Resources.* 11(5):759-769

GONZÁLEZ-VARO JP, Arroyo JM, Jordano P. 2014. Who dispersed the seeds? The use of DNA barcoding in frugivory and seed dispersal studies. *Methods in Ecology and Evolution*. 5(8): 806–814. https://doi.org/10.1111/2041-210X.12212

GOTTWALK J, Zeidler R, Friess N, Ludwig M, Reudenback C, Nauss T. 2019. Introduction of an automatic and open-source radio-tracking system for small mammals. *Methods in Ecology and Evolution*. 10(12):2163-2172

GROSSART H-P, Van den Wyngaert S, Kagami M, Wurzbacher C, Cunliffe M, Rojas-Jimenez K. Fungi in aquatic ecosystems. *Nature Reviews Microbiology*. 17:339-354

HAYDEN E. 2014. The $1,000 genome. *Nature*. 507: 294-295

HEBERT PDN, Cywinska A, Ball SL, deWaard JR. 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society of London B. 270(1512): 313-321.

HILL V, Baele G. 2019. Bayesian estimation of past population dynamics in BEAST 1.10 using the skygrid coalescent model. *Molecular Biology and Evolution*. 36(11):2620-2628

HÖGBERG P, Nordgren A, Buchmann N, Taylor AFS, Ekblad A, Högberg MN, Nyberg G, Ottosson-Löfvenius M, Read DJ. 2001. Large-scale forest girdling shows that current photosynthesis drives soil respiration. *Nature*. 411: 789–792

HOLMES BH, Steinke D, Ward RD. 2009. Identification of shark and ray fins using DNA barcoding. *Fisheries Research*. 95(2-3):280-288

VAN DEN HOOGEN J, Geisen S. et al. 2019. Soil nematode abundance and functional group composition at a global scale. *Nature*. 572:194-198

HOWE A, Lövei GL, Nachman G. 2009. Dummy caterpillars as a simple method to assess predation rates on invertebrates in a tropical agroecosystem. *Entomologia Experimentalis et Applicata*. 131(3):325-329

JELLYMAN DJ, Graynoth E. 2005. The use of fyke nets as a quantitative capture technique for freshwater eels (Anguilla spp.) in rivers. *Fisheries Management and Ecology*.12(4). 237-247

JI Y, Huotari T, Roslin T, Schmidt NM, Wang J, Yu DW, Ovaskainen O. 2019. SPIKEPIPE: A metagenomic pipeline for the accurate quantification of eukaryotic species occurrences and intraspecific abundance change using DNA barcodes or mitogenomes. *Molecular Ecology Resources*. 20(1):256-267

JOHNSON MD, Cox RD, Barnes MA. 2019a. The detection of non-anemophilous plant species using airborn eDNA. *PLoS One*. 14(11):e0225262

JOHNSON MD, Cox RD, Barnes MA. 2019b. Analyzing airborne environmental DNA: A comparison of extraction methods, primer type and trap type on the ability to detect airborne eDNA from terrestrial plant communities. *Environmental DNA*. 1(2):176-185

KAYS R, Crofoot MC, Jetz W. Wikelski M. 2015. Terrestrial animal tracking as an eye on life and planet. *Science*. 348(6240):aaa2478

KING RA, Read DS, Traugott M, Symondson WOC. 2008. Molecular analysis of predation: a review of best practice for DNA-based approaches. *Molecular Ecology*. 17(4): 947-963

KORPELAINEN H, Pietiläinen M. 2017. Biodiversity of pollen in indoor air samples as revealed by DNA metabarcoding. Nordic *Journal of Botany*. 35 (5): 602-608

KOWALCHUK GA. Stephen JR. 2001. Ammonia-oxidizing bacteria: a model for molecular microbial ecology. *Annual Review of Microbiology*. 55: 485–529

LANG D, Tang M, Hu J. Zhou X. 2019. Genome-skimming provides accurate quantification for pollen mixtures. *Molecular Ecology Resources.* 19:1433-1446

LEE P-S, Gan HM, Clements GR, Wilson JJ. 2016. Field calibration of blowfly-derived DNA against traditional methods for assessing mammal diversity in tropical forests. *Genome*. 59(11):1008-1022

LEEMPOEL K, Hebert T, Hadly EA. 2020. A comparison of eDNA to camera trapping for assessment of terrestrial mammal diversity. *Proceedings of the Royal Society of London* B. 287(1918): 20192353

LUCAS A, Bodger O, Brosi BJ, Ford CR, Forman DW, Greig C, Hegarty M, Jones L, Neyland PJ, deVere N. 2018a. Floral resource partitioning by individuals within generalised hoverfly pollination networks revealed by DNA metabarcoding. *Scientific Reports*. 8:5133.

LUCAS A, Bodger O, Brosi BJ, Ford CR, Forman DW, Greig C, Hegarty M, Neyland PJ, deVere N. 2018b. Generalisation and specialisation in hoverfly (Syrphidae) grassland pollen transport networks revealed by DNA metabarcoding. *Journal of Animal Ecology*. 87(4): 1008-1021

MACGREGOR CJ, Kitson JJN, Fox R, Hahn C, Lunt DH, Pocock MJO, Evans DM. 2019. Construction, validation, and application of nocturnal pollen transport networks in an agro-ecosystem: a comparison using light microscopy and DNA metabarcoding. *Ecological Entomology*. 44(1): 17-29

MAKIOLA A, Compson ZG, Baird DJ et al. Key Questions for Next-Generation Biomonitoring. *Frontiers in Environmental Sciences*. 2020. 7:197

MARQUINA D, Esparza-Salas R, Roslin T, Ronquist F. 2019. Establishing arthropod community composition using metabarcoding: surprising inconsistencies between soil samples and preservative ethanol and homogenate from Malaise trap catches. *Molecular Ecology Resources*. 19(6):1516-1530

MURRAY RA, Solomon MG, Fitzgerald JD. 1989. The use of electrophoresis for determining patterns of predation in arthropods. In: Electrophoretic Studies on Agricultural Pests (eds Loxdale HD, den Hollander J), pp. 467–483. Clarendon Press, Oxford

MÜLLER CA, de Mattos Pereira L, Lopes C, Cares J, dos Anjos Borges LG, Giongo A, Graeff-Teixeira C, Morassutti AL. 2019. Meiofaunal diversity in the Atlantic forest soil: a quest for nematodes in a native reserve using eukaryotic metabarcoding analysis. *Forest Ecology and Management*. 453: 117591

NELSON MB, Martiny AC, Martiny JBH. 2016. Global biogeography of microbial nitrogen-cycling traits in soil. Proceedings of the National Academy of Sciences of the United States of America. 113(29): 8033-8040.

NICOLAISEN M, West JS, Sapkota R, Canning GGM, Schoen C, Justesen AF. 2017. Fungal communities including plants pathogens in near surface air are similar across northwestern Europe. Frontiers in Microbiology. 8:1729.

NILSSON RH, Anslan S, Bahram M, Wurzbacher C, Baldrian P, Tedersoo L. 2019. Mycobiome diversity: high-throughput sequencing and identification of fungi. Nature Reviews Microbiology. 17: 95-109.

OPEL T, Meyer H, Wetterish S, Laepple T, Dereviagin A, Murton J. 2018. Ice wedges as archives of winter paleoclimate: a review. Permafrost and Periglacial Processes. 29(3):199-209.

PAGE RDM. 2016. DNA barcoding and taxonomy: dark taxa and dark texts. Philosophical transactions of the royal society B. 371(1702). https://doi.org/10.1098/rstb.2015.0334

POISOT T, Guéveneux-Julien C. Fortin M-J, Gravel D, Legendre P. 2017. Hosts, parasites and their interactions respond to different climatic variables. *Global Ecology and Biogeography*. 26 (8):942-951

POMPANON F, Deagle BE, Symondson WOC, Brown DS, Jarman SN, Taberlet P. 2012. Who is eating what: diet assessment using next generation sequencing. *Molecular Ecology*. 21(8):1931-1950

PORNON A, Escaravage N, Burrus M, Holota H, Khimoun A, Mariette J, Pellizzari C, Iribar A, Erienne R, Taberlet P, Vidal M, Winterton P, Zinger L, Andalo C. 2016. Using metabarcoding to reaveal and quantify plant-pollinator interactions. *Scientific Reports*. 6:27282

PORNON A, Andalo C, Burrus M, Escaravage N. 2017. DNA metabarcoding data unveils invisible pollination networks. *Scientific Reports*. 7:16828

PORRECA AP, Pederson CL, Laursen JR, Colombo RE. A comparison of electrofishing methods and fyke netting to produce reliable abundance an size metrics. Journal of *Freshwater Ecology*. 28(4): 585-590

RATNASINGHAM S, Hebert PDN. 2013. A DNA-based registry for all animal species: the barcode index number (BIN) system. *PLoS One*. 8(7):e66213

ROSLIN T, Majaneva s. 2016. The use of DNA barcodes in food web construction-terrestrial and aquatic ecologists unite! *Genome*. 59(9):603-628

ROSSBERG AG, Uusitalo L, Berg T, Zaiko A, Chenuil A, Uyarra MC, Borja A, Lynam CP. 2017. Quantitative criteria for choosing targets and indicators for sustainable use of ecosystems. *Ecological Indicators*. 72:215-224

RUPPERT KM, Kline RJ, Rahman MS. 2019. Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. *Global Ecology and Conservation*. 17:e00547

SANTORO AE. 2009. Microbial nitrogen cycling at the saltwater-freshwater interface. *Hydrogeology Journal*. 18(1):187-202

SCHINDLER DE, Smits AP. 2017. Subsidies of aquatic resources in terrestrial ecosystems. *Ecosystems*. 20:78-93

SCHNELL IB, Thomsen PF, Wilkinson N, Rasmussen M, Jensen LRD, Willerslev E, Bertelsen MF, Gilbert MTP. 2012. Screening mammal biodiversity using DNA from leeches. *Current Biology*. 22(8): R262–R263

SCHNELL IB, Sollmann R, Calvignac-Spencer S, Siddall ME, Yu DW, Wilting A, Gilbert MTP. (2015). iDNA from terrestrial haematophagous leeches as a wildlife surveying and monitoring tool – Prospects, pitfalls and avenues to be developed. *Frontiers in Zoology*. 12(1): 24

SCHNELL IB, Bohmann K, Schultze SE, Richter SR, Murray DC et al. 2018. Debugging diversity‐A Pan continental exploration of the potential of terrestrial blood-feeding leeches as a vertebrate monitoring tool. *Molecular Ecology Resources*. 18(6): 1282-1298

SEIFERT KA. 2009. Progress towards DNA barcoding of fungi. *Molecular Ecology Resources*. 9(s1):83-89

SHERWOOD AR, Dittbern MN, Johnston ET, Conklin KY. 2017. A metabarcoding comparison of windward and leeward airborne algal diversity across the Ko'olau mountain range on the island of O'ahu Hawai'i. *Journal of Phycology*. 53(2): 437-445

SOININEN J, Bartels P, Heino J, Luoto M, Hillebrand H. 2015. Toward more integrated ecosystem research in aquatic and terrestrial environments. *BioScience*. 65(2):174-182

SRIVATHSAN A, Sha JCM, Vogler AP, Meier R. 2015. Comparing the effectiveness of metagenomics and metabarcoding for diet analysis of a leaf-feeding monkey (*Pygathrix nemaeus*). *Molecular Ecology Resources*. 15(2):250-261

SUN S, Bdgley BD. 2019. Changes in microbial functional genes within the soil metagenome during forest ecosystem restoration. Soil Biology and *Biochemistry*. 135: 163-172

SUNDERLAND KD. 1988. Quantitative methods for detecting invertebrate predation occurring in the field. *Annals of Applied Biology*. 112(1): 201-224

SYMONDSON WOC. 2002. Molecular identification of prey in predator diets. *Molecular Ecology*. 11(4):627-641

TABERLET P, Coissac E, Pompanon F, Brochmann C, Willerslev E. 2012. Towards next-generation biodiversity assessment using DNA metabarcoding. *Molecular Ecology*. 21(8):2045-2050

THOMSEN PF, and Wilerslev E. 2015. Environmental DNA – an emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*. 183:4-18

TREONIS AM, Unangst SK, Kepler RM, Buyer JS, Cavigelli MA, Mirsky SB, Maul JE. 2018. Characterization of soil nematode communities in three cropping systems through morphological and DNA metabarcoding approaches. Scientific *Reports*. 8:2004

VAN DER HEIJDEN MGA, Bardgett RD, van Straalen NM. 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters*. 11(3): 296-310

VĚTROVSKÝ T, Kohout P, Kopecký M. et al. 2019. A meta-analysis of global fungal distribution reveals climate-driven patterns. *Nature Communication*. 10:5142

WHITFORD WG, Rapport DJ, deSoyza AG. 1999. Using resistance and resilience measurements for ‘fitness’ tests in ecosystem health. *Journal of Environmental Management*. 57(1): 21-29

WIRTA HK, Hebert PDN, Kaartinen R, Prosser SW, Várkonyi G, Roslin T. 2014. Complementary molecular information changes our perception of food web structure. *Proceedings of the National Academy of Sciences of the United States of America*. 111(5): 1885-1890

XU S-Z, Li Z-Y, Jin X-H. 2017. DNA barcoding of invasive plants in China: A resource for identifying invasive plants. *Molecular Ecology Resources*. 18(1):128-136

YANG C, Wang X, Miller JA, de Blécourt M, Ji Y, Yang C, Harrison RD, Yu DW. 2014. Using metabarcoding to ask if easily collected soil and leaf-litter samples can be used as a general biodiversity indicator. *Ecological Indicators*. 46:379-389

YOCCOZ NG, Brathen KA, Gielly L et al. 2012. DNA from soil mirrors plant taxonomic and growth form diversity. Molecular Ecology. 21(5) 3647-3655.

ZINGER L, Taberlet P, Schimann H et al. 2019. Body size determines soil community assembly in a tropical forest.

Think-Piece 4: The contribution of DNA-based methods to achieving socio-ecological resilience

Douglas W. Yu[1] and Eleni Matechou[2]

[1] School of Biological Sciences, University of East Anglia, Norwich, UK

[2] School of Mathematics, Statistics and Actuarial Science, University of Kent, Kent, UK

Contents

Tables and Figures

Summary

Lay summary

Socio-ecological resilience is the ability of human institutions to bolster ecosystem resistance to, and recovery from, undesired change. For instance, after a dieback event, forestry professionals could accelerate recovery by replanting a forest with a mix of species that would be more resistant to future pest outbreaks. Dietz et al. (2003) have outlined five elements to build socio-ecological resilience (Table 3). There are two revolutions that are making it more feasible than ever before to achieve the first two elements (Knowledge Generation and Capacity Building).

The first revolution is technological: our rapidly growing ability to use DNA sequencing and/or electronic sensors (drones, satellites, camera traps, and sound recorders) to detect large numbers of species over large numbers of sites and time points. In short, we can now routinely generate high-dimensional data.

The second revolution is analytical: new statistical methods for analysing high-dimensional data, to explain the distribution of species as a result of visible and hidden environmental conditions, local dispersal, and inferred biological interactions, such as predation and competition.

In the long term, we think it will be possible to use high-resolution airborne and satellite remote-sensing imagery to measure the environment in detail, and to combine those images with high-dimensional, point-sample datasets generated using DNA and sensors, to map and track biodiversity continuously over whole landscapes. The contrast with current practice, where restricted lists of 'indicator species' are occasionally censused in small numbers of sites, is the difference between paper maps and satellite navigation.

However, all data sources inevitably include error. In this Think Piece, we survey the kinds of error that arise in DNA-based data, and the available methods that can remove or compensate for that error. Our focus is on estimating abundance, assigning taxonomic identity, and the twin problems of false-negatives and false-positives (failing to detect a species that is indeed present and incorrectly detecting a species that is not truly present).

Finally, we briefly survey six Wildlife Indicators from the UK's 25 Year Environment Plan, and we outline some strategies for combining DNA-based data, remote-sensing layers, and statistical analysis to generate these indicators.

Executive summary

Socio-ecological resilience is the ability of human institutions to bolster ecosystem resistance to, and recovery from, undesired change. For instance, after a dieback event, forestry professionals could accelerate recovery by replanting a forest with a mix of species that would be more resistant to pests. Dietz et al. (2003) have outlined five elements to build socio-ecological resilience: (1) Knowledge Generation, (2) Political

Bargaining, (3) Enforcement, (4) Technological Capacity Building, and (5) Institutional Design and Adaptive Governance. In recent years, two major technical innovations have occurred that together show great promise in helping to lower barriers to achieving socio-ecological resilience, especially in the first element of Knowledge Generation. The first is our growing ability to rapidly generate community inventories via electronic sensors and via high-throughput DNA sequencers. The second is the emergence of new statistical methods for analysing raw biodiversity data, in particular multi-species occupancy models (MSOMs) and (latent-variable) joint species distribution models (jSDMs). **Together, the new technologies and the new statistics make it increasingly feasible to generate high-quality, trustworthy, granular, timely, and understandable information on ecosystem state and change, on levels of uncertainty, and on how human activities affect ecosystem functions and services**.

However, DNA-based data inevitably include error. In this Think Piece, we survey uncertainties in DNA-based data and the laboratory, bioinformatic, and statistical methods that can remove or compensate for error. We focus on errors in abundance estimation, taxonomic assignment, and observation.

For abundance estimation, it is useful to separate within-species from across-species quantification. Within-species quantification means that changes in the abundances of individual species can be tracked from sample to sample, where the samples typically represent a time series or an environmental gradient. This form of quantification contains most of the information needed for community analysis. Across-species quantification allows within-sample estimates of species frequencies, which is required for diet analysis. Methods for achieving within-species and/or across-species quantification include: high-throughput individual barcoding, better PCR primers, quantitative PCR, Unique Molecular Identifiers, and forward and reverse metagenomics.

For taxonomic assignment, errors in query sequences and in reference databases cause paired false-positive and false-negative errors, since incorrect species take the place of correct species. There are multiple, overlapping solutions to this kind of error, including continued expansion of reference databases, curation of reference databases for individual studies, the use of multiple loci, organellar genomes, and whole genome skims for species delimitation, and the PROTAX statistical wrapper to remove assignment bias from existing assignment methods.

For observation, DNA-based methods reduce but do not eliminate species-detection failure (false-negative observation error) and increase the probability of false-positive observation error. Because repeat sampling is typically easier with DNA-based methods, occupancy modelling is very appropriate for correcting for false-negatives, and recent methodological advances are now making it possible to simultaneously correct for false-positives as well. DNA-based data are typically also ‘wider’ than traditional survey methods, meaning that more species are observed per sample. Multi-species occupancy models (MSOMs) can take advantage of these data by allowing rare species to borrow statistical strength from more abundant species, thus allowing more robust occupancy estimates. Joint species distribution models (jSDMs) use species correlations to infer unobserved environmental variables and/or species interactions. MSOMs and jSDMs

provide an excellent foundation but, so far, stop short of providing the required tools because of the need to account for multiple layers of observation error in DNA-based surveys when analysing metabarcoding data. We recommend research investment to combine MSOMs and jSDMs and adapt them to DNA-based data.

Finally, we briefly survey six Wildlife Indicators from Defra's 25 Year Environment Plan, and we outline some strategies for combining DNA-based methods, remote-sensing layers, and MSOMs/jSDMs to generate these indicators, including the inference of metacommunity dynamics, the efficient survey of rare but endangered wildlife species, and the real-time monitoring of ecosystem change via parameterised models to interpret remote-sensing data.

Recommendations for Future Focus

1. Building reference databases for key taxa, such as the freshwater invertebrate taxa used in water-quality biomonitoring.
2. Research on applying multi-species occupancy models (MSOMs) and joint species distributions models (jSDMs) to DNA-based and sensor-based point-sample data, with special attention paid to pipeline additions (e.g. spike-ins) and sampling designs (e.g. repeated samples) that facilitate the inference and removal of errors, especially those that are more likely to occur in DNA-based data, including OTU splitting and lumping, taxonomic misassignment, and loss of across-species abundance data.
3. Re-design of 25YEP Wildlife Indicators to exploit the new data sources and analytical techniques.

Introduction

This Think Piece starts with the assumption that our collective goal as environmental scientists and policymakers is to achieve ***socio-ecological resilience***. *Ecological resilience* is an ecosystem's natural ability to resist and to recover from a perturbation (Isbell et al. 2011). For instance, trees defend against insect herbivores, and a forest regrows after a pest outbreak. *Socio-ecological resilience* is the ability of human actors to bolster ecosystem resistance to, and recovery from, undesirable change (Walker et al. 2004; Hodgson et al. 2015; Spears et al. 2015). This recovery could be to a previous state (*restoration*) or to an alternative state with a different species composition that equals some of the, or even exceeds, previous levels of ecological functioning (*rewilding*) (du Toit and Pettorelli 2019).

Socio-ecological resilience thus builds on ecological resilience by adding *human institutions* and by changing the focus from species composition to *ecosystem functioning*. For simplicity, we use Dietz et al.'s (2003) definition of human institutions as "*ways of organising activities*," which can range from something as simple as a hiking club to something as complex as an international treaty. And we follow GEOBON (2018) in defining ecosystem functioning as "*the collective life activities of plants, animals, and microbes and the effects these activities (e.g. feeding, growing, moving, excreting waste) have on the physical and chemical conditions of their environment*." Ecological functions themselves are the "*biological, geochemical and physical processes that take place or occur within an ecosystem*." Thus, after a tree dieback event, the human institution of professional forestry can plant trees to accelerate the recovery of wood biomass and wildlife habitat, and moreover, foresters might choose to alter the mix of ecological functions and services by planting tree species that are more resistant to pest outbreaks and provide better wildlife habitat and space for recreation, but produce less marketable timber (or the converse). An additional human institution would be to add consultation and conflict-resolution processes to choose the mix of functions of services.

We still have many knowledge gaps regarding the mechanisms of ecological resilience (Oliver et al. 2015), the existence and detection of thresholds (Dakos et al. 2015), and the severity and form of future environmental stressors. However, we have over time collectively built up large and growing knowledge bases on management actions that can restore biodiversity and ecological functioning (www.conservationevidence.com, accessed 14 Dec 2019) and on the 'governance tools' that can deliver those actions (Dietz et al. 2003). In their seminal review, Dietz et al. (2003) outlined the necessary ingredients for achieving socio-ecological resilience, which we summarise here into five elements (1) Knowledge Generation, (2) Political Bargaining, (3) Enforcement, (4) Technological Capacity Building, and (5) Institutional Design and Adaptive Governance (Table 3).

Table 3 Five elements to build socio-ecological resilience (Dietz et al. 2003)

Elements	Action
Knowledge Generation	Produce high-quality, trustworthy, granular, timely, and understandable information on ecosystem state and change, on levels of uncertainty, and on how human activities affect ecosystem functions and services
Capacity Building	Provide the technological and analytical infrastructure to efficiently generate the required information and to efficiently induce compliance
Political Bargaining	Resolve conflict over management alternatives
Enforcement	Induce compliance with management rules
Institutional Design & Adaptive Governance	Implement the above four steps and learn from their outcomes. Create polycentric (overlapping and nested) human institutions to do so

Successfully achieving even one of these five elements poses huge scientific and managerial challenges, and the first element alone, Knowledge Generation, defines the field of conservation biology. However, in recent years, two major technical innovations have occurred that together show great promise in helping to lower barriers to achieving socio-ecological resilience.

The first development is our growing ability to rapidly generate community inventories via electronic sensors such as drones, satellites, camera traps, and sound recorders and via high-throughput DNA sequencers. Coupled with appropriate algorithms and databases, these methods can generate taxon lists quickly and with less dependence on trained observers. The efficiency gains are such that hundreds or even thousands of species can now be detected and quantified (to an extent) in hundreds or even thousands of samples. We have entered the age of Big Community Data.

The second is the emergence of new statistical methods for analysing community and metacommunity data, in particular multi-species occupancy models (MSOMs) and (latent-variable) joint species distribution models (jSDMs). MSOMs allow estimation of the probability of presence at each surveyed site for all species in the assemblage and, hence, inference on the number of undetected species (Dorazio and Royle 2005; Gelfand et al. 2005; Guillera-Arroita et al. 2019; Tingley et al. 2020). jSDMs allow an explicit and flexible explanation of community composition by species' environmental preferences, as well as biotic interactions and spatial autocorrelation (Warton et al. 2015; Bush et al. 2017; Ovaskainen et al. 2017).

Together, the new technologies and the new statistics make it increasingly feasible to generate high-quality, trustworthy, granular, timely, and understandable information on ecosystem state and change, on levels of uncertainty, and on how human activities affect ecosystem functions and services. Community compositions are a measure of ecosystem state, and the increased efficiencies make it feasible to sample repeatedly along environmental and anthropogenic gradients with temporal and spatial granularity, facilitating timely detection of change, estimates of uncertainty, and inference of human effects. It is also possible to infer relationships between community composition and ecological functions and services, such as by sequencing diets of predators and pollinators, by using taxonomic identities to link to our existing storehouse of functional biological knowledge (Janzen et al. 2005), and by motivating follow-up observational and experimental tests that link biodiversity composition with ecological functioning. Point samples can be combined with continuous environmental data layers to generate understandable maps of biodiversity distributions and their environmental drivers (Ovaskainen et al. 2016; Bush et al. 2017).

Given these prospects, our motivating question here is: **How can DNA-based methods help us achieve the Knowledge Generation element of socio-ecological resilience?** It is beyond the scope of this Think Piece to consider in depth the other four elements in Table 3, but we note that because methods are being standardised and raw data are published, third parties can audit or independently collect samples, making results more trustworthy and thus potentially making political conflict more resolvable and enforcement more efficient. Capacity for generating DNA-based data is also easier to build than are natural-history skills, since the labour pool and the underlying technologies draw from molecular biology, medicine, and computing.

DNA-based methods to generate community data and their uncertainties

Here we survey available DNA-based methods for extracting species information from environmental samples. We do not focus on the details of the methods themselves, since there have been several reviews that provide an entry to the methodological literature (Ji et al. 2013, Crampton-Platt et al. 2016, Creer et al. 2016, Bush et al. 2017, Deiner et al. 2017, Somervuo et al. 2017, Axtner et al. 2019, Greenfield et al. 2019, Peel et al. 2019, Piper et al. 2019, Taberlet et al. 2019). **Instead, our interest is in the uncertainties that exist in DNA-based data and the methods that can be applied to remove or at least quantify those uncertainties**. Also, given our goal of applying DNA-derived information to the wildlife indicators in the 25 YEP, we limit our survey to eukaryotic organisms, especially animals and plants.

Bulk-sample vs. environmental DNA

The starting point is the sample, which we divide into two fundamental types: *bulk-sample DNA* and *environmental DNA* (eDNA). Bulk-sample DNA is extracted from whole or nearly whole organisms, comprising many different species. The most common type of bulk sample is mass-collected invertebrates or meiofauna, which are captured with passive (Malaise, pitfall, pan, Berlese, Winkler, flight-intercept, flotation, lure, and light traps) and active methods (sweep and kick netting, insecticide fogging, and vegetation beating). Other taxa that can be sampled in bulk include vascular plants, plankton, diatoms, and algae (Hiiesalu et al. 2012; Visco et al. 2015; Zeng et al. 2015; Pawlowski et al. 2016; Sherwood et al. 2016; Deagle et al. 2018; Wangensteen et al. 2018).

By contrast, eDNA is derived from organismal fragments, including “skin, mucous, saliva, sperm, secretions, eggs, faeces, urine, blood, roots, leaves, fruit, pollen, and rotting bodies” (Bohmann et al. 2014; Bálint et al. 2018; Lacoursière-Roussel and Deiner 2019; Ruppert et al. 2019), which allows eDNA to be collected from water, sediment and soil, air, and from haematophagous and coprophagous invertebrates. The latter category is widely known as iDNA, originally for ‘ingested DNA’ but probably better translated as ‘invertebrate-collected DNA,’ to include vertebrate DNA picked up by contact, such as on the bodies of flies visiting scats (Schubert et al. 2014; Rodgers et al. 2017). [Also, while on terminology, bulk-sample DNA has been called ‘community DNA’ by Creer et al. (2016), but since one can extract community information from eDNA, we consider the term ‘bulk-sample DNA’ to be more direct.]

To our mind, the key distinction between bulk-sample and environmental DNA is that bulk-sample DNA provides a much finer spatial and temporal resolution of species detection: a species detected in a bulk sample was indeed present at the precise location and time of sample collection (albeit with the crucial assumption that one can rule out false-positive detections, see 6.2.2). In contrast, eDNA is transported away from the originating organism and can be preserved in sediment long after an organism has died (known as sedaDNA for sedimentary ancient DNA, Pedersen et al. 2015).

The transport and persistence of eDNA is precisely what makes it so powerful for species detection. For instance, DNA-containing faeces and mucous from a fish dissolves and diffuses through water, allowing the fish to be detected from just a water sample, even if the fish itself is never observed (Jerde et al. 2011; Jerde 2019). However, the downside is that the location and time of a fish's eDNA detection has been displaced from the location and time that the fish released the eDNA, with the absolute spatial and temporal displacement determined by the balance between the velocity of transport and the rates of degradation, settling, and resuspension (Shogren et al. 2017; Hansen et al. 2018). Thus, eDNA can be detected hundreds of metres (to multiple kilometres) downstream of their point sources (Deiner and Altermatt 2014; Jane et al. 2014), whereas fish eDNA detection rates in ponds and lakes drop to zero within tens to hundreds of metres of the source fish, depending in part on the degree of water mixing (Eichmiller et al. 2014; Li et al. 2019). Similarly, airborne pollen and spores could also be transported many kilometres from their originating organisms (Brown and Hovmøller 2002; Brennan et al. 2019), and vertebrate iDNA extracted from a leech can be derived from feeding events up to weeks before leech collection, during which time the host vertebrate could have moved away from the collecting site (Schnell et al. 2012, 2015). *The common effect of these displacements is to reduce the spatial and temporal resolution of eDNA-mediated species detections*, which has a direct consequence for the assumption of closed populations in site-occupancy modelling (Schnell et al. 2015).

Amplicon sequencing

Once samples are in hand, the next challenge is that any genetic sequence used for taxonomic identification, known as the 'marker,' represents only a tiny fraction of the total DNA in a sample. For example, the standard 'DNA-barcode' sequence, which is 658 base pairs of the mitochondrial Cytochrome Oxidase subunit I gene (COI), represents only ~0.01% of the total genomic DNA in bulk arthropod samples (Ji et al. 2019), and Turner et al. (2014) estimated that total mitochondrial DNA per fish species represents only 0.0004% or 0.0000004% of total eDNA in pond and lake water samples, respectively. Similarly, the commonly used 12S 'MiFish' marker for fish (Miya et al. 2015) is only 163-185 bp long, or ≤1% of the fish mitochondrial DNA. The rest of the genomic DNA in eDNA samples is mostly bulk-sample bacterial, fungal, and plant DNA, or in the special case of iDNA, the DNA of the invertebrate collector.

Given this, the dominant approach to extracting species-composition information from bulk-sample and environmental DNA is to use a method called 'amplicon sequencing.' In short, PCR (polymerase chain reaction) is used to make many copies of ('to amplify') the rare target marker, and it is this product, known as the 'amplicon,' that is sequenced on a high-throughput sequencer, most commonly, those manufactured by Illumina (illumina.com). The markers are then clustered by sequence similarity, typically at a level considered to approximate biological species. These clusters are known as Operational Taxonomic Units, or OTUs, and the OTUs are then assigned taxonomies after comparison with reference sequences held in large, online databases.

PCR uses two short DNA 'primer' sequences that are designed to bind directly upstream and downstream of the target marker sequence, and this is what directs the DNA

polymerase enzyme to copy the target. Amplicon sequencing imposes several requirements, of which three key ones are:

1. The primer binding regions should be evolutionarily conserved, that is, similar across all the species within the target taxon (e.g. fish, mammals, arthropods, vertebrates, diatoms, plants, eukaryotes) so that one pair of primer sequences can bind to and thus amplify all the target species present in a sample.
2. In contrast, the marker region *between* the binding regions should be evolutionarily labile, evolving at just the right rate so that it varies a lot *across* species but varies little *within* species.
3. The amplicon cannot be longer than what the sequencer can sequence through. In the case of Illumina, the marker plus primers cannot be longer than ~550 bp. For instance, the most widely used COI primers are 26 bp each and amplify a marker region of 313 bp (Leray et al. 2013), for a total amplicon of 365 bp.

It should not be a surprise that the first two requirements are never perfectly met, especially given the third, Illumina-imposed length constraint. These and other sources of error in amplicon-sequencing pipelines (Table 4) collectively result in *false negatives* (failures to detect target taxa that in fact are in the sample: 'drop-outs'), *false positives* (taxon detections that are in fact artefacts: 'drop-ins'), *poor quantification* of target-taxa abundances or biomasses, and *incorrect assignment of taxonomies* to sequences, which in turn also result in false positives and false negatives.

Table 4 Four classes of metabarcoding errors and their causes. Not included are software errors caused by incorrect usage, bugs, or algorithmic mistakes and general laboratory and field errors like mislabelling, DNA degradation, and sampling biases or inadequate effort. Reprinted from Yang et al. (2020)

Main Errors	Possible Causes	References
False positives ('Drop-ins,' OTU sequences in the final dataset that are not from target taxa)	Sample contamination in the field or lab	Champlot et al. 2010; De Barba et al. 2014
	PCR errors (substitutions, indels, chimeric sequences)	Deagle, Jarman, Coissac, Pompanon, & Taberlet, 2014
	Sequencing errors	Eren, Vineis, Morrison and Sogin, 2013

	Incorrect assignment of sequences to samples (‘tag jumping’)	Esling, Lejzerowicz and Pawlowski, 2015; Schnell, Bohmann and Gilbert, 2015
	Intraspecific variability across the marker leading to multiple OTUs from the same species	Bohmann et al. 2018; Virgilio, Backeljau, Nevado and De Meyer, 2010
	Incorrect classification of an OTU as a prey item when it was in fact consumed by another prey species in the same gut	Hardy et al. 2017
	Numts (nuclear copies of mitochondrial genes)	Bensasson, 2001
False negatives (‘Drop-outs,’ failure to detect target taxa that are in the sample)	Fragmented DNA leading to failure of PCR to amplify	Ziesemer et al. 2016
	Primer bias (interspecific variability across the marker)	Alberdi, Aizpurua, Gilbert, and Bohmann, 2018; Clarke, Czechowski, Soubrier, Stevens and Cooper, 2014; Piñol, Mir, Gomez-Polo and Agustí, 2015
	PCR inhibition	Murray, Coghlan and Bunce, 2015
	PCR stochasticity	Piñol et al. 2015
	PCR runaway	Polz and Cavanaugh, 1998

	Predator and collector DNA dominating the PCR product and causing target taxa (e.g. diet items) to fail to amplify	Deagle, Kirkwood and Jarman, 2009; Shehzad et al. 2012
	Too many PCR cycles in the metabarcoding PCR leading to loss of sequences with low starting DNA	Piñol et al.2015
Poor quantification of target species abundances or biomasses	PCR stochasticity	Deagle, Jarman, Coissac, Pompanon and Taberlet, 2014
	Primer bias	Piñol et al. 2015; Piñol et al. 2019
	Polymerase bias	Nichols et al. 2018
	PCR inhibition Too many cycles in the metabarcoding PCR	Murray et al. 2015
Taxonomic assignment errors (a class of error that can result in false positives or negatives, depending on the nature of the error)	Intra-specific variability across the marker leading to multiple OTUs with different taxonomic assignments Incomplete reference databases	Clarke, Soubrier, Weyrich and Cooper, 2014

Nonetheless, starting a decade ago (Fonseca et al. 2010; Hajibabaei et al. 2011; Hiiesalu et al. 2012; Thomsen et al. 2012; Yoccoz et al. 2012; Yu et al. 2012; Ji et al. 2013), many studies have now shown that amplicon-sequencing pipelines and morphology-based surveys produce highly correlated data on species distributions, with the prospect that DNA-based data will result in management decisions that are as or more reliable as those made using conventional surveys, given best-practice protocols (Hering et al. 2018; Ruppert et al. 2019), while costing much less to produce.

Here are three examples from a burgeoning literature.

1. Ji et al. (2013) and Edwards et al. (2014) showed that amplicon sequencing of arthropod bulk samples and morphologically identified tropical-forest indicator species (e.g. dung beetles, spiders, ants, and birds) both came up with very similar policy conclusions for a restoration experiment and for a systematic conservation planning exercise.
2. Lejzerowicz et al. (2015), Cordier et al. (2017), Pawlowski et al. (2016), and Aylagas et al. (2018) amplicon-sequenced bulk samples of metazoans or of foraminifera sampled from the marine benthos. Since taxonomies of marine-benthic species are incomplete, both morphological analyses and DNA-reference databases assign taxonomies mostly at higher ranks (e.g. phylum, class, order, family, morphospecies), and environmental-quality indices are calculated with these taxonomies. Despite low taxonomic overlap between the morphologically identified and amplicon-sequence datasets, these studies showed that the two methods produced strongly correlated environmental-quality indices used for classifying pollution severity in marine waters.
3. Perhaps most impressive, multiple studies have demonstrated high correspondence between eDNA-derived data and conventional abundance surveys of marine and freshwater fish. The tightest correlations between eDNA copy numbers and fish counts have been produced by species-specific quantitative PCR (Lacoursière Roussel et al. 2016; Doi et al. 2017; Levi et al. 2019; Salter et al. 2019; Pochardt et al. 2020), but amplicon-sequencing datasets have also shown positive correlations of eDNA read proportions or counts with estimates of occupancy and fish biomass (Hänfling et al. 2016; Thomsen et al. 2016; Lawson Handley et al. 2019). Local accuracy is enhanced by apparently rapid eDNA degradation and/or settling rates in water (Thomsen et al. 2012; Shogren et al. 2017; Li et al. 2019).

These and many other studies comparing conventional with amplicon-sequencing datasets have motivated a complementary line of research to design field, laboratory, and bioinformatic pipelines that extract more information while preventing and mitigating errors (e.g. Bengtsson-Palme et al. 2015; Murray et al. 2015; Boyer et al. 2016; Callahan et al. 2016; Zepeda Mendoza et al. 2016; Heeger et al. 2018; Axtner et al. 2019; Curd et al. 2019, https://github.com/VascoElbrecht/JAMP, accessed 15 Dec 2019). The methodological details of these pipelines are beyond the scope of this review, but two recent and magnificent surveys are Piper et al. (2019) and Taberlet et al. (2019).

Abundance estimation in amplicon sequencing

Two key dimensions of ecological community structure are composition and abundance (Vellend 2010). While the studies referenced in the previous section, plus many others, demonstrate that DNA-based data can now reliably extract species *composition* information (which species are present in which samples) from bulk-sample and environmental DNA, the same studies also show that species *abundances* are much less accurately estimated.

This is not surprising, given that species differ in body size, genome size, mitochondrial copy number, DNA extraction efficiency, PCR amplification efficiency, and what we might call survival probability in bioinformatic pipelines (Amend et al. 2010; Yu et al. 2012; Piñol et al. 2015, 2018; Bell et al. 2017; Elbrecht et al. 2017; McLaren et al. 2019; Pauvert et al. 2019). For instance, closely related species might be clustered together into one species,

under some parameter choices (Pauvert et al. 2019). With eDNA, we can add species differences in shedding, transport, and degradation rates. For example, Levi et al. (2019) reported that small, so-called ‘jack’ coho salmon appear to be less detectable by qPCR of aquatic eDNA than are normal-sized coho adults.

Furthermore, there is also considerable noise in amplicon-sequencing pipelines, partly caused by the inherent stochasticity of PCR and also because several steps along the wet-lab pipeline likely result in ‘species drift,’ as only small aliquots are passed from one step to the next: extracted DNA to PCR, PCR amplicon to sequencing library prep, and libraries to sequencing plates.

Sequencing runs also differ in their output and quality (Ji et al. 2019). Finally, even though samples differ in their absolute DNA biomasses, it is typical for researchers and sequencing centres to equalise the amount of sequence data per sample, which is typically achieved by equalising the amount of input DNA per sample that is added to the sequencer (known as “pooling samples in equimolar concentration” after the library prep stage). (N.B. all these sources of error are in addition to errors at the field sampling stage.)

The outcome of species-specific biases, pipeline noise, and sample equalisation is that **read numbers per OTU in a sample are not accurate measures of species biomass or abundance in those samples** (McLaren et al. 2019). At this point, it is useful to differentiate two kinds of quantification error: *within*-species and *across*-species.

1. *Within-species* quantification error means that if species A is represented by 200 reads in sample 1 and by 100 reads in sample 2, it is not reliable to conclude that species A is twice as abundant in sample 1 than in sample 2 (Gloor et al. 2017), since it might just be the case that *sample* 1 generated twice as many reads as sample 2, due to some combination of pipeline noise (creating artefactual differences across samples) and sample equalisation (erasing true differences across samples). It is not possible therefore to accurately track, say, how a species’ biomass changes across a set of samples representing a time series or an environmental gradient.

This might seem to be fixable with rarefaction, equalising total read numbers across samples by random subsampling, but rarefaction is an ‘inadmissible’ method in the words of McMurdie and Holmes (2014) because if the distribution of species read counts differs across samples, which they will always do, the samples with longer tails will lose more species (Willis 2019).

2. *Across-species* quantification error means that if species A is represented by 200 reads and species B is represented by 100 reads *in the same sample*, it is not reliable to conclude that species A has twice the biomass or abundance as species B in the sample, due to species-specific biases along the amplicon pipeline (Figure 13).

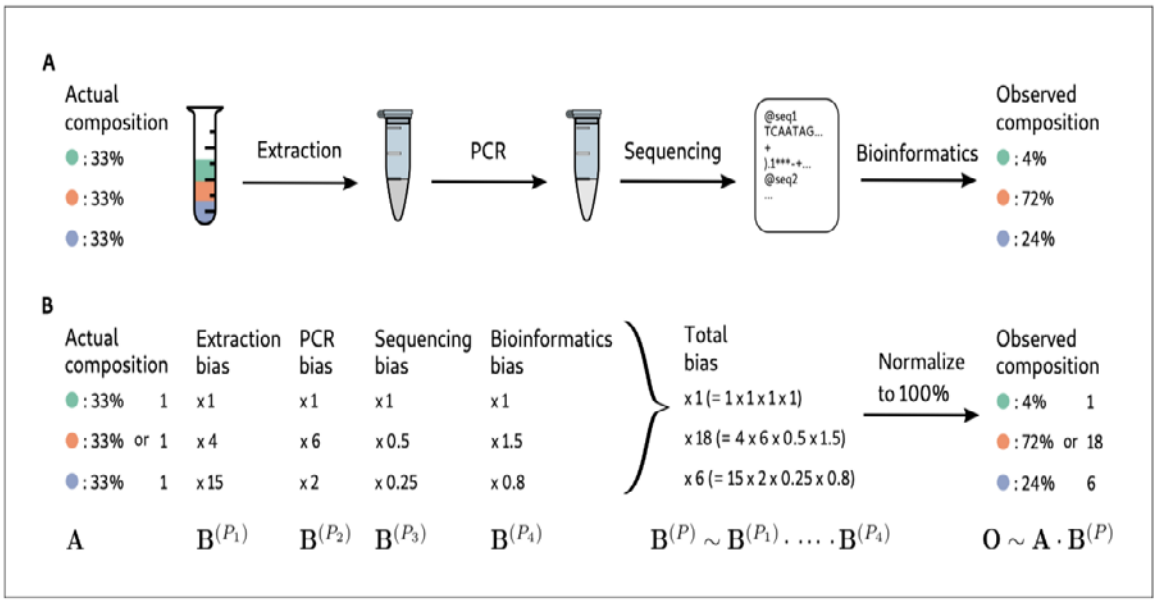

Figure 1. Bias arises throughout an MGS workflow, creating systematic error between the observed and actual compositions. Panel **A** illustrates a hypothetical marker-gene measurement of an even mixture of three taxa. The observed composition differs from the actual composition due to the bias at each step in the workflow. Panel **B** illustrates our mathematical model of bias, in which bias multiplies across steps to create the bias for the MGS protocol as a whole.

DOI: https://doi.org/10.7554/eLife.46923.002

Figure 13 Bias in amplicon sequencing pipelines. From McLaren et al. (2019). Reproduced under the article's CC-BY license (https://elifesciences.org/terms)

So how can we extract quantitative information from DNA-based data? Several approaches have been proposed.

High-throughput individual barcoding. The most straightforward way to estimate abundances is to barcode individual organisms separately and just count them up. This approach assumes that the target taxon has body sizes large enough that individuals can be sorted and their DNA separately extracted and tracked. The basic idea is to separately PCR each individual and tag it uniquely before pooling and sequencing *en masse* on an Illumina (Meier et al. 2016; Creedy et al. 2019; Ratnasingham 2019) or PacBio (Hebert et al. 2018), with costs <<$1 per sample if large numbers are processed. The output is a table of species counts by sample. Bees are an excellent candidate taxon for this method (Creedy et al. 2019; Gueuning et al. 2019). However, many taxa are some combination of being too small, numerous, soft-bodied, or primarily sampled via eDNA, which makes this method unsuitable. This method achieves both within-species and across-species quantification.

Design better PCR primers. In some cases, the target taxon is largely uniform in body size and DNA-extraction efficiency. In this case, it might be possible to design PCR primers that exhibit low species-specific amplification bias. For instance, nematodes are such a taxon, and Schenk et al. (2019) have reported that primers for the 28S D3-D5 and 18S V4

regions return species read frequencies that accurately recover species biomass frequencies and thus return across-species quantification within a sample. This method can achieve across-species quantification but not within-species quantification (i.e. it does not correct for sample-to-sample stochasticity).

Spike-in DNA. To correct for sample-to-sample stochasticity, researchers have advocated adding a fixed amount of a short DNA sequence to each sample between the digestion and the DNA extraction steps, a 'spike-in' sequence that does not match any species that could be in the samples and is flanked by primer binding sequences that match the primers used (Smets et al. 2016; Deagle et al. 2018; Tkacz et al. 2018). Because each sample receives the same amount of spike-in, all samples should return the same number of spike-in reads. However, this never happens, with some samples returning more spike-in reads, meaning that their datafile sizes are too large, and some samples returning fewer spike-in reads, meaning that their datafile sizes are too small. The correction step is simple: divide each sample's OTU sizes by the number of spike-in reads in that sample. Samples with a high number of spike-in reads must have had a low amount of template DNA, so dividing shrinks OTU sizes accordingly. This method corrects sample-to-sample stochasticity and thus achieves within-species quantification, but it does not remove species-specific biases, so across-species quantification is not achieved.

We can see the effect of spike-in correction in this unpublished dataset from Yu's group (Figure 14). Fifty two arthropod OTUs were individually DNA-extracted, and their COI gene copy numbers were quantified using qPCR. DNA from the 52 OTUs were then pooled so that each OTU was represented by the same amount of COI copy number, and a spike-in was added. The sample was then serially diluted six times (0.8 each step) to produce a 7-step dilution series.

In the top figure, each line represents one of the 52 OTUs, and within each OTU ('within-species'), we see a strong correlation between spike-corrected read number and the amount of input DNA (as measured by COI gene copy number), where each point is a different sample in the dilution series. Spike-correction thus achieves *within*-species quantification, allowing us to track how the (DNA) abundance of each OTU varies from sample to sample. However, each OTU also has a different intercept, meaning that the same (spike-corrected) read number predicts a different amount of input DNA for each species, even though the input DNA amounts were the same. These differences represent species-specific biases, especially PCR, along the amplicon-sequencing pipeline. Thus, spike-correction does not achieve *across*-species quantification, meaning that within a sample, we cannot use read numbers to compare species frequencies.

In the bottom figure, we show the same experiment, but without applying spike-in correction. Sample-to-sample stochasticity is high, and we cannot recover the dilution series. And of course, the different intercepts indicate the persistence of species-specific biases.

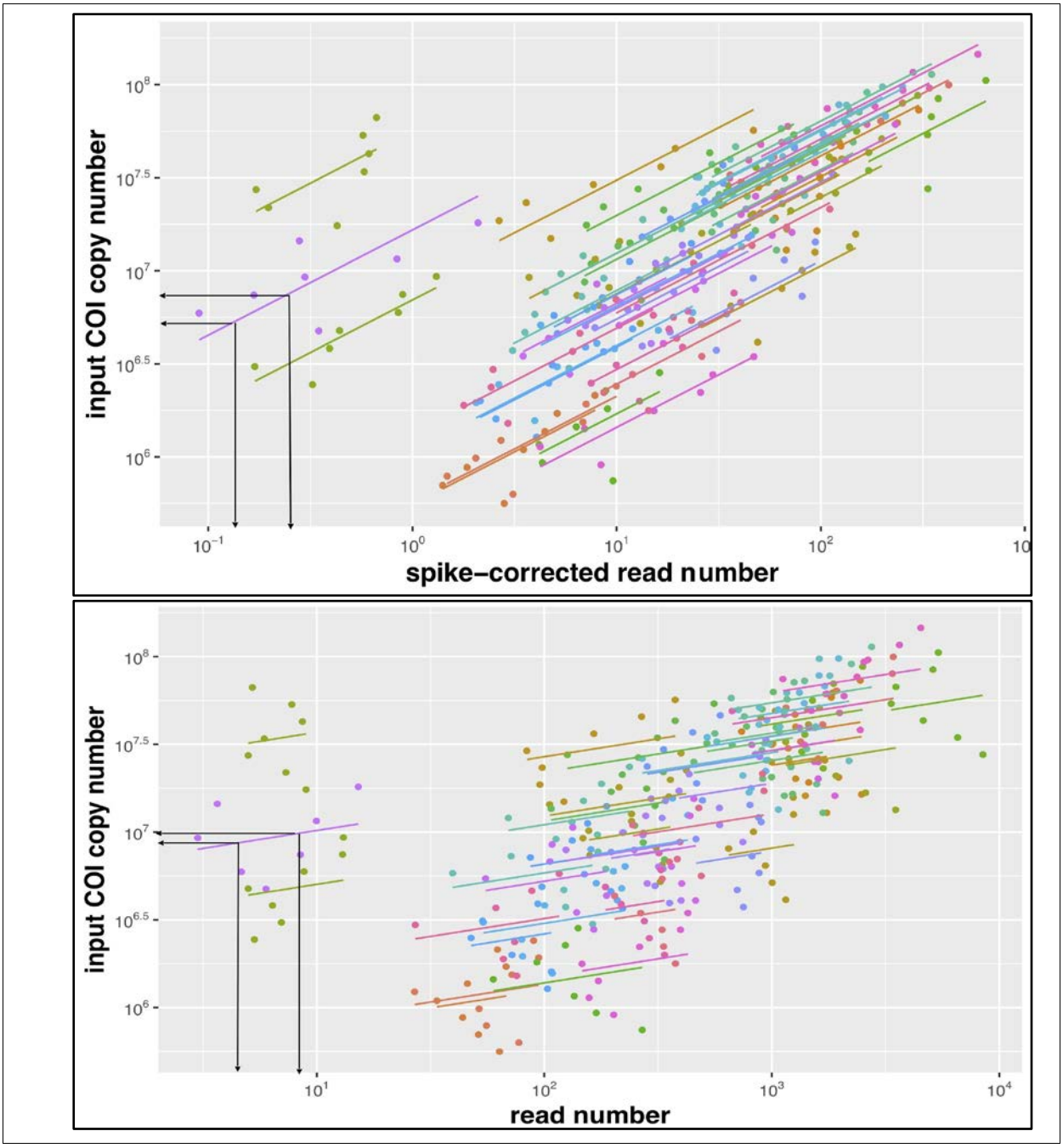

Figure 14 Improving quantification information using spike-in DNA (Top: with spike-in correction, Bottom: without spike-in correction)

qPCR. Quantitative PCR can be used to estimate the DNA concentration of one species per assay and is typically applied to aquatic eDNA. This paper will not review the application of qPCR to eDNA, except to note two recent developments. (1) Studies are now showing that it is possible to apply single-species qPCR to aquatic eDNA to extract usefully accurate information on within-species abundance change over space and time, provided that one corrects for water flux ((Figure 15) Cai et al. 2017; Carraro et al. 2018; Levi et al. 2019; Pochardt et al. 2020). (2) Williamson et al. (2019) have proposed using qPCR to estimate absolute abundances of a subset of species within amplicon-sequenced datasets, and using the observed ratios between that subset and the other species in the dataset to estimate absolute abundances for all species.

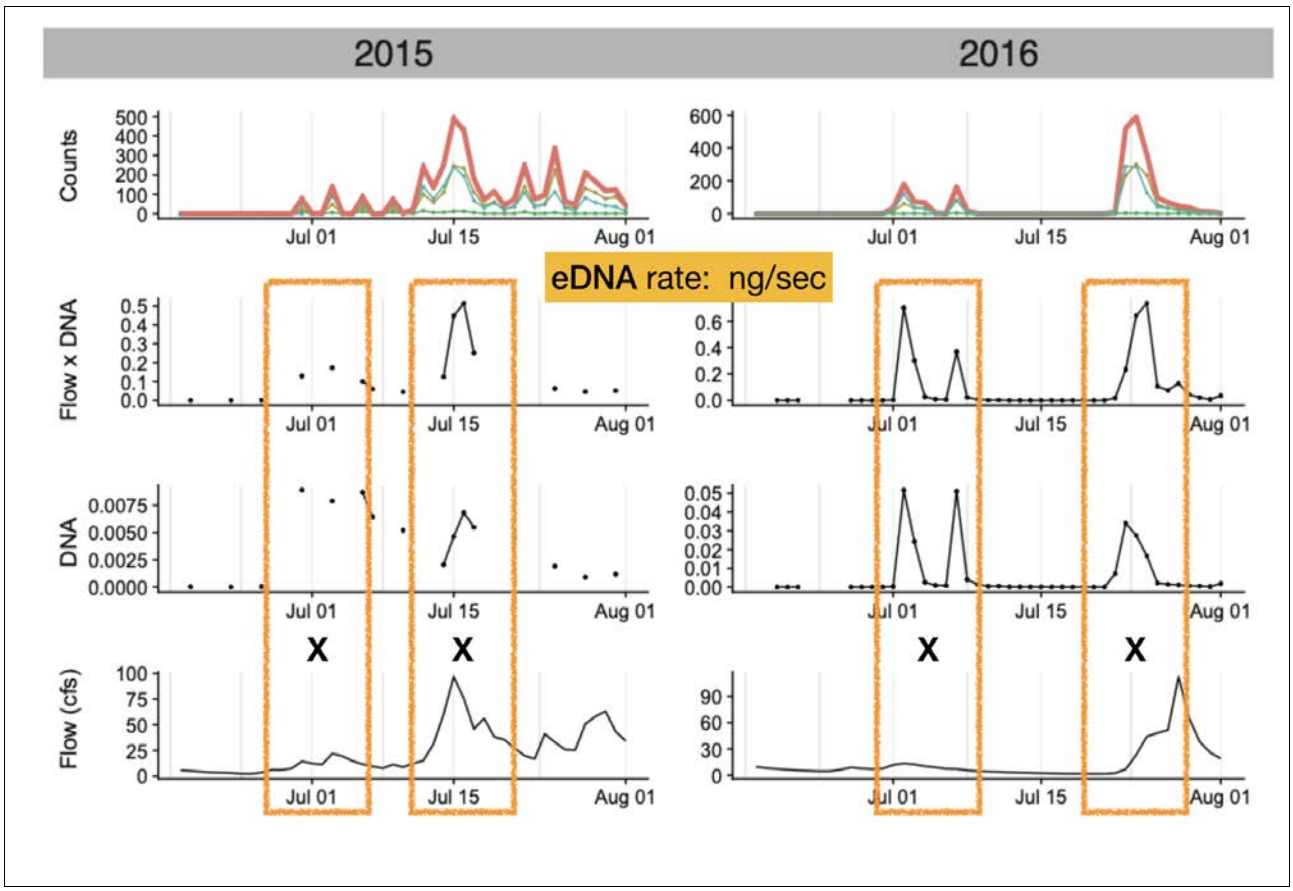

Figure 15 Accurate enumeration of salmon eDNA qPCR corrected for water flux. Timeline of adult sockeye salmon counts, flux-corrected eDNA rate (ng/µl x cfs), uncorrected eDNA concentration (ng/µl), and stream flux (cfs, cubic-feet/s). eDNA measures from consecutive days are connected by lines. Male and female salmon are denoted by yellow-brown and blue lines, respectively and jacks are denoted by green lines. Total adult sockeye salmon counts are denoted by thick red lines (Levi et al. 2019)

Unique Molecular Identifiers (UMIs). UMIs are an alternative method for quantifying species DNA frequencies in amplicon sequencing. A UMI is a series of ~8-12 random bases, denoted as 'NNNNNNNNNNN', which is typically inserted into the forward primer to act as an ultra-high diversity tag (Yourstone et al. 2014; Hoshino and Inagaki 2017; Fields et al. 2020; Karst et al. 2020). A stretch of, say, 12 Ns produces 4^{12} = 16 777 216 uniquely identified forward primer molecules. Species contributing abundant DNA to a sample will capture many primer molecules and thus amplify many different UMI sequences, while species contributing scarce DNA will amplify a low number of UMIs. The relationship between UMI richness and DNA abundance is roughly linear, but likely to asymptote for species with very high DNA abundance (unless UMI richness is also very high) (Hoshino and Inagaki, 2017).

After sequencing, a bioinformatic step clusters reads within each UMI (Yourstone et al. 2014; Chen et al. 2018). Since we expect each UMI to have captured only one template DNA molecule, all reads with the same UMI are expected to be identical, and any differences are attributed to PCR or sequencing error. The most abundant sequence within a UMI is the most likely to be correct and is used to represent that UMI (Fields et al. 2020). Finally, the UMI representative sequences (one per UMI) are clustered into OTUs,

and the size of each OTU should correlate with the starting amount of DNA per species in that sample. This method thus corrects PCR biases and partially achieves across-species quantification (in units of DNA mass per species). Other biases will still exist, such as differences in DNA-extraction efficiencies and mitochondrial densities. Also, UMIs do not correct for sample-to-sample stochasticity and thus do not achieve within-species quantification, but a spike-in can of course be applied at the same time. (N.B. UMIs should not be confused with the similar technique of using short nucleotide sequences to tag primers (Binladen et al. 2007) so that multiple PCR products can be pooled into one sequencing job).

Abundance estimation in metagenomic sequencing

Another way to eliminate species-specific biases caused by PCR is to avoid PCR altogether. In 2013, Zhou et al. (2013) showed that whole-genome-sequencing (WGS) datasets from Illumina sequencers (15.5 Gbp, ~77M reads) were large enough that 36 of 37 insect species in a mock sample could be detected by mapping the 100-bp-long reads to a reference dataset of their DNA-barcode sequences, which were only 658 bp long. Moreover, across-species quantification was partially achieved, with read number per species showing a positive, curvilinear relationship with species biomass (R^2 = 63%). This pioneering work presaged several papers mapping WGS reads to barcodes and to mitochondrial genomes (a kind of superbarcode, Crampton-Platt et al. 2015, 2016). For instance, Tang et al. (2015) mapped shotgun-sequenced bees to a set of mitogenomes and reported a linear-regression R^2 of 25%. Gómez-Rodríguez et al. (2015) mapped shotgun-sequenced leaf beetles to both mitogenomes and barcodes and reported an R^2 of 64% for a linear log-log regression, and Bista et al. (2018) mapped shotgun-sequenced freshwater invertebrates to mitogenomes and returned R^2 values between 45% and 87%, using logistic or linear models to fit different species.

Most recently, Ji et al. (2019) mapped shotgun-sequenced insects to mitogenomes and barcodes and achieved both high accuracy (mapping to barcodes R^2 = 93%, mitogenomes R^2 = 95%) and almost direct proportionality between mapped reads and input DNA-mass (slopes near 1 (0.9) on a log-log scale). The high accuracy was achieved by applying multiple correction factors: a spike-in to remove sample-to-sample stochasticity, a small number of technical replicates to control for sequencing-run stochasticity, and a percent-coverage filter to remove the small number of incorrectly mapped reads (Figure 16).

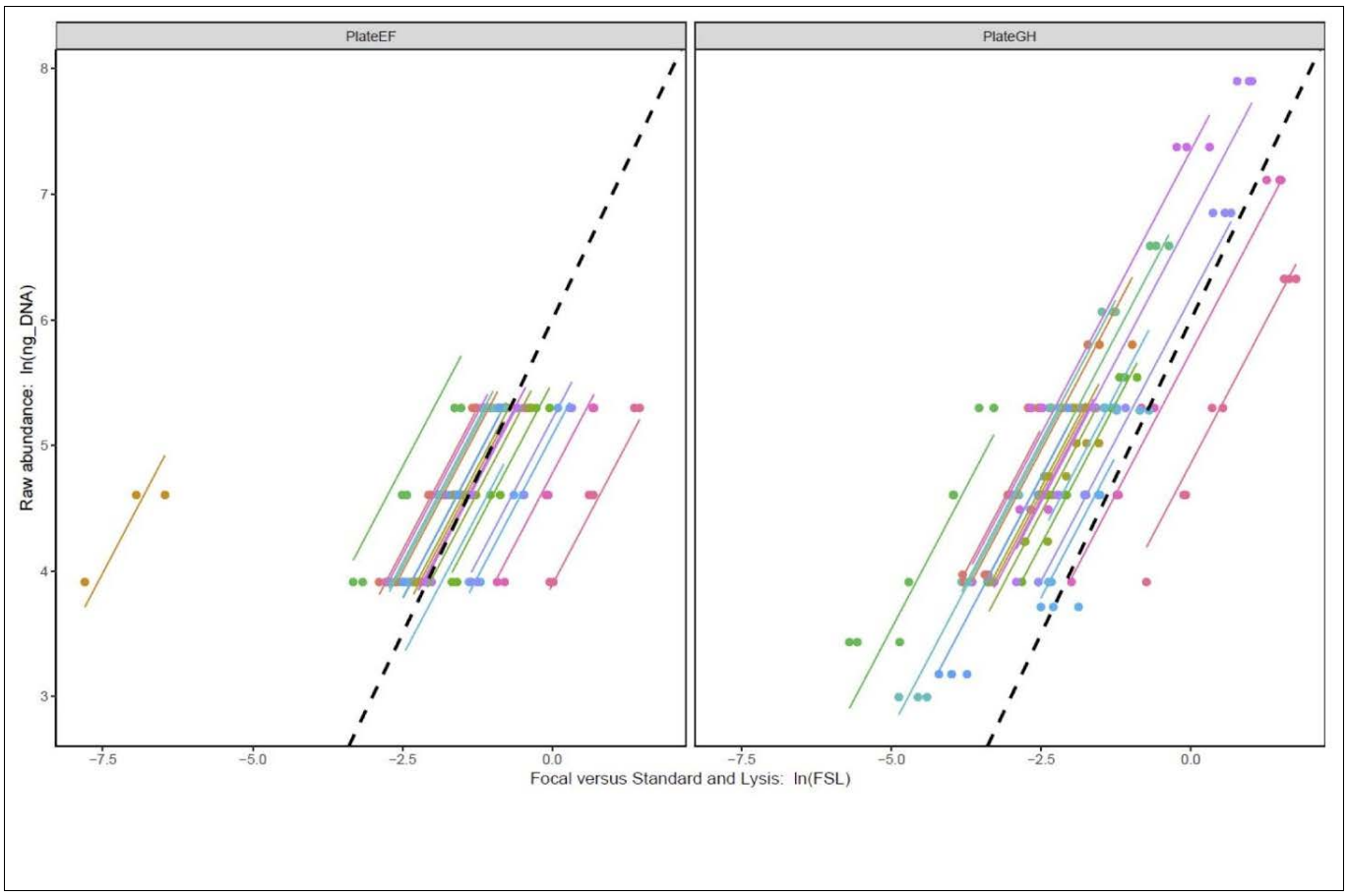

Figure 16 Achieving within-species quantification using multiple correction factors. Taken from Figure S1 in Ji et al. (2019). Each line represents a different species, and the two panels represent different sequencing runs. The X-axis is the number of mapped reads to a species after spike-in correction (FSL, where F is the number of reads mapped to a focal species (unit: sequence count), S is the "spike" (unit: sequence count/DNA mass), and L is the fraction of lysis buffer represented by the aliquot (unitless)), and the Y-axis is the input DNA-mass (ln(ng)). The reference line (black, dashed) in both panels has slope 1 and intercept 6 and shows that the read-to-DNA-mass (ng) relationship is nearly directly proportional but that there is an effect of sequencing run (In the PlateGH run (right), fewer reads predict a given amount of input DNA, relative to the PlateEF run (left). The farthest-left species is the Arctic wolf spider *Pardosa glacialis*. All other species are in the Diptera

It should be emphasised that these control measures only achieved accurate *within*-species quantification, and downstream statistical analysis should use normalised read numbers (unit variance, zero mean). *Across*-species quantification would require additional empirical work to estimate species correction factors (to account for the different intercepts in Figure 16).

Figure 17 shows a schematic of the SPIKEPIPE workflow. Three samples, with different absolute biomasses and species richnesses are first digested by adding lysis buffer to wet biomass at a fixed ratio. A fixed aliquot of the lysis buffer is then removed, and a fixed amount of spike-in DNA is added (orange). After shotgun sequencing, the sample datafile sizes do not reflect the original sample biomasses (boxes with grey dashes). The reads are mapped to barcodes or mitogenomes, and mappings with low percentage coverage are removed. Finally, two corrections are made: the numbers of mapped reads per

species are divided by (1) the number of mapped reads to the spike-in sequences and (2) the ratio of the lysis buffer aliquot to the sample's original total lysis buffer volume. The final datasets recover their original absolute biomasses, allowing recovery of *within*-species abundance information.

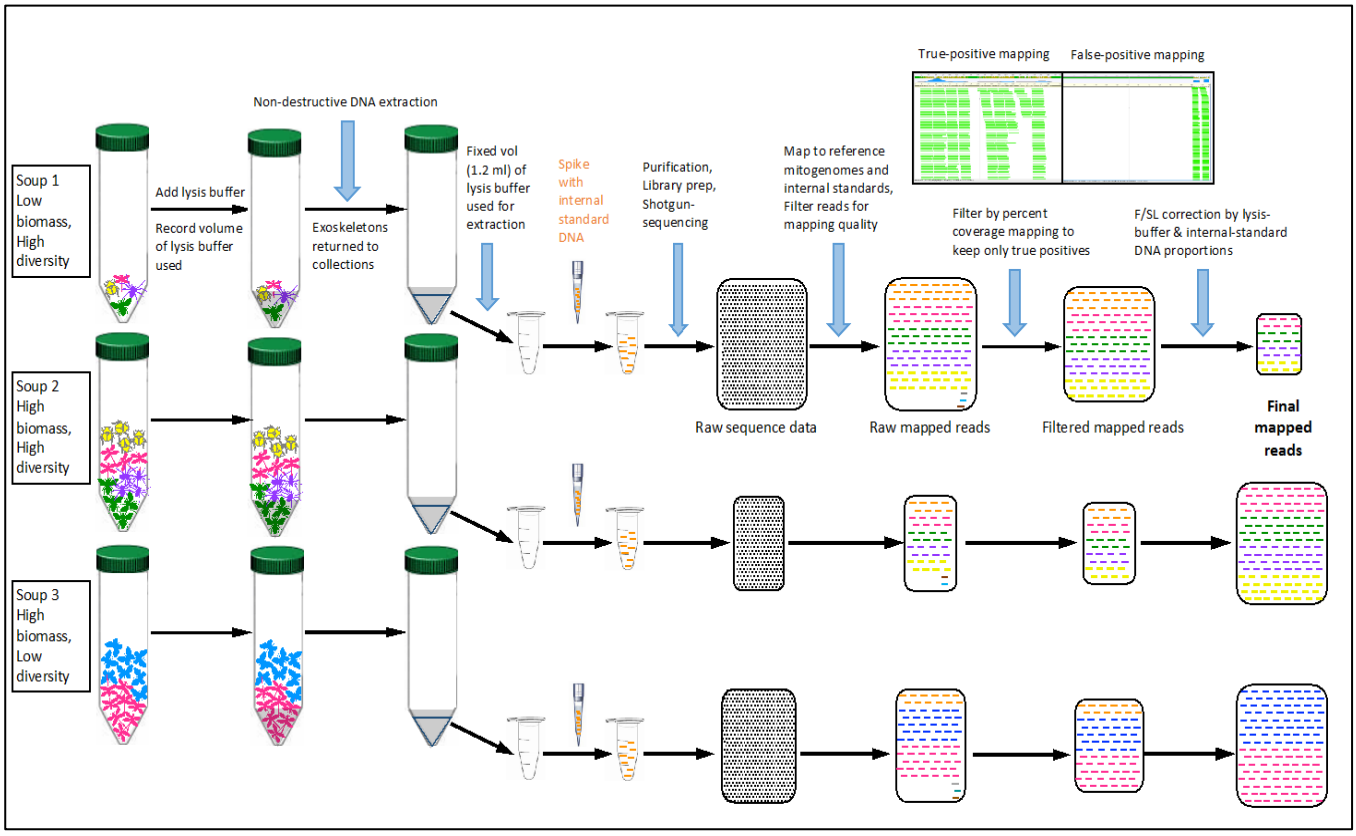

Figure 17 Schematic of the SPIKEPIPE workflow

Building reference databases. Metagenomic sequencing for bulk samples of eukaryotes has much to recommend it: reduced laboratory workload because PCR is avoided, a simpler bioinformatic pipeline, robustness to sample contamination, and, as we have seen, recovery of *within*-species abundance information.

However, metagenomic approaches require a reference database of genes or (mito)genomes against which to map reads, in contrast to amplicon-sequencing, which does not. Building a reference database can add considerable work. For instance, SPIKEPIPE relied on a DNA-barcoding campaign (Wirta et al. 2014) for its barcode database and on nearly 300 'genome skims' (low-coverage shotgun sequencing) for its mitogenome database (Ji et al. 2019).

However, two recent developments promise to decrease the workload of building reference databases. First, Greenfield et al. (2019) have developed the Kelpie package, which uses targeted assembly to mimic PCR on whole metagenome sequencing datasets (Figure 18).

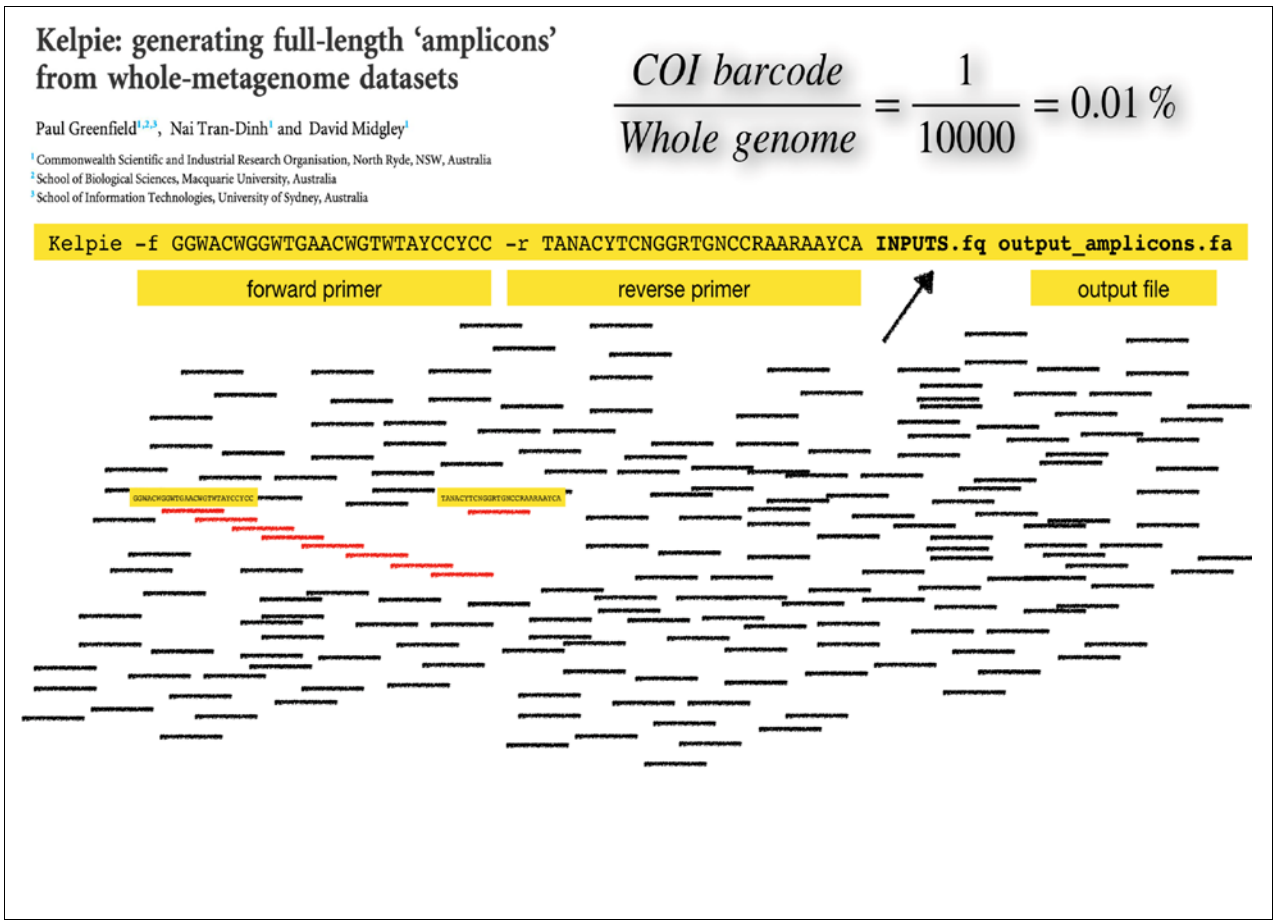

Figure 18 Schematic of the Kelpie approach (Greenfield et al. 2019). A single command includes the forward and reverse primers. Kelpie finds reads in whole metagenome datasets that match the primer sequences and then finds other reads that bridge the gap, to produce a metagenomic amplicon. This process is repeated until the fastq file is depleted

Kelpie is given a forward and a reverse primer sequence, searches for reads that match the primers, and then step-by-step assembles reads from the forward to the reverse read, producing metagenomic 'amplicon' sequences that can be clustered into OTUs and used as a reference for mapping reads, like in the SPIKEPIPE workflow. Targeted assembly continues until the reads are depleted. Given that the COI region typically makes up only 0.01% of a WGS dataset (Ji et al. 2019), species represented by few reads are unlikely to be Kelpie-assembled. On the other hand, contamination risk is also low.

An approach that uses the whole genome has been provided by Peel et al. (2019), who describe a 'reverse metagenomic' pipeline that exploits the long but error-prone reads produced by the MinION sequencers from Oxford Nanopore Technologies (ONT) (Figure 19).

With plants, individual DNA-barcodes are more ambiguous than with animals, and solid identification typically relies on two markers (CBOL Plant Working Group 2009), which reduces the taxonomic resolution of plant metabarcoding datasets, since metabarcoding of mixed-species samples cannot link two markers separately amplified from the same individual. Assembled genomes are also still rare for plants. However, Peel et al. show that unassembled genome skims of individual plant species, sequenced at between 0.3 to

1.0X coverage, can be used as reference databases, at an estimated cost of £50 per species on the Illumina NovaSeq 6000. Such datasets are also being produced in genome-assembly campaigns around the world. The mixed-species query samples, such as pollen or root balls, are separately sequenced on a MinION, generating reads of thousands of base pairs long. Each query long read is mapped to by every reference skim, and the long reads are assigned to the species whose skim mapped with the highest percent coverage.

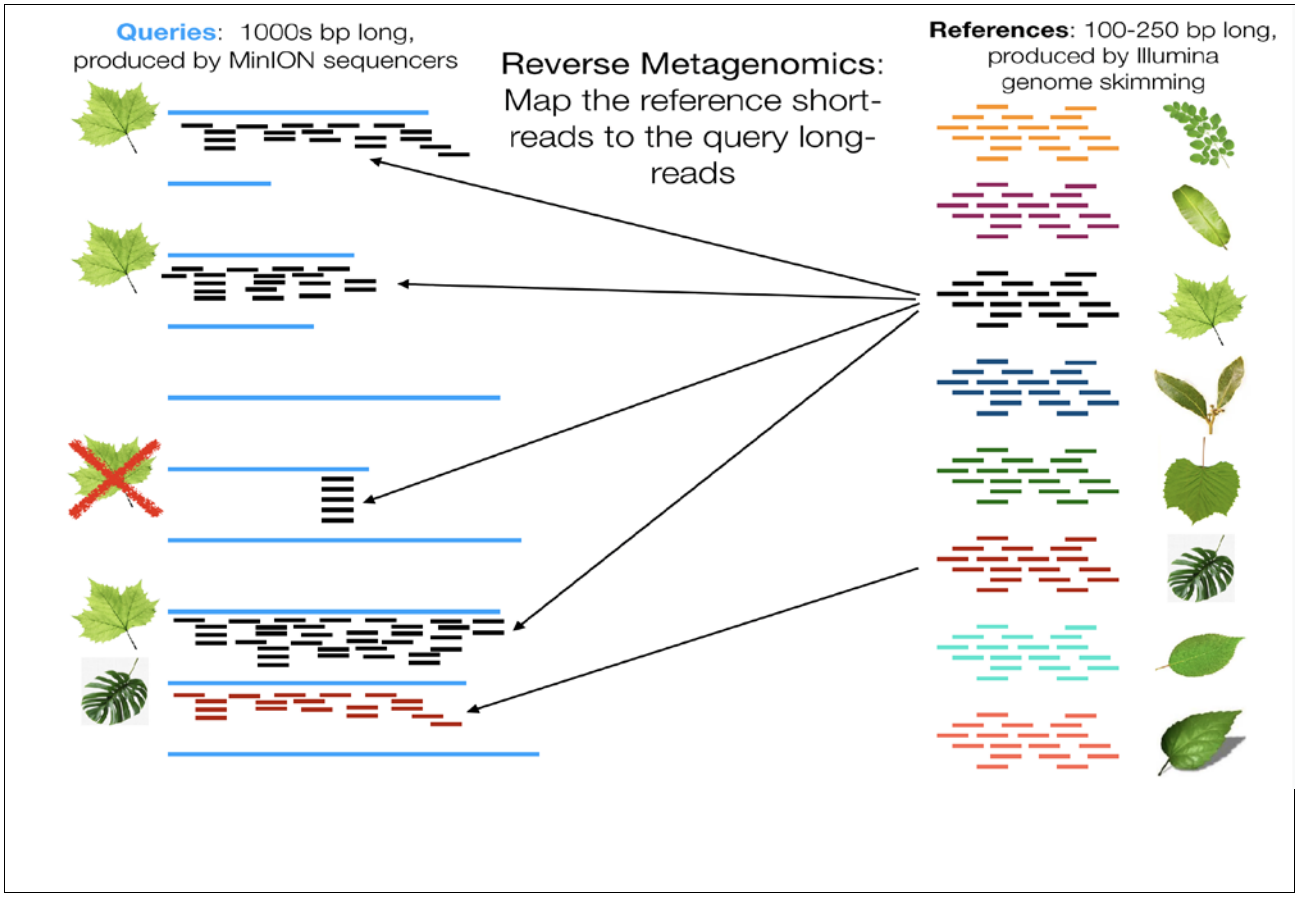

Figure 19 Reverse metagenomics pipeline exploiting long reads produced by the MinION sequencers from Oxford Nanopore Technologies

Peel et al. showed that not only could a reverse metagenomic pipeline identify species, it was also able to achieve *across*-species quantification in mixed-species pollen samples, meaning that biomass-dominant species could be identified (Figure 20). In this figure of six mock samples, each triplet of bars represents input DNA frequencies per species (leftmost) and the estimates of two RevMet technical replicates (centre and right bars).

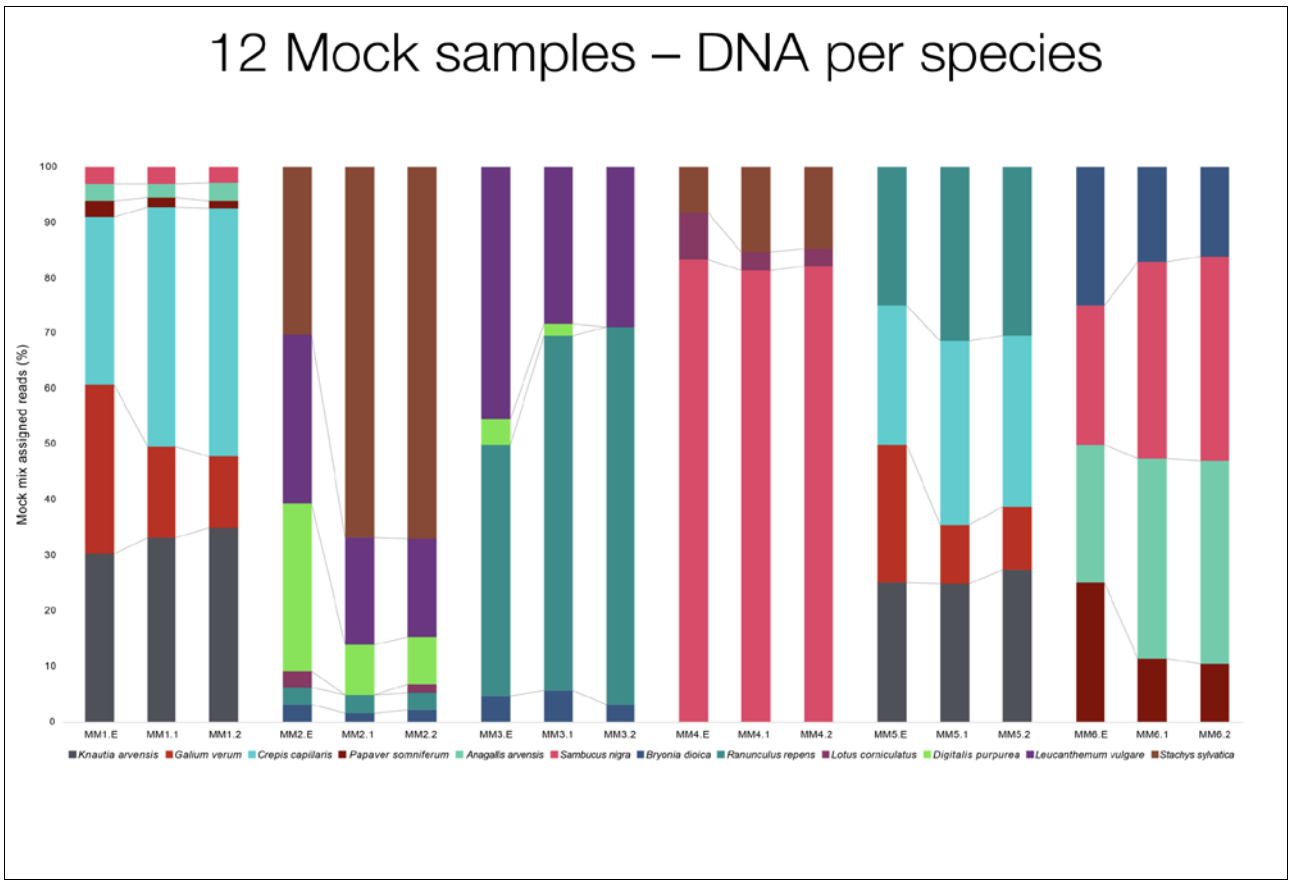

Figure 20 Reverse metagenomic pipeline used to achieve across-species quantification in mixed-species pollen samples using mock communities

Finally, Lang et al. (2019) have shown that the forward metagenomic method also accurately recovers across-species quantitative information from pollen. They produced genome skims of mixed pollen samples and mapped them to a reference database of chloroplast genomes assembled from individual-species WGS. After mapping only to variable protein-coding regions in the chloroplast genomes, and correcting the number of mapped reads per species by the different lengths of their protein-coding regions, Lang et al. found that read frequency correlated strongly and linearly with pollen-grain frequency (R^2 = 86.7%, linear regression).

Summary of abundance estimation

Estimating species abundances in DNA-based data is considerably more difficult than estimating species compositions. It is helpful to differentiate two kinds of abundance information: *within*-species and *across*-species.

Within-species abundance information estimates how a species' abundance varies from sample to sample, such as along a time series or an environmental gradient. Estimating this requires correction for sample-to-sample stochasticity caused by the pipeline, which can be achieved by propagating absolute-abundance information from a subset of species (Williamson et al. 2019) or with a common absolute standard in the form of a DNA spike-in (Ji et al. 2019). *Within*-species abundance information should therefore be normalised to

unit variance and mean zero to reflect the lack of reliable *across*-species abundance information.

Across-species abundance information estimates species frequencies within a sample, such as is used for diet analysis. Estimating this is more difficult because an unknown number of factors work together to obscure this information, including species differences in the efficiencies of DNA extraction, PCR amplification, and to a lesser extent, sequencing, OTU clustering, and bioinformatic treatment. UMIs can recover some of this information but by its nature cannot correct for species differences in DNA-extraction efficiency. For plant pollen, both forward and reverse metagenomic pipelines (Lang et al. 2019; Peel et al. 2019) appear to be able to recover species frequencies, and these methods should be explored for other sample types. We note however that forward metagenomics does not recover species frequencies from mixed-arthropod samples (Ji et al. 2019).

Finally, for some sample types, it is straightforward to estimate both within- and across-species abundance information. For instance, high-throughput individual barcoding is proposed for estimating within-species population trends and species frequencies in pan-trap sampled bees (Gueuning et al. 2019), and nematodes appear to be uniform enough that even unspiked amplicon-sequencing has recovered across-species abundance information (Schenk et al. 2019).

Taxonomic assignment

One of the two major efficiency gains from DNA-based biodiversity datasets is in taxonomic assignment (the other is sampling). In principle, with a long enough and variable enough DNA sequence, it should usually be possible to confidently assign a taxonomy to a specimen (or to its eDNA). In practice, there still exist many causes of erroneous taxonomic assignments, which in turn result in paired false positives and false negatives since erroneous assignments cause incorrect species to take the place of correct species (Wright et al. 2020). These include:

- Sequence error, especially from amplicon-sequencing pipelines.
    - The DNA sequences amplified from the samples do not contain enough taxonomic information because they are too short and too conserved to differentiate species.
    - The sequences also contain errors caused by PCR and sequencing.
    - Some sequences are amplified incorrectly from non-target taxa (e.g. bacteria) and/or from defunct nuclear copies of mitochondrial genes (known as Numts for Nuclear Mitochondrial DNA).
    - Sequence divergence is not congruent with taxonomic hierarchy. For instance, even within a given gene, species barcodes do not differ by at least 2% (or 1% or 3%). Similarly there is no uniform increase in sequence divergence as one travels up the taxonomy hierarchy (e.g. it is not correct to say that sequences in sister families differ between, say 5 and 8%).
- Reference database error.

- Reference databases are incomplete, especially for less widely used markers, such as mt16S and mt12S. Incompleteness takes the form of outright absent taxa and of inadequate coverage of sequence diversity in the taxa that are present.
- Reference databases contain sequences that have been assigned
- incorrect (including synonymised) taxonomies (Nilsson et al. 2006; Kozlov et al. 2016).

There are a number of overlapping solutions to these problems. The most important now is to continue building up barcode reference databases, such as SILVA (Quast et al. 2013), UNITE (Nilsson et al. 2019) and BOLD (Ratnasingham and Hebert 2007). Continuous efficiency improvements are key to this. For instance, Hebert et al. (2018) have shown that COI DNA-barcode-sequencing costs can be reduced to $0.20 per sample on PacBio sequencers, with further cost declines expected. Large-scale barcoding (e.g. ibol.org/programs/bioscan) and genome-assembly (e.g. www.earthbiogenome.org, www.darwintreeoflife.org) campaigns will help finance the full costs of database expansion: sample acquisition, DNA extraction, PCR (in some pipelines), sequencing, and informatics.

The second solution is for individual studies to curate existing reference databases. For instance, Axtner et al. (2019) describe a several-step bioinformatic protocol that starts with the MIDORI database (Machida et al. 2017) and curates 12S and 16S reference databases for the Tetrapoda (amphibians, reptiles, mammals), including removal of sequences that do not have a species-level assignment, do not overlap with the target gene, or contain ambiguities. Names are then updated to the most current taxonomic nomenclature. Finally, to detect mis-assigned sequences, the sequences are used to make a phylogenetic tree, and the software package SATIVA (Kozlov et al. 2016) is used to remove sequences that do not group with their congeners or confamilials, likely indicating misclassifications.

The third solution is to use multiple loci per specimen or sample. For instance, plant barcoding has long relied on at least two markers (CBOL Plant Working Group 2009), Bourke et al. (2013) demonstrate that a multi-locus COI-ITS2 barcode is needed to differentiate a closely related group of *Anopheles* species, and Axtner et al. (2019) advocate requiring both 12S and 16S markers to indicate the confident presence of vertebrate species in environmental DNA. Continuing this line of logic, organellar genomes (Nie et al. 2019) and, more powerfully, genome skims provide high resolving power for species delimitation and phylogeny estimation (Straub et al. 2012; Coissac et al. 2016; Ondov et al. 2016; Peel et al. 2019; Sarmashghi et al. 2019; Zielezinski et al. 2019; Bohmann et al. 2020; Nevill et al. 2020).

The fourth solution is to use the PROTAX software package (Somervuo et al. 2016, 2017) as a statistical wrapper around other taxonomic assignment methods (e.g. BLAST, phylogenetic placement, LAST). All other assignment methods work only with available reference sequences. However, when carrying out morphological taxonomy, it is standard practice that when a specimen does not fall into the concepts of any known species, a new morphospecies is temporarily erected. That is, the specimen is provisionally assigned to

an unknown species (“cf. sp. nov.”) within some higher taxon. Similarly, PROTAX is given a set of reference sequences and their taxonomies and fits a model that assigns four taxonomic ranks (species, genus, family, order) with their associated probabilities to a barcode sequence. These assignments include the possibility that a sequence be assigned to an unknown taxon (e.g. a morphospecies within a known genus, with associated probabilities). PROTAX’s model-fitting procedure avoids using a universal standard of sequence similarity to delimit taxa. Instead, the fitted model estimates the degree of sequence similarity is needed to assign membership to a given rank within a given taxon. In summary, PROTAX reduces overconfidence in assignment.

Summary of taxonomic assignment

The COI animal DNA barcode was introduced 17 years ago (Hebert et al. 2003), before the advent of genomics. The age of ubiquitous genomes lies some years ahead. In the meantime, the community makes steady, albeit uneven, progress in building general and targeted reference datasets, in expanding barcode loci up to whole genomes, and in devising statistical methods for the unbiased estimation of confidence in taxonomic assignments. The latter approach is particularly important because it formally acknowledges and quantifies error in taxonomic assignment, and this error can then be propagated in downstream statistical analysis, analogous to the process of propagating sampling error. For instance, Wright et al. (2020) use an independent expert classification of bat calls to estimate a confusion matrix of correct and incorrect classifications by automated software. Because each false-positive identification is also a false-negative classification, Wright *et al*.’s model corrects both kinds of errors in their occupancy model.

Statistical inference from DNA-based data

As we describe above, and similar to surveys that require direct observation of species, DNA-based surveys are prone to **observation error.** Unless accounted for within a statistical framework, this observation error may give rise to incorrect conclusions regarding species presence and community structure.

Practitioners and researchers in the field are accustomed to accounting for the probability of a **false-negative observation error**, i.e. the possibility that the species is present but is not detected, in traditional surveys. On the other hand, the probability of a **false-positive error** is typically considered negligible in traditional surveys but not in DNA-based surveys. This means that some practitioners may treat the results of DNA-based surveys with suspicion, unless these results are also confirmed using alternative sampling methods. An example demonstrating this issue is described by Jerde (2019): eDNA surveys repeatedly suggested the presence of the invasive Asian carp in the Great Lakes, but repeated attempts to physically catch any carp failed. The eDNA results were treated with suspicion by decision-makers, leading a fisheries manager, tasked with preventing Asian carp spread into the Great Lakes, to say, "*I will never believe an eDNA positive detection until we capture a fish*".

We argue that even though we can never assign 100% confidence to DNA-based results, by using statistical methods, we can quantify our (un)certainty around conclusions based on DNA-based surveys, giving us the tools required for effective decision-making.

The Asian carp case study demonstrates that even though DNA-based methods are not error-free, they likely benefit from lower rates of a false-negative error than do traditional survey methods, especially in the case of rare or difficult-to-catch species. Nevertheless, also in this case study, not all eDNA samples collected from the Great Lakes were positive for the presence of Asian carp. Therefore, as is the case for traditional survey data, such as occupancy data discussed in the next section, DNA-based data should be treated as imperfect and modelled using appropriate statistical methods that account for the probabilities of observation error.

Estimation of species richness and diversity

Species richness is defined as the number of species in an assemblage (Gotelli and Chao 2013). Since the number of species is typically unknown, statistical methods need to be employed to estimate the number of unseen species, and hence species richness, from a sample. The sample may consist of (relative) **abundance data**, recording the number of individuals detected from each species — or in the case of microbial data, the frequencies of all observed operational taxonomic units (OTUs). Alternatively, the sample may consist of **incidence data**, recording the number of sampling units in which the species has been detected. Commonly employed estimators of the minimum number of unseen species are those developed by Chao (1984) for abundance data and by Chao (1987) for incidence data. Bunge et al. (2014) provide a review of methods for estimating the number of unseen

species, or obtaining an upper bound for it, covering parametric and non-parametric approaches, and apply them to microbial samples.

However, a simple measure of species richness does not account for other community properties (Buckland et al. 2012), such as **evenness** or **similarity**. Several **biodiversity measures** have been proposed to account for both species richness *and* evenness (see Maurer and McGill 2011 for a recent review), with **Simpson's index** (Simpson 1949) and **Shannon's entropy** (Shannon 1948), the most widely used measures.

Recently, Leinster and Cobbold (2012) have developed a measure that accounts for species **similarity**. Their criticism of existing measures of diversity is summarised in the following sentence: "... a community of six dramatically different species is said to be no more diverse than a community of six species of barnacle." Their measure takes in as data both the **relative abundance of all detected species** in the assemblage and **all pairwise measures of similarity**. This similarity can be for example a genetic notion, which "will lead to a **measure of genetic diversity**" and could be especially useful in multi-species eDNA surveys where species delimitation is inherently uncertain. Depending on the clustering algorithm used (or no clustering, in the DADA2 pipeline (Callahan et al. 2016)), OTU number can overestimate or underestimate biological species richness. However, similarity-corrected richness should be robust to such errors in clustering. For instance, Wang et al. (2019) compared species richnesses in different types of plantation forests, and one of their comparisons compared phylogenetic diversities (PD) across forest types, the idea being that closely related OTUs (including oversplit species) cluster together and therefore contribute less to PD than do distantly related OTUs.

However, all the aforementioned diversity measures, including the Leinster and Cobbold (2012) measure, do not account for the number of unseen species and their corresponding abundances. To that effect, Willis (2019) argues that to minimise the risk of reaching incorrect conclusions on taxonomic richness, diversity measures should be **adjusted for measurement error** and updated to **account for the number of unseen species** before they can be used to compare assemblages.

α-diversity versus β-diversity

Species richness, as defined above, is often referred to as **α-diversity,** which is a measure of diversity within a sample (Dornelas et al. 2014). There is growing evidence (Vellend et al. 2013; Dornelas et al. 2014; Magurran et al. 2015) that the general consensus that local α-diversity metrics do not track the global decrease in biodiversity is correct. As Dornelas et al. (2014) argue, "...local and regional assemblages are experiencing a substitution of their taxa, rather than systematic loss."

Therefore, a more appropriate measure of biodiversity change is given by **β-diversity**, "which measures change in community composition over time (or space)" (Magurran et al. 2015). As reported by Dornelas et al. (2014) who analysed 100 time series of biomes across earth, no consistent negative trend could be detected in species richness using α-diversity, whereas measures of β-diversity exhibited consistent long-term changes.

Generating causal hypotheses of biodiversity response to environmental change

Single-species occupancy models

Single-species occupancy data are collected by repeatedly surveying sites and recording whether at least one individual of the species of interest has been detected. **Single-species qPCR data** can be seen as occupancy data, with the corresponding eDNA score, that is the number of positive qPCR for each site, representing the number of times the species has been detected at that site, which can also be equal to 0.

Single-species occupancy models, first introduced by MacKenzie et al. (2002), account for the probability of failing to detect a species that is present (**a false-negative observation error**) and estimate the probability of species presence at surveyed sites. These occupancy models have been extended to also account for the probability of falsely detecting a species that is not present (**a false-positive observation error**) by Royle and Link (2006). However, the Royle and Link (2006) model is not identifiable in that it does not provide a unique solution in terms of the probability of species presence. Instead, two solutions in terms of the probability of species presence (probability = ψ and probability = $1 - \psi$) are equally supported. Royle and Link (2006) suggest employing a constraint that sets the **probability of a true positive to be greater than the probability of a false positive**, which is expected to be true both in traditional and in DNA-based surveys.

An attempt to estimate observation error for single-species eDNA data was made by Dorazio and Erickson (2018), who defined a **two-level occupancy model** with one level for each stage in eDNA surveys, namely the field stage and the lab stage. They implemented their model, together with the option of comparing different models in terms of covariates, within a Bayesian framework, in the *ednaoccupancy* R-package. However, **they assumed that the probability of a false-positive error is zero, which is unrealistic for DNA-based surveys**.

This assumption was relaxed by Guillera-Arroita et al. (2017) who extended the model by Royle and Link (2006) to model single species qPCR data by **accounting for the probability of a false positive or false negative observation error in both stages of eDNA surveys** (Figure 21).

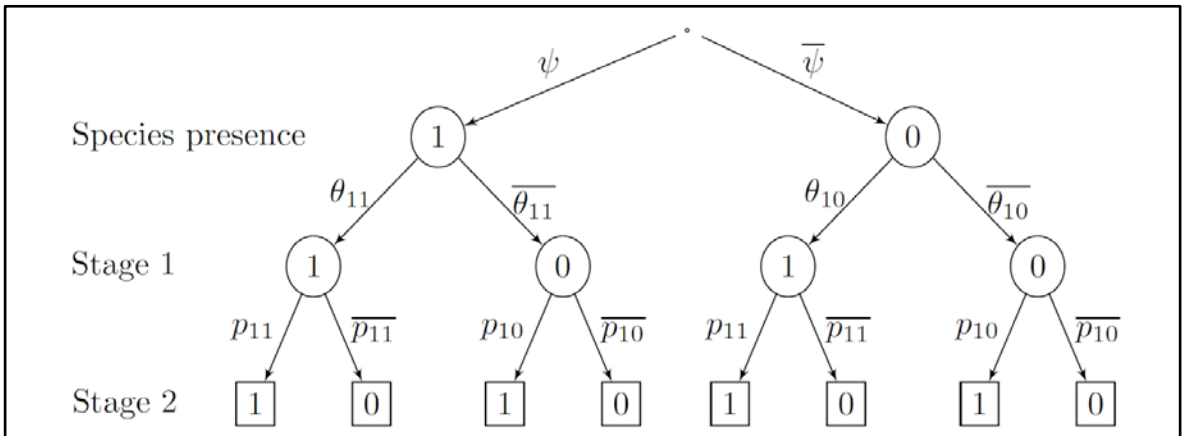

Figure 1: Schematic representation of the Guillera-Arroita et al. (2017) model. The probability of species presence is ψ. Conditional on species presence, the probability that DNA of the species is included in the water sample is θ_{11} (probability of a stage 1 true positive observation), while conditional on species absence, the corresponding probability is θ_{10} (probability of a stage 1 false positive observation). Conditional on DNA of the species being present in the water sample, the probability of detecting it in a PCR run is p_{11} (probability of a stage 2 true positive observation) while the corresponding probability conditional on DNA absence in the water sample is p_{10} (probability of a stage 2 false positive observation). The complements for all parameters are denoted by an overline, i.e. $\psi + \overline{\psi} = 1$ etc.

Figure 21 Schematic representation of the Guillera-Arroita et al. (2017) occupancy model

However, as was the case for the original Royle and Link (2006) model, the Guillera-Arroita et al. (2017) model is also non-identifiable, and in fact the model has four solutions that are equally supported (these can be seen by following the four different branches that lead to the final outcome of a positive PCR run (1) in figure 21. To overcome this identifiability issue, **Guillera-Arroita et al.** (2017) **augmented their eDNA data with data collected using two types of traditional survey techniques**, and for the four species of frog that they considered, they reported the range of estimates for the probabilities of an observation error in stages 1 and 2 (Table 5).

Table 5 Range of observation error estimates obtained for four species by Guillera-Arroita et al. (2017)

Probability	Range
Stage 1 false-positive error	0-7%
Stage 1 false-negative error	40-62%
Stage 2 false-positive error	3-5%

Stage 2 false-negative error	4-14%

Clearly, these results highlight that **the probabilities of observation error are not negligible at either stage**. The Guillera-Arroita et al. (2017) model provided an important insight into the properties of eDNA surveys, but the applicability of the model is limited by the need to also collect traditional survey data, which can be infeasible in large scale, long-term studies. Also, the model does not account for the effect of any covariates on the probability of species presence or the probabilities of observation error and hence does not allow inference on the factors that affect any of these important quantities.

Recently, the Guillera-Arroita et al. (2017) model was extended by Griffin et al. (2019) to **account for environmental covariates that affect the probabilities of species presence or the probabilities of observation error at either stage**. In addition, Griffin et al. (2019) formulated their model within a Bayesian framework, which allowed them to incorporate prior knowledge on the observation errors in each stage. They formalised the suggestion by Royle and Link (2006) that the probability of a true positive is expected to be greater than the probability of a false positive observation. This **allows estimation of the probabilities of species presence without needing to augment eDNA data with additional sources of information**, which had not been possible using the Guillera-Arroita et al. (2017) model. The model and algorithm, including efficient Bayesian variable selection to identify important predictors for all model parameters, is implemented in a freely-available R-Shiny app https://seak.shinyapps.io/eDNA/.

When analysing eDNA data on great crested newts, collected by Natural England, Griffin et al. (2019) estimated that the probability of a stage 1 false positive error is 15%, the probability of a stage 1 false negative error is 27%, while the corresponding probabilities for stage 2 are 5% and 19%, respectively.

Multi-species occupancy models (MSOMs)

Dorazio and Royle (2005) developed a **multi-species occupancy model** by essentially "stacking" single-species occupancy models. This model can be used to infer **species richness at the surveyed sites** (that is the total number of species detected at least once + the total number of species present at any of the sites but not detected).

However, **the Dorazio and Royle** (2005) **model assumes that species are independent of each other**, which is generally unrealistic, and has been shown to give rise to unreliable estimates of the total number of species, even when the model assumptions hold (Guillera-Arroita et al. 2019). The assumption of independence is relaxed by Rota et al. (2016) who explain that modelling the latent occupancy state of each species at each site as a multivariate Bernoulli random variable allows them to test different hypotheses regarding the effect of environmental covariates and species interactions on species presence.

For alternative models that account for species interactions, but limited to two species, while still accounting for imperfect detection see references in Rota et al. (2016), while for

models that account for species interactions but ignore observation error see next section on joint species distribution modelling.

Joint species distribution models (jSDMs)

jSDMs are hierarchical (typically Bayesian) models for community data (Ovaskainen et al. 2017). They do not account for uncertainty in the data, as do occupancy models, but are more flexible in **modelling species occurrence and co-occurrence in ecological communities**. Using jSDMs, one can **account for the effect of environmental covariates** on species richness, for any unexplained variation using latent covariates as well as **for phylogenetic relationships between species** and **for species traits**. jSDMs provide an estimate of a **correlation between all pairs of species in the assemblage**, indicating whether a pair is likely or not to co-occur, *after accounting for the effect of environmental covariates*. This flexibility has made jSDMs popular modelling tools for presence-absence data, as they can be used to answer important ecological questions (see Table 6).

Table 6 Questions that can be asked with joint species distribution models (jSDMs) (slightly simplified Table 1 of Ovaskainen et al. (2017)), reprinted under the article's CC-BY license)

Question type	Question
Fundamental Question 1	How much variation in species occurrence is due to environmental filtering, biotic interactions and random processes, and how do these impacts vary across spatial and temporal scales? Assess the explanatory power of models and by variance partitioning among fixed and random effects at different scales
Fundamental Question 2	How do species' traits and phylogenetic relationships correlate with ecological niches? Model responses to environmental covariates as a function of species' traits and phylogenetic correlations
Fundamental Question 3	What are the structures of species interaction networks? Estimate the species-to-species association matrices
Fundamental Question 4	How does community similarity depend on environmental similarity and/or geographic distance? Decompose community similarity into similarity due to responses to environmental covariates and/or spatial covariance

Fundamental Question 5	How does community structure change over time due to predictable succession or stochastic ecological drift? Include time since environmental perturbation as a predictor, or by including temporally varying random effects
Applied Question 1	Do some species indicate the presence of others? Test how much the predictive power of the model increases for a focal species when accounting for the occurrences of other species
Applied Question 2	How can geographic areas be classified into communities of common profile? Cluster predicted communities based on their similarity
Applied Question 3	Which processes have been central in determining the response of a community to environmental change? Decompose the response to environmental change to components related to species niches and random effects
Applied Question 4	How can species be classified in terms of their response to abiotic environment? Cluster parameters or predictions measuring the species responses to environmental covariates
Applied Question 5	How does community structure change over time due to predictable succession or stochastic ecological drift? Include time since environmental perturbation as a predictor, or by including temporally varying random effects

However, to date, as also acknowledged by Ovaskainen et al. (2017), jSDMs have not been extended to account for observation error and hence, as they stand, are not directly applicable to metabarcoding data. In our view, model development for eDNA data should now focus on combining the flexibility of jSDMs with the ability of occupancy models to account for observation error.

Future directions in terms of statistical developments

Single species qPCR data are widely collected and will continue to contribute substantially to the monitoring of protected species, such as great crested newts. The model by (Griffin et al. 2019) and corresponding software has provided the required tools for analysing such data and obtaining reliable estimates of site-specific probabilities of species presence and for identifying important predictors of species presence.

On the other hand, no statistical models have been developed for metabarcoding data, even if such data are now increasingly replacing the use of single species data in monitoring surveys. MSOMs and jSDMs provide an excellent foundation but, so far, stop short of providing the required tools because of the need to account for multiple layers of observation error in DNA-based surveys when analysing metabarcoding data.

Similarly, high-throughput individual barcoding data have substantial potential in allowing us to overcome the challenges, at least for some taxa, of estimating abundance from DNA-based data that we outlined above. This would require tailoring the models that are widely used for modelling count data (Royle 2004; Gomez et al. 2018) obtained by direct observation of species to account for the unique features of DNA-based monitoring data.

For DNA-based methods to achieve their full potential in conservation biology and hence in achieving the first element (*Knowledge generation*) required for socio-ecological resilience (Table 3), technological advances that result in new types of data need to be combined with methodological advancements in statistical models that are specifically tailored for such data.

Potential Contributions of DNA-based methods to the 25YEP Wildlife Indicators

Here we briefly survey six Wildlife Indicators in the UK's 25 Year Environment Plan (Defra 2019) and signpost opportunities to combine DNA-based data, remote sensing, and statistical analysis.

D1 Quantity, quality and connectivity of habitats

"This indicator will measure changes in extent, condition, connectivity and function of terrestrial and freshwater habitats in England….Data are available to measure some aspects of this indicator such as extent and condition of some habitats, but *further work is required to assess habitats beyond protected sites, and reliable methods for measuring ecological connectivity need to be further tested.* Some indicators of aspects of ecosystem functions and processes are available but these are not comprehensive. *New methods of Earth Observation (EO) together with development of measures of favourable conservation status and long-term site-based monitoring offer good opportunities to develop this indicator*."

D2 Extent and condition of protected sites – land, water and sea

"Protected sites are areas of land, inland water and the sea that have special legal protection to conserve important habitats and species in England...This indicator has 2 components: (a) extent (hectares) of protected sites on land, water and at sea and *(b) condition of protected sites on land, water and at sea*. *Condition for terrestrial sites is assessed against relevant common standards agreed by the UK conservation agencies. Condition methodology for MPAs is currently under development.*"

D4 Relative abundance and/or distribution of widespread species

"This indicator will use regularly collected data to track changes in relative abundance and/or distribution of species which are widespread and characteristic of different broad habitats in England including birds, bats, butterflies, moths and plants. *The expectation is that this indicator will be expanded to include more species groups and habitat types*. Trends in abundance or distribution of wild birds, bats, butterflies and moths are already published and methods for analysing trends in plants are being developed. Further work is needed to determine how best to combine and present trends for different species groups and habitats within this indicator."

D7 Species supporting ecosystem functions

"All species have a functional role within ecosystems such as photosynthesis, respiration, decomposition, nutrient cycling, predator-prey and symbiotic relationships such as pollination. *Plants, fungi, algae, invertebrates and soil micro-organisms are particularly important*. The presence, abundance and diversity of species are key factors in

determining the resilience of ecosystems to environmental changes, including climate change and disease, and the maintenance of ecosystem services. Further research is required to develop this indicator, building on the existing pollinator indicator and defining species groups and functions for inclusion."

These four indicators, **D1**, **D2**, **D4**, and **D7**, all involve the large-scale mapping of biodiversity with paired environmental variables, such as land-use class and history, vegetation structure, pollutant and toxin levels, elevation, and climate, and we address them together.

Large-scale, granular, and continuous monitoring of habitat condition and extent is feasible only via remote sensing, but remote-sensing data do not carry direct information on biodiversity composition. We have proposed, however, that biodiversity point samples, including DNA-based data and also direct surveys, bioacoustics, and camera-traps, can be used in conjunction with new statistical methods (to be developed by combining features of multi-species occupancy models and joint species distribution models) to parameterise models that interpret raw remote-sensing data in terms of species composition, thereby producing spatially continuous biodiversity maps that are continuously updated (Bush et al. 2017). A schematic of this plan is shown in Figure 22 (taken from Bush et al. 2017).

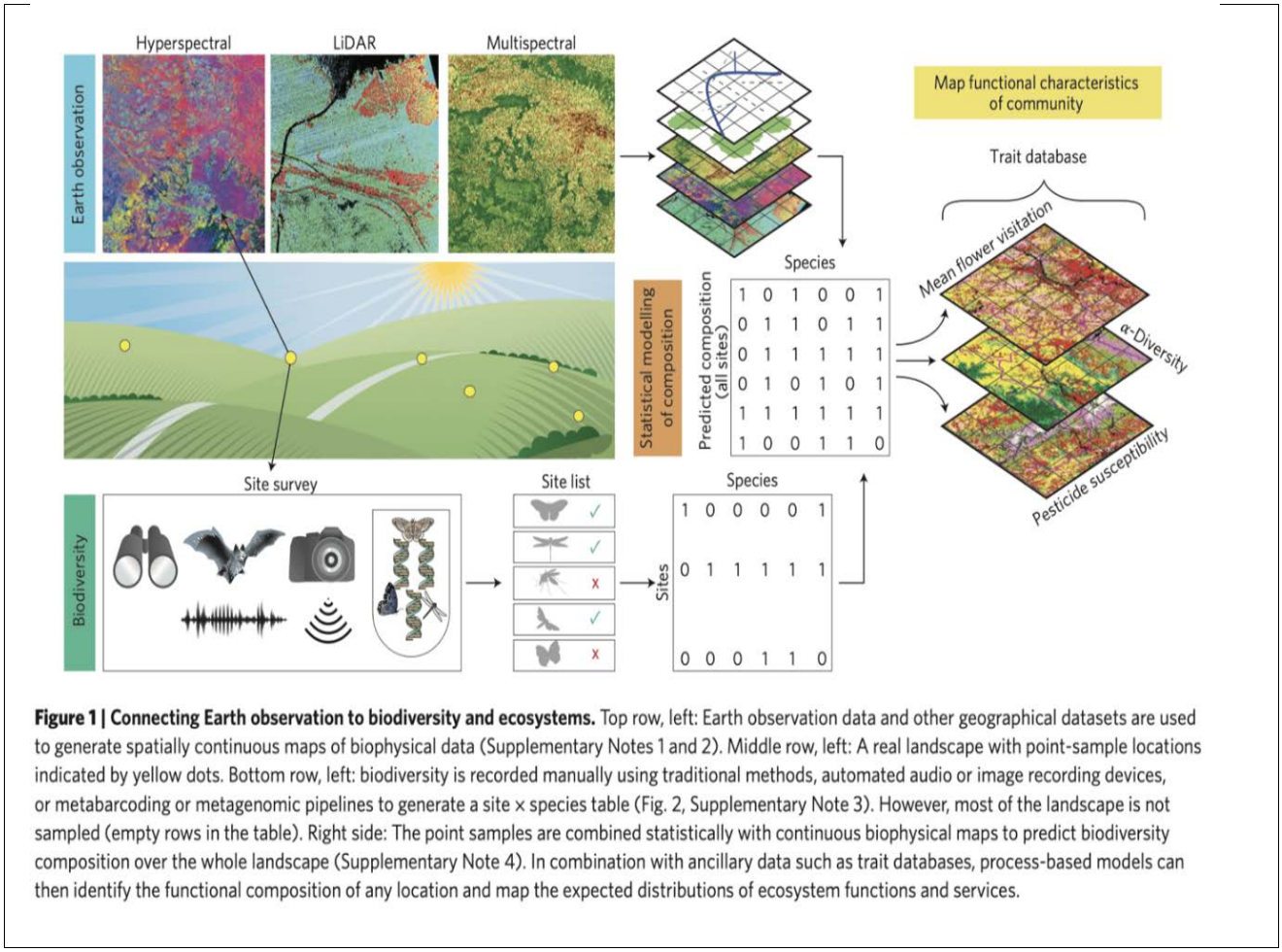

Figure 1 | Connecting Earth observation to biodiversity and ecosystems. Top row, left: Earth observation data and other geographical datasets are used to generate spatially continuous maps of biophysical data (Supplementary Notes 1 and 2). Middle row, left: A real landscape with point-sample locations indicated by yellow dots. Bottom row, left: biodiversity is recorded manually using traditional methods, automated audio or image recording devices, or metabarcoding or metagenomic pipelines to generate a site × species table (Fig. 2, Supplementary Note 3). However, most of the landscape is not sampled (empty rows in the table). Right side: The point samples are combined statistically with continuous biophysical maps to predict biodiversity composition over the whole landscape (Supplementary Note 4). In combination with ancillary data such as trait databases, process-based models can then identify the functional composition of any location and map the expected distributions of ecosystem functions and services.

Figure 22 Connecting Earth Observation data to biodiversity and ecosystems

We see two ways to translate OTU tables to ecological functions. The first is to assign taxonomies to OTUs, and the taxonomies can then be linked with our vast storehouse of functional biological knowledge (Janzen et al. 2005) (e.g. pollinators, food plants, predators of pests, disease resistance, decomposition, water purification) and also mapped. The second is to simultaneously measure ecological functions in the field (e.g. pollination efficacy, pest control, carbon sequestration) and to correlate them with the mapped species inventories.

We note also that DNA-based approaches increase sampling options. Placed traps represent only one set of options. Cars can be fitted with capture devices for flying invertebrates (Kosakovsky Pond et al. 2009), air filters can be used to sample plant pollen and fungal spores (Abrego et al. 2018; Brennan et al. 2019), and citizen scientists can sample from water bodies (PondNet eDNA Monitoring for GCN 2019 Update: https://freshwaterhabitats.org.uk/pondnet-edna-monitoring-for-gcn-2019-update/).

A very interesting additional possibility is to infer *metacommunity dynamics* from the same kinds of datasets (Ovaskainen et al. 2019; Ponisio et al. 2019). Roughly speaking, metacommunities are individual communities linked by long-distance dispersal. Distances, local habitat features, and species traits interact to determine the distribution of species across a metacommunity. Over ten years, Ponisio et al. (2019) repeatedly collected and morphologically identified a total of 10 491 wild bees from multiple transects taken across several-hundred sq. km agroecosystem landscape. Ponisio et al. also used remote-sensing layers to identify hedgerows and potential remnant habitat patches capable of supporting wild-bee populations, within a matrix of otherwise unsuitable agricultural land. The remnant habitats and hedgerows form the potential metacommunity, and different bee species have different capacities for dispersing through, colonising, and persisting in the linked patches. A multi-species occupancy model found that florally diverse hedgerows and remnant habitat contribute to wild-bee dispersal and persistence, with their relative contributions dependent on the diet breadth of the bee species, as inferred from taxonomy and floral observations. A DNA-based approach would use high-throughput individual barcoding to produce the same type of dataset (possibly freeing up resources for more sampling or a wider taxonomic breadth), and bee diet breadth could be measured by metabarcoding or metagenomics of collected pollen. In sum, DNA-based data, remote-sensing data, and multi-species occupancy modelling allow the estimation of ecological connectivity, habitat condition (from the point of view of focal taxa), and the spatial distribution of pollinator function.

D5 Conservation status of our native species

“This indicator will track changes in the conservation status of terrestrial, freshwater and marine species using established international (IUCN 12) categories and criteria. Species will be classified in several categories including: least concern; near threatened; vulnerable; endangered; critically endangered; and, regionally extinct. A simple index will be constructed to summarise the changes in numbers of species in each category. Baseline assessment data for approximately 10,000 species are available. This includes birds, mammals, reptiles, amphibians, some invertebrates, vascular plants, bryophytes,

lichens and some fungi. Assessments are currently undertaken for Great Britain. *Further assessments are required for a wider range of species, and will need to be repeated (4 to 6 year intervals) in order to detect change in extinction risk for individual species and native species as a whole*."

D6 Abundance and distribution of priority species in England

"Priority species are defined by the Secretary of State under Section 41 of the Natural Environment and Rural Communities Act 2006 as species which are of principal importance for the purpose of conserving biodiversity in England. This indicator has 2 components: (a) changes in the relative abundance of those priority species for which abundance data are available; and, (b) changes in distribution of priority species (i.e. changes in the number of one kilometre grid squares in which species are recorded in any given year) for those priority species for which only distribution data are available."

The next two indicators, **D5** and **D6**, both require tracking species-level population trends, and we address them together. D5 and D6 are focused on pre-defined sets of species, many of which will be protected and/or commercially valuable animal species (especially the latter for marine protected areas, MPAs). DNA-based methods can contribute as long as the relevant species can be sampled. For aquatic species, distributions and abundances can be estimated by eDNA, although it will be necessary to control for the effects of variation in water flow and volume and for observation errors, as we discuss above. For semi-aquatic and terrestrial animals, aquatic eDNA (Sales et al. 2020) (and perhaps soil eDNA, Leempoel et al. 2020) can also provide some distribution information, but observation error will be higher, measures will likely be limited to incidence instead of abundance, and some species will require supplementary detection methods such as camera traps.

In addition, there is considerable scope for using invertebrate samplers of vertebrate DNA, known as 'iDNA' (Bohmann et al. 2013; Calvignac-Spencer et al. 2013; Schnell et al. 2015). As an example, Ji et al. (2020) contracted 163 park rangers to collect leeches in 172 patrol areas, resulting in 30,468 total leeches, divided over 893 replicate samples. The replicate sampling allowed the use of a multispecies occupancy model to correct for false negatives and to let data-deficient species borrow strength from species with more detection events. Species-level occupancies were estimated for 86 vertebrate species, mostly frogs and mammals. Sites at lower elevations and closer to the reserve edge were estimated to have higher occupancies of domestic species (cows, sheep, goats), whereas most large-mammal wildlife (e.g. sambar, black bear, serow, tufted deer) showed the opposite pattern: greater occupancy at higher elevations and closer to the reserve interior (Figure 23). In the UK, the likely sources of iDNA are flies, midges, and mosquitoes. We are not advocating exclusive reliance on iDNA; it is known that combining camera-traps and iDNA can produce more confident occupancy estimates (Abrams et al. 2019; Tilker et al. 2020).

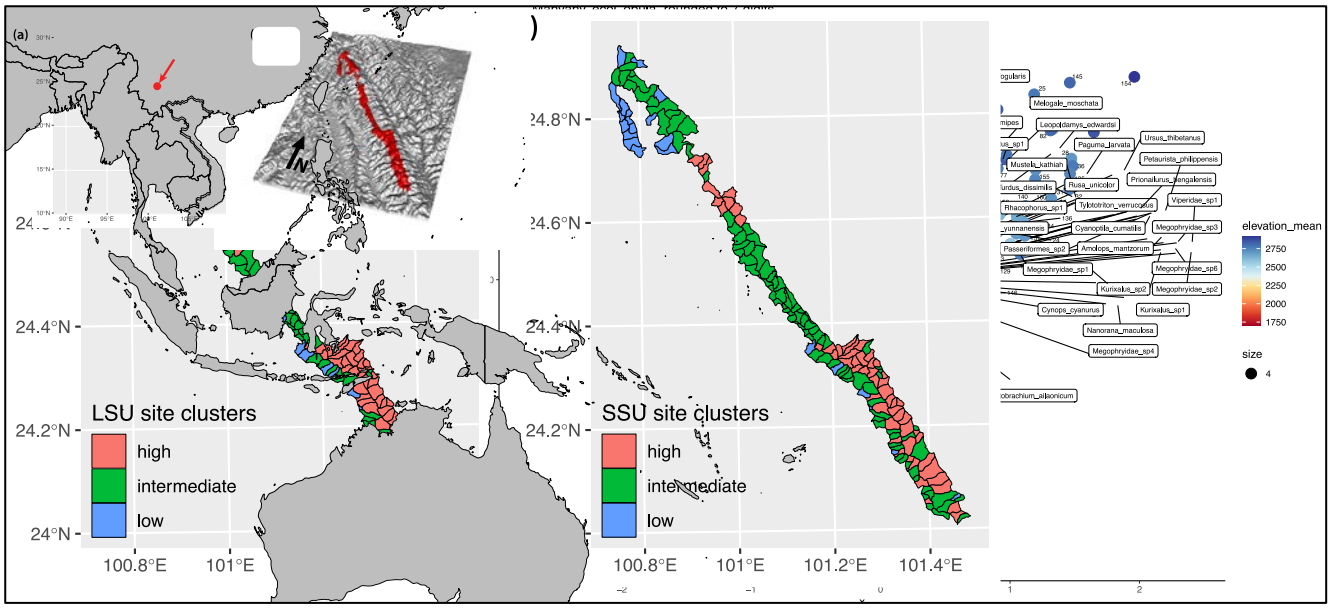

Figure 23 Spatial distribution of 86 vertebrate species censused by leech iDNA. Adapted from Ji et al. (2020). Left, Ailaoshan nature reserve, with patrol areas coloured by membership in one of three community clusters produced by hierarchical clustering of mean Jaccard dissimilarities. Right, ecoCopula ordination of observed vertebrate species distributions. Each point is a patrol area, coloured by elevation. Both methods show that community composition is structured by elevation.

Finally, even more than the previous indicators, indicators D5 and D6 will benefit from bolstered reference databases: continued taxonomic revisions, curation of existing reference sequences, assembled mitochondrial and chloroplast genomes, whole genome skims, and thorough sampling of intra-specific variation. Particularly for protected species and their close relatives, it is beneficial to have multiple diagnostic loci in order to reduce false-positive detections (Axtner et al. 2019).

References

ABRAMS, J. F., L. Hörig, R. Brozovic, J. Axtner, A. Crampton-Platt, A. Mohamed, S. T. Wong, et al. 2019. Shifting up a gear with iDNA: from mammal detection events to standardized surveys. *The Journal of applied ecology*

ABREGO, N., V. Norros, P. Halme, P. Somervuo, H. Ali-Kovero, and O. Ovaskainen. 2018. Give me a sample of air and I will tell which species are found from your region: Molecular identification of fungi from airborne spore samples. Molecular ecology resources 18:511–524

AMEND, A. S., K. A. Seifert, and T. D. Bruns. 2010. Quantifying microbial communities with 454 pyrosequencing: does read abundance count? *Molecular ecology* 19:5555–5565

AXTNER, J., A. Crampton-Platt, L. A. Hörig, A. Mohamed, C. C. Y. Xu, D. W. Yu, and A. Wilting. 2019. An efficient and robust laboratory workflow and tetrapod database for larger scale environmental DNA studies. *GigaScience* 8:646–617

AYLAGAS, E., Á. Borja, I. Muxika, and R.-E. Naiara. 2018. Adapting metabarcoding-based benthic biomonitoring into routine marine ecological status assessment networks. *Ecological indicators* 95:194–202

BÁLINT, M., M. Pfenninger, H.-P. Grossart, P. Taberlet, M. Vellend, M. A. Leibold, G. Englund, et al. 2018. Environmental DNA Time Series in Ecology. T*rends in ecology and evolution* 33:945–957

BELL, K. L., J. Fowler, K. S. Burgess, E. K. Dobbs, D. Gruenewald, B. Lawley, C. Morozumi, et al. 2017. Applying pollen DNA metabarcoding to the study of plant-pollinator interactions. *Applications in plant sciences* 5

BENGTSSON-PALME, J., M. Hartmann, K. M. Eriksson, C. Pal, K. Thorell, D. G. J. Larsson, and R. H. Nilsson. 2015. metaxa2: improved identification and taxonomic classification of small and large subunit rRNA in metagenomic data. *Molecular ecology resources*

BINLADEN, J., M. T. P. Gilbert, J. P. Bollback, F. Panitz, C. Bendixen, R. Nielsen, and E. Willerslev. 2007. The use of coded PCR primers enables high-throughput sequencing of multiple homolog amplification products by 454 parallel sequencing. *PLoS One* 2:e197

BISTA, I., G. R. Carvalho, M. Tang, K. Walsh, X. Zhou, M. Hajibabaei, S. Shokralla, et al. 2018. Performance of amplicon and shotgun sequencing for accurate biomass estimation in invertebrate community samples. *Molecular ecology resources*

BOHMANN, K., A. Evans, M. T. P. Gilbert, G. R. Carvalho, S. Creer, M. Knapp, D. W. Yu, et al. 2014. Environmental DNA for wildlife biology and biodiversity monitoring. *Trends in ecology & evolution* 29:358–367

BOHMANN, K., S. Mirarab, V. Bafna, and T. Gilbert. 2020. Beyond DNA barcoding: The unrealised potential of genome skim data in sample identification. Authorea Preprints

BOHMANN, K., I. B. Schnell, and M. T. P. Gilbert. 2013. When bugs reveal biodiversity. *Molecular ecology* 22:909–911

BOURKE, B. P., T. P. Oliveira, L. Suesdek, E. S. Bergo, and M. A. M. Sallum. 2013. A multi-locus approach to barcoding in the Anopheles strodei subgroup (Diptera: Culicidae). *Parasites & vectors* 6:111

BOYER, F., C. Mercier, A. Bonin, Y. Le Bras, P. Taberlet, and E. Coissac. 2016. OBITOOLS: a UNIX-inspired software package for DNA metabarcoding. *Molecular ecology resources* 16:176–182

BRENNAN, G. L., C. Potter, N. de Vere, G. W. Griffith, C. A. Skjøth, N. J. Osborne, B. W. Wheeler, et al. 2019. Temperate airborne grass pollen defined by spatio-temporal shifts in community composition. *Nature ecology & evolution* 3:750–754

BROWN, J. K. M., and M. S. Hovmøller. 2002. Aerial dispersal of pathogens on the global and continental scales and its impact on plant disease. *Science* 297:537–541

BUCKLAND, S. T., S. R. Baillie, J. M. P. Dick, and D. A. Elston. 2012. How should regional biodiversity be monitored? Environmental and ecological statistics 19 (4) doi: 10.1007/s10651-0202-7

BUNGE, J., A. Willis, and F. Walsh. 2014. Estimating the Number of Species in Microbial Diversity Studies. *Annual Review of Statistics and Its Application* 1(1):427-445 doi: 10.1146/annurev-statistics-022513-115654

BUSH, A., R. Sollmann, A. Wilting, K. Bohmann, B. Cole, H. Balzter, C. Martius, et al. 2017. Connecting Earth observation to high-throughput biodiversity data. *Nature Ecology & Evolution* 1:0176–0149

CAI, W., Z. Ma, C. Yang, L. Wang, W. Wang, G. Zhao, Y. Geng, et al. 2017. Using eDNA to detect the distribution and density of invasive crayfish in the Honghe-Hani rice terrace World Heritage site. *PLoS One* 12:e0177724–13

CALLAHAN, B. J., P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson, and S. P. Holmes. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nature methods* 13:581–583

CALVIGNAC-SPENCER, S., F. H. Leendertz, M. T. P. Gilbert, and G. Schubert. 2013. An invertebrate stomach’s view on vertebrate ecology: certain invertebrates could be used as “vertebrate samplers” and deliver DNA-based information on many aspects of vertebrate ecology. *BioEssays: news and reviews in molecular, cellular and developmental biology* 35:1004–1013

CARRARO, L., H. Hartikainen, J. Jokela, E. Bertuzzo, and A. Rinaldo. 2018. Estimating species distribution and abundance in river networks using environmental DNA. *Proceedings of the National Academy of Sciences of the United States of America* 115:11724–11729

CBOL Plant Working Group. 2009. A DNA barcode for land plants. Proceedings of the National Academy of Sciences of the United States of America 106:12794–12797.

Chao, A. 1984. Nonparametric Estimation of the Number of Classes in a Population. *Scandinavian journal of statistics, theory and applications* 11:265–270

CHAO, A. 1987. Estimating the population size for capture-recapture data with unequal catchability. *Biometrics* 43:783–791

CHEN, S., Y. Zhou, Y. Chen, and J. Gu. 2018. fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34:i884–i890

COISSAC, E., P. M. Hollingsworth, S. Lavergne, and P. Taberlet. 2016. From barcodes to genomes: extending the concept of DNA barcoding. *Molecular ecology*

CORDIER, T., P. Esling, F. Lejzerowicz, J. Visco, A. Ouadahi, C. Martins, T. Cedhagen, et al. 2017. Predicting the Ecological Quality Status of Marine Environments from eDNA Metabarcoding Data Using Supervised Machine Learning. *Environmental science and technology* 51:9118–9126

CRAMPTON-PLATT, A., M. J. T. N. Timmermans, M. L. Gimmel, S. N. Kutty, T. D. Cockerill, C. Vun Khen, and A. P. Vogler. 2015. Soup to Tree: The Phylogeny of Beetles Inferred by Mitochondrial Metagenomics of a Bornean Rainforest Sample. *Molecular biology and evolution* 32:2302–2316

CRAMPTON-PLATT, A., D. W. Yu, X. Zhou, and A. P. Vogler. 2016. Mitochondrial metagenomics: letting the genes out of the bottle. *GigaScience* 5:15

CREEDY, T. J., H. Norman, C. Q. Tang, K. Q. Chin, C. Andújar, P. Arribas, R. O'Connor, et al. 2019. A validated workflow for rapid taxonomic assignment and monitoring of a national fauna of bees (Apiformes) using high throughput barcoding. *bioRxiv* 6:1–38

CREER, S., K. Deiner, S. Frey, D. Porazinska, P. Taberlet, W. K. Thomas, C. Potter, et al. 2016. The ecologist's field guide to sequence-based identification of biodiversity. (R. Freckleton, ed.) *Methods in ecology and evolution* 7:1008–1018

CURD, E. E., Z. Gold, G. S. Kandlikar, J. Gomer, M. Ogden, T. O'Connell, L. Pipes, et al. 2019. Anacapa Toolkit: An environmental DNA toolkit for processing multilocus metabarcode datasets. *Methods in ecology and evolution / British Ecological Society* 10:1469–1475

DAKOS, V., S. R. Carpenter, E. H. van Nes, and M. Scheffer. 2015. Resilience indicators: prospects and limitations for early warnings of regime shifts. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* 370:20130263

DEAGLE, B. E., L. J. Clarke, J. A. Kitchener, A. M. Polanowski, and A. T. Davidson. 2018. Genetic monitoring of open ocean biodiversity: An evaluation of DNA metabarcoding for processing continuous plankton recorder samples. *Molecular ecology resources* 18:391–406

Defra. 2019. Measuring environmental change: outcome indicator framework for the 25 Year Environment Plan. Department for Environment Food & Rural Affairs

DEINER, K., and F. Altermatt. 2014. Transport distance of invertebrate environmental DNA in a natural river. *PLoS One* 9:e88786

DIETZ, T., E. Ostrom, and P. C. Stern. 2003. The struggle to govern the commons. *Science* 302:1907–1912

DOI, H., R. Inui, Y. Akamatsu, K. Kanno, H. Yamanaka, T. Takahara, and T. Minamoto. 2017. Environmental DNA analysis for estimating the abundance and biomass of stream fish. *Freshwater biology* 62:30–39

DORAZIO, R. M., and R. A. Erickson. 2018. eDNA occupancy: An r package for multiscale occupancy modelling of environmental DNA data. *Molecular ecology resources* 18:368–380

DORAZIO, R. M., and J. A. Royle. 2005. Estimating Size and Composition of Biological Communities by Modeling the Occurrence of Species. *Journal of the American Statistical Association* 100:389–398

DORNELAS, M., N. J. Gotelli, B. McGill, H. Shimadzu, F. Moyes, C. Sievers, and A. E. Magurran. 2014. Assemblage time series reveal biodiversity change but not systematic loss. *Science* 344:296–299

DU TOIT, J. T., and N. Pettorelli. 2019. The differences between rewilding and restoring an ecologically degraded landscape. (M. Cadotte, ed.)*The Journal of applied ecology* 56:2467–2471

EDWARDS, D. P., A. Magrach, P. Woodcock, Y. Ji, N. T. L. Lim, F. A. Edwards, T. H. Larsen, et al. 2014. Selective-logging and oil palm: multitaxon impacts, biodiversity indicators, and trade-offs for conservation planning. *Ecological applications: a publication of the Ecological Society of America* 24:2029–2049

EICHMILLER, J. J., P. G. Bajer, and P. W. Sorensen. 2014. The relationship between the distribution of common carp and their environmental DNA in a small lake. *PLoS One* 9:e112611

ELBRECHT, V., B. Peinert, and F. Leese. 2017. Sorting things out: Assessing effects of unequal specimen biomass on DNA metabarcoding. *Ecology and evolution* 7:6918–6926

FIELDS, B., S. Moeskjær, V.-P. Friman, S. U. Andersen, and J. P. W. Young. 2020. MAUI-seq: Metabarcoding using amplicons with unique molecular identifiers to improve error correction. *bioRxiv* **doi:** https://doi.org/10.1101/538587

FONSECA, V. G., G. R. Carvalho, W. Sung, H. F. Johnson, D. M. Power, S. P. Neill, M. Packer, et al. 2010. Second-generation environmental sequencing unmasks marine metazoan biodiversity. *Nature communications* 1:98

GELFAND, A. E., A. M. Schmidt, S. Wu, J. A. Silander, A. Latimer, and A. G. Rebelo. 2005. Modelling species diversity through species level hierarchical modelling. *Journal of the Royal Statistical Society. Series C, Applied statistics* 54:1–20.

GEOBON. 2018. Ecosystem Function. GEO BON

GLOOR, G. B., J. M. Macklaim, V. Pawlowsky-Glahn, and J. J. Egozcue. 2017. Microbiome Datasets Are Compositional: And This Is Not Optional. *Frontiers in microbiology* 8:2224

GOMEZ, J. P., S. K. Robinson, J. K. Blackburn, and J. M. Ponciano. 2018. An efficient extension of N-mixture models for multi-species abundance estimation. *Methods in ecology and evolution / British Ecological Society* 9:340–353

GÓMEZ-RODRÍGUEZ, C., A. C. Platt, M. J. T. N. Timmermans, A. Baselga, and A. P. Vogler. 2015. Validating the power of mitochondrial metagenomics for community ecology and phylogenetics of complex assemblages. *Methods in ecology and evolution / British Ecological Society* 6:883–894

GOTELLI, N. J., and A. Chao. 2013. Measuring and Estimating Species Richness, Species Diversity, and Biotic Similarity from Sampling Data. *Encyclopedia of Biodiversity* 195–211

GREENFIELD, P., N. Tran-Dinh, and D. Midgley. 2019. Kelpie: generating full-length “amplicons” from whole-metagenome datasets. *PeerJ* 6:e6174

GRIFFIN, J. E., E. Matechou, A. S. Buxton, D. Bormpoudakis, and R. A. Griffiths. 2019. Modelling environmental DNA data; Bayesian variable selection accounting for false positive and false negative errors. *Journal of the Royal Statistical Society*. Series C, Applied statistics https://doi.org/10.1111/rssc.12390

GUEUNING, M., D. Ganser, S. Blaser, M. Albrecht, E. Knop, C. Praz, and J. E. Frey. 2019. Evaluating next-generation sequencing (NGS) methods for routine monitoring of wild bees: Metabarcoding, mitogenomics or NGS barcoding. *Molecular ecology resources* 19:847–862

GUILLERA-ARROITA, G., M. Kéry, and J. J. Lahoz-Monfort. 2019. Inferring species richness using multispecies occupancy modeling: Estimation performance and interpretation. *Ecology and Evolution*. https://doi.org/10.1002/ece3.4821

GUILLERA-ARROITA, G., J. J. Lahoz-Monfort, A. R. van Rooyen, A. R. Weeks, and R. Tingley. 2017. Dealing with false-positive and false-negative errors about species occurrence at multiple levels. *Methods in Ecology and Evolution*. doi: 10.1111/2041-210X.12743

HAJIBABAEI, M., S. Shokralla, X. Zhou, G. A. C. Singer, and D. J. Baird. 2011. Environmental barcoding: a next-generation sequencing approach for biomonitoring applications using river benthos. *PLoS One* 6:e17497

HÄNFLING, B., L. Lawson Handley, D. S. Read, C. Hahn, J. Li, P. Nichols, R. C. Blackman, et al. 2016. Environmental DNA metabarcoding of lake fish communities reflects long-term data from established survey methods. *Molecular ecology* 25:3101–3119

HANSEN, B. K., D. Bekkevold, L. W. Clausen, and E. E. Nielsen. 2018. The sceptical optimist: challenges and perspectives for the application of environmental DNA in marine fisheries. *Fish and fisheries* 19:751–768

HEBERT, P. D. N., T. W. A. Braukmann, S. W. J. Prosser, S. Ratnasingham, J. R. deWaard, N. V. Ivanova, D. H. Janzen, et al. 2018. A Sequel to Sanger: amplicon sequencing that scales. *BMC Genomics* Mar 27;19 (1):219. doi: 10.1186/s12864-018-4611-3

HEBERT, P. D. N., A. Cywinska, S. L. Ball, and J. R. Dewaard. 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society of London: *Biological Sciences* 270:313–321

HEEGER, F., E. C. Bourne, C. Baschien, A. Yurkov, B. Bunk, C. Spröer, J. Overmann, et al. 2018. Long-read DNA metabarcoding of ribosomal RNA in the analysis of fungi from aquatic environments. *Molecular ecology resources* 18:1500–1514

HERING, D., Á. Borja, J. I. Jones, D. Pont, P. Boets, A. Bouchez, K. Bruce, et al. 2018. Implementation options for DNA-based identification into ecological status assessment under the European Water Framework Directive. *Water research* 1–56

HIIESALU, I., M. Öpik, M. Metsis, L. Lilje, J. Davison, M. Vasar, M. Moora, et al. 2012. Plant species richness belowground: higher richness and new patterns revealed by next-generation sequencing. *Molecular ecology* 21:2004–2016

HODGSON, D., J. L. McDonald, and D. J. Hosken. 2015. What do you mean, “resilient”? *Trends in ecology and evolution* 30:503–506

HOSHINO, T., and F. Inagaki. 2017. Application of Stochastic Labeling with Random-Sequence Barcodes for Simultaneous Quantification and Sequencing of Environmental 16S rRNA Genes. *PLoS One* 12:e0169431

ISBELL, F., V. Calcagno, A. Hector, J. Connolly, W. S. Harpole, P. B. Reich, M. Scherer-Lorenzen, et al. 2011. High plant diversity is needed to maintain ecosystem services. *Nature* 1–5

JANE, S. F., T. M. Wilcox, K. S. McKelvey, M. K. Young, M. K. Schwartz, W. H. Lowe, B. H. Letcher, et al. 2014. Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. *Molecular ecology resources* 15:216–227

JANZEN, D. H., M. Hajibabaei, J. M. Burns, W. Hallwachs, E. Remigio, and P. D. N. Hebert. 2005. Wedding biodiversity inventory of a large and complex Lepidoptera fauna with DNA barcoding. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* 360:1835–1845

JERDE, C. L. 2019. Can we manage fisheries with the inherent uncertainty from eDNA? *Journal of fish biology*. https://doi.org/10.1111/jfb.14218

Jerde, C. L., A. R. Mahon, W. L. Chadderton, and D. M. Lodge. 2011. “Sight-unseen” detection of rare aquatic species using environmental DNA. *Conservation Letters* 4:150–157

JI, Y., L. Ashton, S. M. Pedley, D. P. Edwards, Y. Tang, A. Nakamura, R. Kitching, et al. 2013. Reliable, verifiable and efficient monitoring of biodiversity via metabarcoding. *Ecology letters* 16:1245–1257

JI, Y., T. Huotari, T. Roslin, N. M. Schmidt, J. Wang, D. W. Yu, and O. Ovaskainen. 2019. SPIKEPIPE: A metagenomic pipeline for the accurate quantification of eukaryotic species occurrences and intraspecific abundance change using DNA barcodes or mitogenomes. Molecular ecology resources. 20(2) doi: 10.1111/1755-0998.13057

JI, Y., C. C. M. Baker, Y. Li, V. D. Popescu, Z. Wang, J. Wang, L. Wang, et al. 2020. Large-scale Quantification of Vertebrate Biodiversity in Ailaoshan Nature Reserve from Leech iDNA. bioRxiv **doi:** https://doi.org/10.1101/2020.02.10.941336

KARST, S. M., R. M. Ziels, R. H. Kirkegaard, E. A. Sørensen, D. McDonald, Q. Zhu, R. Knight, et al. 2020. Enabling high-accuracy long-read amplicon sequences using unique molecular identifiers with Nanopore or PacBio sequencing. bioRxiv **doi:** https://doi.org/10.1101/645903

KOSAKOVSKY-POND, S., S. Wadhawan, F. Chiaromonte, G. Ananda, W.-Y. Chung, J. Taylor, A. Nekrutenko, et al. 2009. Windshield splatter analysis with the Galaxy metagenomic pipeline. *Genome research* 19:2144–2153

KOZLOV, A. M., J. Zhang, P. Yilmaz, F. O. Glöckner, and A. Stamatakis. 2016. Phylogeny-aware identification and correction of taxonomically mislabeled sequences. *Nucleic acids research* 44:5022–5033

LACOURSIÈRE-ROUSSEL, A., G. Côté, V. Leclerc, and L. Bernatchez. 2016. Quantifying relative fish abundance with eDNA: a promising tool for fisheries management. *The Journal of applied ecology* 53:1148–1157

LACOURSIÈRE-ROUSSEL, A., and K. Deiner. 2019. Environmental DNA is not the tool by itself. *Journal of fish biology*. https://doi.org/10.1111/jfb.14177

LANG, D., M. Tang, J. Hu, and X. Zhou. 2019. Genome-skimming provides accurate quantification for pollen mixtures. *Molecular ecology resources* 19:1433–1446

LAWSON-HANDLEY, L., D. S. Read, I. J. Winfield, H. Kimbell, H. Johnson, J. Li, C. Hahn, et al. 2019. Temporal and spatial variation in distribution of fish environmental DNA in England's largest lake. *Environmental DNA* 1:26–39

LEEMPOEl, K., T. Hebert, and E. A. Hadly. 2020. A comparison of eDNA to camera trapping for assessment of terrestrial mammal diversity. *Proceedings. Biological sciences / The Royal Society* 287:20192353

LEINSTER, T., and C. A. Cobbold. 2012. Measuring diversity: the importance of species similarity. *Ecology* 93:477–489

LEJZEROWICZ, F., P. Esling, L. Pillet, T. A. Wilding, K. D. Black, and J. Pawlowski. 2015. High-throughput sequencing and morphology perform equally well for benthic monitoring of marine ecosystems. *Scientific reports* 5:13932

LERAY, M., J. Y. Yang, C. P. Meyer, S. C. Mills, N. Agudelo, V. Ranwez, J. T. Boehm, et al. 2013. A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterising coral reef fish gut contents. *Frontiers in zoology* 10:34

LEVI, T., J. M. Allen, D. Bell, J. Joyce, J. R. Russell, D. A. Tallmon, S. C. Vulstek, et al. 2019. Environmental DNA for the enumeration and management of Pacific salmon. *Molecular ecology resources* 19:597–608

LI, J., L. J. Lawson Handley, L. R. Harper, R. Brys, H. V. Watson, C. Di Muri, X. Zhang, et al. 2019. Limited dispersion and quick degradation of environmental DNA in fish ponds inferred by metabarcoding. *Environmental DNA* 1:238–250

MACHIDA, R. J., M. Leray, S.-L. Ho, and N. Knowlton. 2017. Metazoan mitochondrial gene sequence reference datasets for taxonomic assignment of environmental samples. *Scientific data* 4:170027

MACKENZIE, D. I., J. D. Nichols, G. B. Lachman, S. Droege, J. Andrew Royle, and C. A. Langtimm. 2002. Estimating site occupancy rates when detection probabilities are less than one. *Ecology* 83:2248–2255

MAGURRAN, A. E., M. Dornelas, F. Moyes, N. J. Gotelli, and B. McGill. 2015. Rapid biotic homogenization of marine fish assemblages. Nature communications 6:8405.

Maurer, B. A., and B. J. McGill. 2011. Measurement of species diversity. Biological diversity: frontiers in measurement and assessment 55–65.

MCLAREN, M. R., A. D. Willis, and B. J. Callahan. 2019. Consistent and correctable bias in metagenomic sequencing experiments. eLife 8. doi: 10.7554/eLife.46923

MCMURDIE, P. J., and S. Holmes. 2014. Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible. *PLoS computational biology* 10:e1003531

MEIER, R., W. Wong, A. Srivathsan, and M. Foo. 2016. $1 DNA barcodes for reconstructing complex phenomes and finding rare species in specimen-rich samples. Cladistics: *The international journal of the Willi Hennig Society* 32:100–110

MIYA, M., Y. Sato, T. Fukunaga, T. Sado, J. Y. Poulsen, K. Sato, T. Minamoto, et al. 2015. MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. *Royal Society Open Science* 2:150088–150033

MURRAY, D. C., M. L. Coghlan, and M. Bunce. 2015. From benchtop to desktop: important considerations when designing amplicon sequencing workflows. *PLoS One* 10:e0124671–21

NEVILL, P. G., X. Zhong, J. Tonti-Filippini, M. Byrne, M. Hislop, K. Thiele, S. van Leeuwen, et al. 2020. Large scale genome skimming from herbarium material for accurate plant identification and phylogenomics. *Plant methods* 16:1

NIE, R., J. Wei, S. Zhang, A. P. Vogler, L. Wu, A. S. Konstantinov, W. Li, et al. 2019. Diversification of mitogenomes in three sympatric Altica flea beetles (Insecta, Chrysomelidae). *Zoologica scripta* 48:657–666

NILSSON, R. H., R. Henrik Nilsson, M. Ryberg, E. Kristiansson, K. Abarenkov, K.-H. Larsson, and U. Kõljalg. 2006. Taxonomic Reliability of DNA Sequences in Public Sequence Databases: A Fungal Perspective. *PLoS One* https://doi.org/10.1371/journal.pone.0000059

NILSSON, R. H., K.-H. Larsson, A. F. S. Taylor, J. Bengtsson-Palme, T. S. Jeppesen, D. Schigel, P. Kennedy, et al. 2019. The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. *Nucleic acids research* 47:D259–D264

OLIVER, T. H., M. S. Heard, N. J. B. Isaac, D. B. Roy, D. Procter, F. Eigenbrod, R. Freckleton, et al. 2015. Biodiversity and Resilience of Ecosystem Functions. *Trends in ecology and evolution* 30:673–684

ONDOV, B. D., T. J. Treangen, P. Melsted, A. B. Mallonee, N. H. Bergman, S. Koren, and A. M. Phillippy. 2016. Mash: fast genome and metagenome distance estimation using MinHash. *Genome biology* 17:132

OVASKAINEN, O., D. B. Roy, R. Fox, and B. J. Anderson. 2016. Uncovering hidden spatial structure in species communities with spatially explicit joint species distribution models. Methods in ecology and evolution / British Ecological Society 7:428–436.

Ovaskainen, O., J. Rybicki, and N. Abrego. 2019. What can observational data reveal about metacommunity processes? *Ecography* 42:1877–1886

OVASKAINEN, O., G. Tikhonov, A. Norberg, F. Guillaume Blanchet, L. Duan, D. Dunson, T. Roslin, et al. 2017. How to make more out of community data? A conceptual framework and its implementation as models and software. *Ecology letters* 20:561–576

PAUVERT, C., M. Buée, V. Laval, V. Edel-Hermann, L. Fauchery, A. Gautier, I. Lesur, et al. 2019. Bioinformatics matters: The accuracy of plant and soil fungal community data is highly dependent on the metabarcoding pipeline. *Fungal ecology* 41:23–33

PAWLOWSKI, J., P. Esling, F. Lejzerowicz, T. Cordier, J. A. Visco, C. I. M. Martins, A. Kvalvik, et al. 2016. Benthic monitoring of salmon farms in Norway using foraminiferal metabarcoding. *Aquaculture Environment Interactions* 8:371–386

PEDERSEN, M. W., S. Overballe-Petersen, L. Ermini, C. Der Sarkissian, J. Haile, M. Hellstrom, J. Spens, et al. 2015. Ancient and modern environmental DNA. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* 370:20130383–20130383

PEEL, N., L. V. Dicks, Clark, D. Heavens, L. Percival-Alwyn, C. Cooper, R. G. Davies, et al. 2019. Semi-quantitative characterisation of mixed pollen samples using MinION sequencing and Reverse Metagenomics (RevMet). Methods in ecology and evolution. https://doi.org/10.1111/2041-210X.13265

PIÑOL, J., G. Mir, P. Gomez Polo and N. Agustí. 2015. Universal and blocking primer mismatches limit the use of high-throughput DNA sequencing for the quantitative metabarcoding of arthropods. *Molecular ecology resources* 15:819–830

PIÑOL, J., M. A. Senar and W. O. C. Symondson. 2018. The Choice Of Universal Primers And The Characteristics Of The Species Mixture Determine When DNA Metabarcoding Can Be Quantitative. *Molecular Ecology Notes* 28:407–419

POCHARDT, M., J. M. Allen, T. Hart, S. D. L. Miller, D. W. Yu, and T. Levi. 2020. Environmental DNA facilitates accurate, inexpensive, and multiyear population estimates of millions of anadromous fish. *Molecular ecology resources* 20:457–467

PONISIO, L. C., P. de Valpine, L. K. M'Gonigle, and C. Kremen. 2019. Proximity of restored hedgerows interacts with local floral diversity and species' traits to shape long-term pollinator metacommunity dynamics. *Ecology letters* 22:1048–1060

QUAST, C., E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, et al. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research* 41:D590–6

RATNASINGHAM, S. 2019. mBRAVE: The Multiplex Barcode Research And Visualization Environment. Biodiversity Information *Science and Standards* 3:e37986

RATNASINGHAM, S., and P. D. N. Hebert. 2007. BOLD: The Barcode of Life Data System (www.barcodinglife.org). *Molecular ecology notes* 7:355–364

RODGERS, T. W., C. C. Y. Xu, J. Giacalone, K. M. Kapheim, K. Saltonstall, M. Vargas, D. W. Yu, et al. 2017. Carrion fly-derived DNA metabarcoding is an effective tool for mammal surveys: Evidence from a known tropical mammal community. *Molecular ecology resources* 215:403–413

ROTA, C. T., M. A. R. Ferreira, R. W. Kays, T. D. Forrester, E. L. Kalies, W. J. McShea, A. W. Parsons, et al. 2016. A multispecies occupancy model for two or more interacting species. *Methods in Ecology and Evolution*. https://doi.org/10.1111/2041-210X.12587

ROYLE, J. A. 2004. N-mixture models for estimating population size from spatially replicated counts. *Biometrics* 60:108–115

ROYLE, J. A., and W. A. Link. 2006. Generalized site occupancy models allowing for false positive and false negative errors. *Ecology* 87:835–841

RUPPERT, K. M., R. J. Kline, and M. S. Rahman. 2019. Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. *Global Ecology and Conservation* 17:e00547. DOI: 10.1016/j.gecco.2019.e00547

SALES, N. G., M. B. McKenzie, J. Drake, L. R. Harper, S. S. Browett, I. Coscia, O. S. Wangensteen, et al. 2020. Fishing for mammals: Landscape-level monitoring of terrestrial and semi-aquatic communities using eDNA from riverine systems. (B. Mosher, ed.)*The Journal of applied ecology*. https://doi.org/10.1111/1365-2664.13592

SALTER, I., M. Joensen, R. Kristiansen, P. Steingrund, and P. Vestergaard. 2019. Environmental DNA concentrations are correlated with regional biomass of Atlantic cod in oceanic waters. *Communications Biology* 2:461

SARMASHGHI, S., K. Bohmann, M. T. P Gilbert, V. Bafna, and S. Mirarab. 2019. Skmer: assembly-free and alignment-free sample identification using genome skims. *Genome biology* 20:34.

SCHENK, J., S. Geisen, N. Kleinboelting, and W. Traunspurger. 2019. Metabarcoding data allow for reliable biomass estimates in the most abundant animals on earth. *Metabarcoding and Metagenomics* 3:e46704. https://doi.org/10.3897/mbmg.3.46704

SCHNELL, I. B., R. Sollmann, S. Calvignac-Spencer, M. E. Siddall, D. W. Yu, A. Wilting, and M. T. P. Gilbert. 2015. iDNA from terrestrial haematophagous leeches as a wildlife surveying and monitoring tool – prospects, pitfalls and avenues to be developed. *Frontiers in zoology* 12:302

SCHNELL, I. B., P. F. Thomsen, N. Wilkinson, M. Rasmussen, L. R. D. Jensen, E. Willerslev, M. F. Bertelsen, et al. 2012. Screening mammal biodiversity using DNA from leeches. *Current biology*: CB 22:R262–R263

SCHUBERT, G., M. Stockhausen, C. Hoffmann, K. Merkel, L. Vigilant, F. H. Leendertz, and S. Calvignac-Spencer. 2014. Targeted detection of mammalian species using carrion fly-derived DNA. *Molecular ecology resources* 15:285–294

SHANNON, C. E. 1948. A Mathematical Theory of Communication. *Bell System Technical Journal* 27:379–423

SHERWOOD, A. R., M. N. Dittbern, E. T. Johnston, and K. Y. Conklin. 2016. A metabarcoding comparison of windward and leeward airborne algal diversity across the Koʻolau mountain range on the island of Oʻahu, Hawaiʻi. *Journal of phycology*. 53 (2) :437-445. doi: 10.1111/jpy.12502

SHOGREN, A. J., J. L. Tank, E. Andruszkiewicz, B. Olds, A. R. Mahon, C. L. Jerde, and D. Bolster. 2017. Controls on eDNA movement in streams: Transport, Retention, and Resuspension. *Scientific reports* 7:5065

SIMPSON, E. H. 1949. Measurement of Diversity. *Nature* 163:688–688

SMETS, W., J. W. Leff, M. A. Bradford, R. L. McCulley, S. Lebeer, and N. Fierer. 2016. A method for simultaneous measurement of soil bacterial abundances and community composition via 16S rRNA gene sequencing. *Soil biology and biochemistry* 96:145–151

SOMERVUO, P., S. Koskela, J. Pennanen, R. Henrik Nilsson, and O. Ovaskainen. 2016. Unbiased probabilistic taxonomic classification for DNA barcoding. *Bioinformatics* 32:2920–2927

SOMERVUO, P., D. W. Yu, C. C. Y. Xu, Y. Ji, J. Hultman, H. Wirta, and O. Ovaskainen. 2017. Quantifying uncertainty of taxonomic placement in DNA barcoding and metabarcoding. *Methods in ecology and evolution / British Ecological Society* 8:398–407

SPEARS, B. M., S. C. Ives, D. G. Angeler, C. R. Allen, S. Birk, L. Carvalho, S. Cavers, et al. 2015. Effective management of ecological resilience - are we there yet? *The Journal of applied ecology* 52:1311–1315

STRAUB, S. C. K., M. Parks, K. Weitemier, M. Fishbein, R. C. Cronn, and A. Liston. 2012. Navigating the tip of the genomic iceberg: Next-generation sequencing for plant systematics. *American journal of botany* 99:349–364

TANG, M., C. J. Hardman, Y. Ji, G. Meng, S. Liu, M. Tan, S. Yang, et al. 2015. High-throughput monitoring of wild bee diversity and abundance via mitogenomics. *Methods in ecology and evolution / British Ecological Society* 6:1034–1043

THOMSEN, P. F., J. Kielgast, L. L. Iversen, C. Wiuf, M. Rasmussen, M. T. P. Gilbert, L. Orlando, et al. 2012. Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular ecology* 21:2565–2573

THOMSEN, P. F., P. R. Møller, E. E. Sigsgaard, S. W. Knudsen, O. A. Jørgensen, and E. Willerslev. 2016. Environmental DNA from Seawater Samples Correlate with Trawl Catches of Subarctic, Deepwater Fishes. *PLoS One* 11:e0165252

TILKER, A., J. F. Abrams, A. Nguyen, L. Hörig, J. Axtner, J. Louvrier, B. M. Rawson, et al. 2020. Identifying conservation priorities in a defaunated tropical biodiversity hotspot. (L. Maiorano, ed) *Diversity and distributions* 10:100331

TINGLEY, M. W., C. P. Nadeau, and M. E. Sandor. 2020. Multi-species occupancy models as robust estimators of community richness. (A. Baselga, ed) *Methods in ecology and evolution / British Ecological Society* 11:265

TKACZ, A., M. Hortala, and P. S. Poole. 2018. Absolute quantitation of microbiota abundance in environmental samples. *Microbiome* 6:110

TURNER, C. R., M. A. Barnes, C. C. Y. Xu, S. E. Jones, C. L. Jerde, and D. M. Lodge. 2014. Particle size distribution and optimal capture of aqueous macrobial eDNA. (M. Gilbert, ed.)Methods in ecology and evolution / British Ecological Society 5:676–684.

Vellend, M. 2010. Conceptual synthesis in community ecology. *The Quarterly review of biology* 85:183–206

VELLEND, M., L. Baeten, I. H. Myers-Smith, S. C. Elmendorf, R. Beauséjour, C. D. Brown, P. De Frenne, et al. 2013. Global meta-analysis reveals no net change in local-scale plant biodiversity over time. *Proceedings of the National Academy of Sciences of the United States of America* 110:19456–19459

VISCO, J. A., L. Apothéloz-Perret-Gentil, A. Cordonier, P. Esling, L. Pillet, and J. Pawlowski. 2015. Environmental Monitoring: Inferring the Diatom Index from Next-Generation Sequencing Data. *Environmental science and technology* 49:7597–7605

WALKER, B., C. S. Holling, S. R. Carpenter, and A. P. Kinzig. 2004. Resilience, adaptability and transformability in social–ecological systems. Ecology and Society 9:5.

Wangensteen, O. S., E. Cebrian, C. Palacín, and X. Turon. 2018. Under the canopy: Community-wide effects of invasive algae in Marine Protected Areas revealed by metabarcoding. *Marine pollution bulletin* 127:54–66

WANG, X., F. Hua, L. Wang, D. S. Wilcove, and D. W. Yu. 2019. The biodiversity benefit of native forests and mixed-species plantations over monoculture plantations. Diversity and Distributions https://doi.org/10.1111/ddi.12972

WARTON, D. I., F. G. Blanchet, R. B. O'Hara, O. Ovaskainen, S. Taskinen, S. C. Walker, and F. K. C. Hui. 2015. So Many Variables: Joint Modeling in Community Ecology. *Trends in ecology & evolution* 30:766–779

WILLIAMSON, B. D., J. P. Hughes, and A. D. Willis. 2019. A multi-view model for relative and absolute microbial abundances. bioRxiv. doi: https://doi.org/10.1101/761486

WILLIS, A. D. 2019. Rarefaction, Alpha Diversity, and Statistics. *Frontiers in microbiology* 10:2407

WIRTA, H. K., P. D. N. Hebert, R. Kaartinen, S. W. Prosser, G. Várkonyi, and T. Roslin. 2014. Complementary molecular information changes our perception of food web structure. *Proceedings of the National Academy of Sciences of the United States of America* 111:1885–1890

WRIGHT, W. J., K. M. Irvine, E. S. Almberg, and A. R. Litt. 2020. Modelling misclassification in multi-species acoustic data when estimating occupancy and relative activity. (N. Yoccoz, ed) *Methods in ecology and evolution / British Ecological Society* 11:71–81

YANG, C.Y., Bohmann, K., Wang, X.Y., Wang, C., Wales, N., Ding, Z.L., Gopalakrishnan, S., Yu, D.W. Biodiversity Soup II: A bulk-sample metabarcoding pipeline emphasizing error reduction. *bioRxiv*. doi: https://www.biorxiv.org/content/10.1101/2020.07.07.187666v3

YOCCOZ, N. G., K. A. Bråthen, L. Gielly, J. Haile, M. E. Edwards, T. Goslar, H. Von Stedingk, et al. 2012. DNA from soil mirrors plant taxonomic and growth form diversity. *Molecular ecology* 21:3647–3655

YOURSTONE, S. M., D. S. Lundberg, J. L. Dangl, and C. D. Jones. 2014. MT-Toolbox: improved amplicon sequencing using molecule tags. *BMC bioinformatics* 15:284

YU, D. W., Y. Ji, B. C. Emerson, X. Wang, C. Ye, C. Yang, and Z. Ding. 2012. Biodiversity soup: metabarcoding of arthropods for rapid biodiversity assessment and biomonitoring. *Methods in ecology and evolution / British Ecological Society* 3:613–623.

ZENG, W., B. Zhou, P. Lei, Y. Zeng, Y. Liu, C. Liu, and W. Xiang. 2015. A molecular method to identify species of fine roots and to predict the proportion of a species in mixed samples in subtropical forests. *Frontiers in plant science* 6:313

ZEPEDA MENDOZA, M. L., K. Bohmann, A. Carmona Baez, and M. T. P. Gilbert. 2016. DAMe: a toolkit for the initial processing of datasets with PCR replicates of double-tagged amplicons for DNA metabarcoding analyses. *BMC research notes* 9:255

ZHOU, X., Y. Li, S. Liu, Q. Yang, X. Su, L. Zhou, M. Tang, et al. 2013. Ultra-deep sequencing enables high-fidelity recovery of biodiversity for bulk arthropod samples without PCR amplification. *GigaScience* 2:4

ZIELEZINSKI, A., H. Z. Girgis, G. Bernard, C.-A. Leimeister, K. Tang, T. Dencker, A. K. Lau, et al. 2019. Benchmarking of alignment-free sequence comparison methods. *Genome biology* 20:144

Think-Piece 5: Technology solutions to evaluate ecosystem function

Iain Perry and Peter Kille

Cardiff School of Biosciences, Cardiff University, Sir Martin Evans Building, Cardiff, CF10 3AX.

Contents

Tables

Summary

Lay Summary

Isolation of genetic material from environmental samples (soil, water sediment), so called eDNA is now routine. These samples provide insight into the macro and micro ecology of the ecosystem from which they are isolated. Targeting specific sequences from specific species allows non-invasive sampling of organisms of interest. Information derived from DNA can be used to support conservation efforts and it also allows the tracking of pathogens and invasive species. More generic approaches allow us to profile bacterial, fungal, algal, plant or animal species. From these data we can perform multi-dimensional analysis, addressing key environmental questions. For example, a bacterial profile can report on the presence of pathogens or the impact of pollutants. Quantitative genetic approaches and Next Generation Sequencing technology that supports the use of eDNA, represents a mature technology with validated applications throughout the healthcare industry. New innovations will provide additional, enhanced utility and cost efficiencies, supporting increased spatial and temporal resolution monitoring with the potential to provide real time surveillance. Currently, these eDNA approaches have been deployed as adjuncts to established approaches limiting the potential benefits gained. Our recommendation is for a new generation of biomonitoring approaches to be adopted based on the full potential proved by eDNA. The potential of these novel eDNA approaches combined with good ecological knowledge and interpretation will provide the tools needed to realise ‘A Green Future’, and deliver the “Plan to Improve the Environment”.

Since the technology is well established, the major hurdles to exploiting eDNA tools are in transferring from research tools to regulatory and industrial implementation. Our recommendation therefore are focused around a four step process to fast-track this objective:

1. Define and specify the explicit need or question.
2. Establish a transparent ‘AGILE’ assessments and validation processes.
3. Liaise between UKRI and BEIS to establish funding pipeline from research concept to product.
4. Engage with the industrial sectors to deliver products and services for the environmental sector.

Executive summary

DNA-based approaches are pervasive diagnostic tools in the arena of human health. Here we address the question of whether there are equivalents that can be applied to environmental health, either for proactive protection or for routine monitoring. The current reorganisation of the environmental legislation associated with the break from the European Union, together with advances in DNA-based environmental research, provide us with a timely opportunity to embrace new technologies in attaining a ‘green future’ for the UK. The regulatory acceptance of DNA based tools targeting single species has been

firmly establish, as exemplified by the Great Crested Newt assay demonstrating the utility of such technologies to support the monitoring and protection of the environment, whilst begging the question as to the barriers that are preventing further tests from being deployed. Rapid developments in Next Generation Sequencing (NGS) Technologies have provide a ‘technology push’ whilst validation of the diagnostic strengths of these approaches is starting to result in a ‘market pull’ for new endorsed and ratified tests. The presence of an emerging private sector SME base combined with inward investment by government agencies to establish DNA based expertise and facilities, indicates the future economic potential of this sector. The ability to exploit environmental DNA (eDNA) for single species detection is transforming our ability to monitor threatened and invasive non-native (alien) species and survey for vectors of disease. Unlocking the potential for these non-invasively collected samples to provide valuable quantitative information associated with biodiversity levels, from fish stocking levels to metrics that can be used within Environmental Quality Surveys, is the current area of substantial research activity. DNA based approaches have the capability to provide SMART metrics that support ‘effects-based monitoring’, linking environmental changes to their functional consequences, assisting management and supporting mitigation strategies. Fundamental research has delivered a surfeit of DNA based environmental tools; regulators now need to establish a transparent fast-track process for adoption and accreditation for mature assays whilst challenging the research community to deliver innovative approaches to priority challenges.

Introduction

Mankind is both intentionally and unintentionally re-shaping the world resulting in impacts at global and local levels. Changes in the composition of our atmosphere have a direct link to climate change and ocean acidification. Intensive farming practices threaten soil structure, whilst increased water abstraction driven by the needs of a growing population, together with the associated agriculture, is reshaping water availability across terrestrial ecosystems. These global drivers are overlaid at a local level, with changes in chemical and nutrient inputs stemming from diffuse and point source inputs of both agriculture and industry whilst many systems are still dealing with legacy contaminates released when regulation and monitoring was less stringent. These challenges are to be expected given the global social economic and industrial changes. How we respond is key, embracing technological advances to better manage and mitigate environmental change is essential if we are to deliver solutions to support the 'Environmental revolution' that values nature and the services it can deliver. From before globalisation, human travel has resulted in easy dispersal of biological material, resulting in rapid distribution of pathogens together with the introduction of organisms into non-native biomes, which in this context is representing invasive non-native 'alien' species (INNS) with the potential to disrupt delicate balanced ecologies. As a society we are becoming acutely aware of the vulnerability of our native species, many of which due to their intrinsic life history and scarceness are increasingly difficult to monitor.

The growing realisation embodied by the "One World - One Health" concept articulates the link between environmental and human health beyond the direct threats posed by infectious diseases and antimicrobial resistance. Direct regulatory frameworks, such as that represented by the Water Framework Directive, together with government policy documents such as the Defra's 25 Year Environment Plan, e.g. Defra's *A Green Future* (Department for Environment Food & Rural Affairs 2018), The Environment (Wales) Act 2016 (Welsh Government 2016) and the Scotland's *State of the Environment* report (Critchlow-Watton *et al.* 2014), communicate the importance of environmental protection to the health of the nation, but also empower the governmental bodies charged with this responsibility to improve the process and surveillance. The rate and degree of challenges to our ecosystem posed by a changing world together with rapid social realisation of its importance has not been mirrored by the implementation of new technologies to support this agenda. Whilst the medical and environmental research fraternities have reaped huge benefits from the DNA (molecular and genomics) revolution, the environmental regulatory sector has only a modest (but increasing) exploitation of these tools. The current article aims to explore the opportunities provided by current DNA technologies, the challenges (sampling to data interpretation) and the opportunities presented by this rapidly developing area. The objective is to assess technological solutions, being mindful of their 'technology readiness level (TRL)' (Council 2020), identifying the barriers that need to be overcome to accelerate their deployment and offer the regulatory community SMART solutions for a changing world.

A Question of Scale

DNA-based tools can be applied to evaluate an ecosystem at any level of scale, from community structure analysis reflecting a holistic read-out of environmental health, through to the response for a specific gene within a specified species as a diagnostic for the presence or functional impact of a pollutant of interest. Many of the core DNA methodologies are used widely in human health diagnostics, however, the effective integration of these methods into monitoring workflows to address specific environmental protection needs are at very different TRLs. Furthermore, there is a misplaced assumption that the DNA-based data generated provides direct and transparent ecosystem information, rather than the reality of requiring expert analysis and ecological judgement to generate an informative focused output. Here we will evaluate the DNA-based approaches stratified by 'scale' from techniques that attempt to evaluate ecosystems holistically, to those that address very specific species centric functional questions. Each individual sub-section will end by identifying the 'gaps' and 'R&D' related to sampling or processing, validation, reference data sets and data processing requirements.

Tools for ecosystem level analysis

Biodiversity analysis: Metabarcoding

The quantitative concept and workflow underlying metabarcoding is conceptually very straightforward and can be summarised in five steps; i) Environmental sampling, ii) DNA extraction, iii) Isolation of species specific 'barcodes' (short sequence of DNA diagnostic of a species), iv) High Throughput Sequencing, and; v) Barcode quantitation. However, the apparent transparency conceals critical issues ranging from sampling strategies through to concepts associated with our fundamental understanding and definition of 'species'.

Environmental sampling: For a 'metabarcoding' approach to provide a truthful representation of a community, you must first be able to representatively sample that community. This difficulty is not unique to DNA-based approaches. Ecosystems are (mostly) heterogeneous so sampling strategies have developed to address this issue and many of which can be integrated into DNA-based approaches. Whether sampling soil incorporating the established 'W' pattern or deploying appropriate matrix of terrestrial 'pitfalls', the same care to ensure representation must be taken for DNA as for no-DNA-based approaches. There are some ecological methodologies that are challenging to transfer – for example kick-sampling, a technique used for sampling stream invertebrates since it was developed in 1961 (Hynes 1961), liberates both a range of biotic and abiotic material. DNA extraction from this 'slurry' of material is impractical since it not only represents a large volume, but also many of the abiotic (sediment, small stones etc.) and dead plant matter can inhibit DNA extraction and processing. Although sorting the biotic matter normally employed for ecosystem assessment would generate an appropriate starting material, this process with appropriate expertise, would represent a significant contribution to the normal processing of the samples, therefore making any efficiency gained from subsequent DNA analysis minimal. However, this overlooks the value-added

data that DNA barcoding may provide due to the taxonomic insights which are extremely challenging to derive by hand, together with the removal of ‘assessor’ bias, resulting in a different skill-level in species identification.

There are sampling approaches that are unique to DNA approaches and provide significant value-added utility such as environmental DNA (eDNA) and invertebrate (gut) DNA (iDNA) that allow fragments of DNA to be sampled from the environment either mechanically or by using the natural native feeding behaviour of an invertebrate. Currently, eDNA is being extensively used in research with a specific application focus on detection of a threated species that has led to legislative regulatory tools for monitoring great crested newts (Defra - Great Crested Newt Detection WC1067 [4]) (Biggs et al. 2014), an approach that is discussed in Section “Targeting single species monitoring”. The sampling methodology described provides a valuable template, although, its application to community analysis needs careful consideration. If the technique is being used to indirectly evaluate invertebrate or vertebrate communities, it must be recognised that DNA released from each organism is externally varied and influenced by species specific physiology, i.e. malting or spawning. Furthermore, the physical and chemical properties of the environmental samples such as the hydrodynamics (flowrate, total volume, temperature etc.) of waterbodies, underlying geology, sediment properties and level of organic matter all influence DNA availability. Despite these issues eDNA analysis provides a rapid technique to evaluate diversity and if applied against appropriate comparative scenarios, e.g. evaluating up and down stream of a potential pollution point source or for long-term monitoring of a habitat, they can have significant value. A significant advantage of eDNA sampling approaches is that, in addition to indirectly providing insight into the macro invertebrate and vertebrate communities, they directly capture organisms representing other tropic levels including the prokaryotic microbiota and the micro-eukaryotes representing the primary producers. This allows a single eDNA sample to be used to provide metrics at multiple tropic levels. However, the sampling regime, volumes and temporal/spatial replication, that ensure ‘true’ representation, require appropriate validation. One example of a post sampling, pre DNA extraction process requiring such industry consolidation and validation is the means for preservation of samples between processes which can depend on the specific end use (Gray et al. 2013). Recent emergence of iDNA sampling represents the indirect sampling of biodiversity by exploiting the ‘feeding’ habits of an invertebrate which then has its ‘gut’ contents analysed to determine its diet is a surrogate for species presence (Siegenthaler et al. 2019). This approach exploits the natural range and dietary habits of the invertebrate, so selection and knowledge of the normal biology of the ‘sentinel’ species is essential. However, by selecting appropriate species, a wide geographical range may be monitored indirectly. The use of DNA profiling of ‘gut’ and faeces for biodiversity monitoring is further explored in Section “Food webs and functional units”.

DNA extraction: Methodologies have been developed to liberate DNA from extremely challenging matrices including sediments that are thousands of years old (Smith *et al.* 2015). Furthermore, Next Generation Sequencing (NGS) approaches can exploit significantly fragmented DNA and allow the exploitation of degraded DNA. However, the optimal extraction method depends on the substrate. Therefore, extracting DNA from a

complex mixture is often a matter of compromise. A simple example may be represented by a mixture between sporulate and non-sporulate. The spores require an aggressive process, both physical and chemical, to liberate the DNA they contain, whilst exposure of the non- sporulate material to the same condition may significantly degrade the DNA from this source. Therefore, for the use of DNA-based approaches to be used within a regulatory environment, the DNA extraction approach must be consistent within a specific application range – one size does not fit all (each procedure will require an optimised approach) but once fixed, the method must be employed in a rigorously reproducible manner. In human diagnostics this approach is assisted through use of robotics many of which can be modified to ensure reproducibility of the environmental sample processing and ensuring that any bias is consistent between samples.

Isolation of species specific 'barcodes' (short sequence of DNA diagnostic of a species): DNA accumulates changes over-time, a property that allows us to derive the evolutionary relationship between organisms by comparing the similarity of their DNA sequences – species with a closer phylogenetic relationship having more similar DNA. However, not all DNA sequences change at the same rate and evolutionary rates differ between taxonomic groups. Furthermore, for a sequence to have good utility as a 'barcode' it must provide sufficient species discrimination over a short region (~150-300 bp fragment) and this region should contain or be flanked by conserved sequences that allow sequence alignment and sites for primers to facilitate amplification (essential for many protocols). The most used workflow involves the amplification of an appropriate 'barcode' region prior to sequencing. Research communities representing different taxonomic groups have invested significant efforts in identifying appropriate primers that allow 'unbiased' amplification of 'barcodes' which allow genus/species resolution within specific taxonomic groups. The bacterial community exploit the variable loops and conserved stem of 16S ribosomal RNA (rRNA); the mycology (fungal) community have focused efforts on dual loci barcoding of the internal transcribed spacer (ITS) between 18S and 5.8S rRNA genes and TEF1a (Badotti *et al.* 2017; Hoang *et al.* 2019); the algal community use the 3' UTR of the gene encoding the Rubisco Large Subunit (3RbcL), whilst the invertebrate and vertebrate community use regions of the Mitochondrial Cytochrome Oxidase I (COI) and ribosomal 12S (Table 7).

A number of technical issues are intrinsic to assays exploiting PCR based amplification. These include issues of good laboratory practice such as the presence of appropriate negative controls to eliminate contamination – whilst always critical they have a key role in analysis for bacterial metabarcoding as bacteria are pervasive in most reagents and are impractical to exclude from processing which means that negative controls represent a background community derived from the technical process that need to be excluded from the analysis (Hornung *et al.* 2019). In addition, PCR has issues associated with initial primer hybridisation bias as well as quantitative issues occurring after logarithmic amplification is completed. The first of these is commonly addressed by combining triplicate primary PCRs as well as using 'hot-start' Taq enzymes, whilst the second can be addressed by restricting the number of amplification cycles used to the minimum required to deliver appropriate quality of amplified material (Paul *et al.* 2010; Kebschull and Zador 2015; Hornung *et al.* 2019).

Table 7 Commonly used COI and 12S primer pairs

Primer pair	Amplification gene	Author
LCO1490 & HC02198	COI	(Folmer et al. 1994)
Uni-MinibarF1 & Uni-MinibarR1	COI	(Meusnier et al. 2008)
ZBJ-ARTF1c & ZBJ-ARTR2c	COI	(Zeale et al. 2011)
MlCOIintF & MlCOIintR	COI	(Leray et al. 2013)
III_C_F & III_B_R	COI	(Shokralla et al. 2015)
BF1 & BF2	COI	(Elbrecht and Leese 2017)
BR1 & BR2	COI	(Elbrecht and Leese 2017)
ArF5 & ArR5	COI	(Gibson et al. 2014)
jgLCO1490 & jgHCO2198	COI	(Geller et al. 2013)
L499 & H2123d	COI	(Van Houdt et al. 2010)
MarVer1F & MarVer1R	12s	(Valsecchi et al. 2020)
MarVer3F & MarVer3R	12s	(Valsecchi et al. 2020)
MiFish-E-F & MiFish-E-R	12s	(Miya et al. 2015)
12s-V5F & 12s-V5R	12s	(Riaz et al. 2011)

Multiplexing, the ability to analyse multiple samples at the sample time, is universally used to ensure that metabarcoding approaches are cost effective. Multiplexing is achieved by the introduction of short DNA sequences specific to a sample known as ‘indexes’ (originally called molecular identifier (MID) and now more commonly referenced as unique molecular identifiers (UMIs) (Kivioja *et al.* 2011; Hong and Gresham 2017)). Two parallel approaches are used to incorporate these indexes; a) engineered as 5’-extensions to the barcode specific primer (one step index incorporation) or b) a common adapter is incorporated as a 5’-extension to the barcode specific primer which is used in a second round of amplification which incorporates the sequence primer and index as 5’ extensions

to the adapter (two step index incorporation). Although indexes have been refined to remove sequences that introduce bias such as those that introduce harpins and >400 indexes are now in common use (and as many as 9,216 different index combination are available (Lexogen 2019)), the two contrasting amplification approaches do have differing potential to introduce bias into the analysis. Inclusion of indexes directly into the primers used for the primary amplification may introduce bias since the index sequences can contribute differently to hybridisation. The two-step process is more costly and time consuming and has the potential to include bias associated with overamplification – something that can be addressed with appropriate optimisation (most commonly used is Illumina's Nextera protocol (Illumina 2016).

The use of long-amplicon barcodes and non-amplification capture based approaches are two emerging technologies that should be considered. The emergence of 3rd generation single molecule sequencing based on PacBio and Nanopore technologies, support long reads (>10 kb) and allows for much longer segments of DNA to be amplified heralding the potential for increased phylogenetic resolution. The Achilles heel of both 3rd generation technologies is the accuracy of the initial base pair read, although both technologies have improved significantly in the last few years. These issues can be addressed through various 'on system' approaches associated such as PacBio's 'circular consensus sequencing' approach (Ardui *et al.* 2018), which generates multiple sequences from the same molecule and generates a highly accurate consensus, as well as informatic approaches that combine multiple independent reads to generate a post sequencing consensus. For phylogenetic characterisation, the accuracy issue can be compensated for by the increased length of read, although the length of read is restricted by amplicon length although there are optimised protocols for complete 16S and 16-28S (Jiao *et al.* ; Johnson *et al.* 2019). One key advantage of this approach is the 'one chip' informatic processing provided through the MinION Nanopore Platform (Krehenwinkel *et al.* 2020; Li *et al.* 2020). This classifies and quantitates the phylogenetic composition of a sample dynamically as it is sequenced allowing real time detection and community structure to be derived. The limitations of these long-read approaches are that they are more expensive (per sample) and that the number of observations (reads per sample) is much lower than the short read approaches, however, for many applications the number of reads produced by the short read system significantly exceeds that required to address the question.

The final emerging approach is 'sequence capture'. This relies on a substantial reference library being available for the species 'barcode' in question. However, the 'barcode' can be of any length and can represent genomic and organelle (mitochondrial or plastid). This reference database is used to design a suite of 'capture' oligonucleotides, commonly 50 bp long and overlapping by 25 bp. These capture probes are biotinylated to facilitate capture onto a solid phase, commonly a magnetic bead and then mixed with the target DNA to enrich for the target barcode. It is claimed they will capture any sequence with >70% identity to the capture probe. Enriched material is then used to generate an NGS compatible read library, including appropriate indexing. This method removes primer and amplification bias, therefore, depending on the representation of the reference database, truthfully represents the diversity of the source DNA (Shokralla *et al.* 2016). The issue with this approach is associated with the availability of appropriate reference sets and the

informatic processing required for data interpretation. However, commercial capture sets are available for both 16S, whole mtDNA and plants (Compositae and Angiosperms) (ArborBiosciences 2020c, a, b). These capture approaches have been successfully deployed in amongst others, bacteria, lepidoptera, and arachnida (Faircloth 2017; Gasc *et al.* 2017)

High through-put sequencing: Next Generation Sequencing platforms have matured considerably with a number of technologies having been withdrawn from the market. Reviews of the key developments in this field have been excellently reviewed by others (Levy and Myers 2016; Amarasinghe *et al.* 2020). For metabarcoding approaches the key platforms are the short-read Illumina MiSeq and iSeq platforms (Illumina 2020) and the BGI platform equivalents (DNBSEQ-G50) (BGI 2020). The key developments are around delivering appropriate capacity and accessibility for the Miseq v2 Nano and the iSeq (generating ~1 Million read pairs of 2x250 bp and 4 Million 2x150 bp respectively). As mentioned previously, PacBio and Nanopore platforms provide long read alternatives which provide various opportunities. The developments that will have the greatest impact are the combination of new highly portable Nanopore sequencing platforms (MinION and MinIT or the MinION Mk1C) incorporating on-board informatic processing. The Ion torrent's Genexus system aims to provide a single day load-to-report solution, with library preparation and sequencing all automated as one process (generating 60 Million read pairs) of up to 32 libraries, thus removing a large segment of human variability. Potential new developing methods include hybrid sequencing platforms such as Quantapore that aims to combine nucleotide emission with pore-based technologies.

Barcode quantitation: In its most basic form the concept of barcode quantification is extremely straightforward as it represents counting the number of times each species barcode is observed. However, embedded in this simplicity are some complex mathematics and conceptual evolutionary questions. There are some substantive reviews of the workflows associate with the process of metabarcoding data (Murray *et al.* 2015; Tedersoo *et al.* 2015; Corse *et al.* 2017) and this article will not repeat these authoritative articles. However, key to exploiting these approaches for environmental analysis requires three key concepts to be identified: i) Denoising and Operational Taxonomic Unit clustering, ii) Lowest (nearest) common ancestor assignment and iii) Quantitative sampling. The primary barcode sequences generated will vary based on technical error associated with NGS as well as natural genetic variation. This is addressed by initialling denoising the sequences by defining a threshold and 'error models' associated with the technology together and using this to correct stochastic sequence error. Subsequently, a threshold of biological variation expected in the sequence between 'taxonomic units' is defined and used to group sequences together yielding a representative sequence and an observation count. The expected variation between 'taxonomic units' will depend on what barcode has been used and the group of organisms being profiled, together with the resolution required by the analysis – however the majority of analysis use 99% (i.e. 1% variation). There are many debates on what level is relevant to any particular analysis, but as long as it is defined and justified, it more relates to the use of the data. OTUs are not species but are informatically defined groupings. To associate these with species we need to 'match' them to a database of known species. This is usually done using relatively

simple homology matching algorithms which associate species with the most homologous reference sequence with the sequence. This can be highly problematic if the degree of homology is low (<95%). Therefore, Lowest (nearest) common ancestor assignment is being used where integration of the representative OTU sequence into a phylogenetic tree is used to perform the taxonomic assignment and provide confidence in the level of the assignment. Finally, there are qualitative parameters that need to be considered such as impact of sequencing depth (rarefaction), Jaccard distance and Pilous evenness.

Food webs and functional units

Extraction of DNA from faecal material or digestive tract contents has become increasing routine (Symondson 2002; King *et al.* 2008) allowing insight into food-webs and ecological networks that were previously inaccessible. The value of 'hard-part' dietary analysis of predators to define complex species interdependency is well recognised (Jeanniard du Dot *et al.* 2017) with the limitations of application associated with 'soft-part' and use in invertebrates self-evident. The DNA-based methodology addresses these questions allowing access to previously obscure dietary behaviours whilst also allowing application across trophic levels. The potential information gleaned from these analyses provides a wealth of information relating to the interconnectedness of the ecological systems. Analysis of a spectrum of invertebrate diets (iDNA) across an ecosystem has the potential to provide unprecedented insight into the intrinsic interactions and robustness of the ecosystem against species declines and extinctions. The derivation of Ecological Networks from iDNA may therefore be crucial in defining functional 'units' which may determine ecosystem robustness and identifying critical species both for protection and as key restoration pathways (Pocock *et al.* 2012).

It is important to recognise that there are specific technical challenges associated with iDNA linked both to organism specific gut chemistry and removal of host specific signal. DNA has been successfully extracted from the digestive systems of beetles (von Berg *et al.* 2008) to birds (Titulaer *et al.* 2017), however, the precise conditions associated with the extraction are host specific. Furthermore, host DNA predominates the extracted material which then interferes with isolation (amplification) of the dietary signal. This can be address either through a bioinformatic brute-force approach where excess sequence data is 'filtered' to remove the host signal or by elegant primer design and the use of 'blocking' primer to exclude the amplification of host material. These latter approaches are valuable but can compromise analysis where prey items are taxonomically closely related to host.

Supporting 'higher-level' function

Metabarcoding provides a rapid and cost-efficient approach to evaluating biodiversity but any functional conclusions are derived indirectly from established knowledge concerning the functional contribution of the organisms detected. For macro-eukaryotes the link between detection and implied role within an ecosystem is a reliable supposition, however, this assertion cannot be made for microorganisms. Microbial genomes are comprised of core and accessory genetic material, with the core genome being vertically inherited and therefore is aligned to the phylogeny whilst the accessory genome is to various degrees 'mobile' being horizontally transmitted between organisms (Koonin *et al.* 2003). These

accessories represent many of the functionally important pathways of interest from essential bio-geochemical cycles, such as nitrification (van Kessel *et al.* 2015), through to genetic cassettes encoding Antimicrobial Resistance (AMR) (McMillan *et al.* 2019). The implication of the functional importance of the accessory genome is that it is extreme challenging to imply all but the basic properties of microbial function from metabarcoding data. The solution is to perform metagenomic analysis which will provide both diversity and functional potential. It is important to recognise that this is a 'potential' since the presence of a functional pathway within a metagenome does not signify that it is active. To evaluate processes that are functionally active metatranscriptomics can be performed, either as a complement to genome studies or independently (although the latter has reduced analytical power). Although knowledge of gene-expression substantiates pathway activity it does not directly provide read-out of pathway productivity since this is influenced by post-translational modification, together with metabolic flux. Targeting RNA over DNA presents technical challenges associated with sample preservation and processing. A number of commercial products are now available that provide good RNA preservation (RNALater, Zymo Research RNA shield, LifeGuard Soil Preservation Solution) and allowing purification and ribosomal depletion of bacterial RNA (Direct-zol RNA MiniPrep, RNeasy mini kit, RiboMinus, MICROBExpress and Ribo-Zero)(Petrova *et al.* 2017), however, these products significantly increase the cost of sample processing. Current developments in nanopore sequencing have the potential to allow direct sequencing of RNA (https://store.nanoporetech.com/direct-rna-sequencing-kit.html#modal=nanopore-currency-notice), a development that promises to enhance the process.

Data resources have been established as repositories for metagenomic data which perform basic analysis, categorising the functional pathways identifiable within the data. Metagenomic databases and tools have been reviewed (Wilke *et al.* 2016; Shi *et al.* 2019b; EMBL-EBI 2020) but examples include EBI MGnify and MG-RAST. Although, in terms of NGS platforms, microbial genome size is small (2-5 Mbp) and both the diversity and skewed representation (common microbes dominate the analysis) necessitate a high sequencing depth if functional representation is to be reliable. This being said, the cost of sequencing is falling to make metagenomics a tractable routine approach.

Microbial ecology affords additional considerations. Functionally important microbial niches can be extremely specific, as exemplified by critical biogeochemical process that occur at the plant-soil interface (the rhizosphere), raising the question of strategies for microbial sampling. Microbial communities (microbiomes) associated with plants and invertebrates contribute significantly to functional properties of soils. Their characterisation and manipulation provide key opportunities for remedial action whether that be maintaining nutrient balance (Zipfel and Oldroyd 2017) or for pollutant removal (SM *et al.* 2020). This is an expanding area that may have significant implications for both evaluation of environmental processes as well as designing possible remedial strategies.

There is increasing realisation of the importance of the environment in the selection of new AMR genes, their transmission and their role as reservoirs for AMR. The release of AMR into the environment either as a waste product after being employed as clinical (Harris *et al.* 2014) or veterinary (Agga *et al.* 2015) medicine or at chemical production facilities

(Singer 2017) may lead to selection of novel AMR pathways. Human exposure, through recreational or other activities, my lead to the incorporation of AMR into human microbiomes and thus their appearance into clinical settings. The longevity of these pathways with environmental reservoirs is also poorly understood. The analytical pathways for detecting AMR pathways from genomic data are well established (Feldgarden *et al.* 2019; Shi *et al.* 2019a) and approaches for targeted analysis of specific AMR genes by qPCR are also available (Waseem *et al.* 2019).

A limitation of metagenome and metatranscriptome approaches is associated with the inability to link the functional pathways explicitly to the microbe encoding them. The use of long read NGS for genomic application can improve this linkage (although currently the costs is significantly higher) since it leads to more contiguous fragments of DNA. The key technology in unravelling the organism-function relationship is single-cell sequencing where organisms are separated and sequencing performed individually using a single-amplified genome (SAG) strategy (Bowers *et al.* 2017). New platforms exploiting microfluidic and emersion-based encapsulation now allow thousands of individual genomes to be characterised separately allowing direct association of pathway to organism but also allow us to unlock the critical interactions and interdependencies between microbes (Woyke *et al.* 2017; Nicholls *et al.* 2019). Intriguingly, similar functional flexibility is observed in the primary producers (algae and fungi). The derivation and functional dissection of algal and mycological networks are in their infancy which is a surprising oversight since these microbes play key roles in nutrient cycling and carbon fixation.

Targeted single species monitoring

Species-specific eDNA signatures provide a highly tractable approach that can be applied to perform population monitoring non-invasively (without the need for direct observation), allowing qualitative and quantitative tracing of a species through an ecosystem. Compared to conventional ecological surveys, DNA-based species detection provides significant benefits, is more cost effective and reduces ecosystem impact of monitoring (Lawson-Handley *et al.* 2017). Although these techniques can deliver abundance data that is needed to inform biodiversity indices, this measurement is influenced by both the physical characteristics of the environment being sampled and the biology of the target organism. The stability and concentration of DNA in the environment is influenced by UV, temperature and water column dilution (river flow rate / rain fall) whilst the quantity of DNA shed into the environment is influenced by the target organism's physiology i.e. reproductive status, life stage and diet. Although the quantity of DNA highly correlates to species biomass, the relationship will be ecosystem and species specific. However, site-occupancy rates, a metric which DNA-based tools deliver high efficacy, can be used within standard ecological models for monitoring populations as a proxy for abundance (Lawson-Handley *et al.* 2017)(pp38).

Single species DNA monitoring approaches have been developed for >100 species representing the full taxonomic range of macro-organisms and applied across the full breadth of ecosystems (terrestrial, marine and fresh water) (Lawson-Handley *et al.*

2017)(pp33). Questions addressed range from detection of invasive non-native species (INNS), conservation (protection and monitoring), population management and disease surveillance. This was first applied by Ficetola *et al.* (2008) and is extensively reviewed by Lawson-Handley et al (2017). A sub-selection of the studies performed rigours evaluation against classical non-DNA-based approaches with a minority recording detection limits and environmental persistence parameters. The clear conclusion is that the DNA assays outperformed routine monitoring and that through the rigorous use of controls and standardised operating procedures (SOPs) to determine detection and persistence levels these 'research' tools can be converted into highly robust and defensible instruments to support environmental forensics. However, the development of robust SOPs together with their validation is exceptionally limited, this is only currently achieved in the UK for (Defra - Great Crested Newt Detection WC1067 (Biggs *et al.* 2014), with a clear gap between 'research tools' and 'legislator instruments' representing the division between 'research' and 'regulation'. This does not prevent research tools being deployed as advisory information to inform regulators, Environmental Management and Assessment (IEMA) and UK environmental goods and services sector (EGSS).

Technologies supporting single species monitoring

DNA-based tools are readily available, but the cost of development and deployment of individual tests depend highly on the detection limits and quantitative precision required. Clinical genetics has driven the development of an array of amplification and detection-based systems from PCR (combined with agarose-based gel electrophoresis) through qPCR to dip-stick lateral flow devices (Jauset-Ribio *et al.* 2016) and electrical based DNA sensors (Huang *et al.* 2019). Unfortunately, many of these technologies require significant up-front investment to be customisable against a specific target and the commercial drivers have not been present within the environmental sector to drive this customisation. The consequence is that within the environmental sector this has led to adoption of 'research tools' rather than the development of more robust tailored commercial solutions.

The majority of studies targeting single species have employed PCR (single or nested), quantitative PCR (qPCR) and digital PCR (ddPCR) as a basis for developing single species detection / quantitation. A detailed disruption of these approaches, together with a comparison of their strengths and limitation is provided by Lawson-Handley *et al.* (2017). In summary, qPCR and ddPCR provide additional sensitivity of standard PCR but require additional optimisation. Whilst probe based qPCR has more specificity than generic 'dye' based methods, it is also affected more by the purity of the DNA employed – something that is often highly variable with environmental samples. The capital costs and maintenance of qPCR platforms restricts the use to well established molecular laboratories and has led to people employing multiple rounds of PCR to address the sensitivity / specific limitation of standard PCR. Unfortunately, these 'nested' PCR approaches have significant technical challenges related to the increased frequency of false positives stemming from the workflow as well as environmental contamination. All these approaches are currently restricted to being performed in the laboratory and therefore remotely (temporally and physically) from the monitoring site. It is possible to establish mobile laboratories on ships or highly customised vehicles, but this is not routine.

Intriguingly, many innovations that are in development to support clinical DNA point-of-care assessment have the potential to provide cheaper and more robust end-point single species surveying tools. Combining loop-mediated isothermal amplification (LAMP) coupled to novel fluorescent and luminescent based detection or isothermal amplification combined with lateral flow or electronic detection has yielded a range of point-of-care assays for the detection of disease-based targets (Roy *et al.* 2016; Shirato *et al.* 2018). Transfer of these technologies into the environmental sector for species detection will reduce cost and deliver field-ready technologies, however, due to the proprietary nature of these technologies this will require industrial input and clear-commercial drivers from the regulators.

Workflow employed for single species monitoring

Although, the intrinsic information contained within an organism's DNA makes it an ideal species-specific diagnostic tool there are two major consideration – fragment size and sequence specificity. If the material used in an assay is fragmented – either eDNA, or extracted DNA from hair/faeces, then it is essential to understand the stability of the diagnostic DNA fragment in the environment – the larger the target, the more rapid its degradation, and the variation in the DNA that leads to species discrimination. It is very straight-forward to identify a large fragment of DNA which is species specific but far harder to identify small environmentally long-lived fragments with covered primer sites for amplificant that has the required discriminatory power.

DNA tools are commonly employed using three workflows ranging from qualitative to quantitative.

1. **Amplification and sequence confirmation**: DNA extraction from organism derived material (blood, hair (with root) or faecal matter), amplification of generic barcode regions (COI for most animals) followed by Sanger sequencing and reference database analysis. Provided appropriate negative controls to ensure no cross-sample contamination, this is highly robust and will lead to species identification.
2. **Species specific amplification**: DNA extraction from environmental sample followed by amplification of small fragment (150-300bp) using species specific primers followed by detection of fragment by molecular size analysis (Capillary Electrophoresis or agarose gel). This can be implemented as a semi-quantitative technique and can be validated through Sanger sequencing of fragment if required.
3. **Quantification of species-specific amplicon:** DNA extraction from environmental samples, followed by quantification using species specific barcode fragment (150-300 bp). Commonly these methods use qPCR or ddPCR. Other detection methods are available but rarely used. Additional levels of specificity can be incorporated into assays by combining probe-based detection with PCR (hybridisation or hydrolysis -based probes). Quantification has additional challenges associated with translating barcode quantification into an environmental meaningful measurement.

Metabarcoding as a generic workflow for species monitoring

The economics of deploying a generic workflow or a specific species approach needs to be evaluated. The extraction of DNA from an environmental sample, such as an eDNA filter, with subsequent metabarcoding analysis may facilitate informatic interrogation for a specific DNA signature. Alternatively, the eDNA sample may be used as a generic template for targeted analyses. Against this is the potential to develop highly optimised assays towards a specific target which may be a more cost-effective approach for that individual target but would not support parallel analyses for other targets of interest. For example, do we want a dedicated low-cost assay for one endangered species or a more generic workflow that would allow a range of question to be answered immediately and even generate an archival sample that could be employed subsequently if additional analyses was required?

DNA Knowledge based requirements

The design and implementation of species-specific DNA assays together with our ability to interrogate a metagenomic profile for specific species is wholly dependent on the genetic knowledge base associated with the taxonomic group in question. The bar code of life project (BOLD) was establish with the overt objective to generate a database representing a common fragment of DNA from all eukaryotes – the fragment used is the mitochondrial Cytochrome Oxidase gene (commonly referred to as COI or COX I). To date 10.5 million DNA barcode sequences representing over 307,078 species have been deposited. Although this is an impressive endeavour it represents <4% of the conserved estimate of 8.7 billon eukaryotic species on the planet. Furthermore taxonomic coverage is not consistent with larger macro-fauna being overrepresent in comparison to micro-eukaryotes with many species represented by short fragments of sequences. Additionally, analysis reveals that for particular taxonomic groups, COI does not contain the degree of phylogenetic resolution required to perform species classification leading to a database being established for different regions for specific taxonomic groups – mycology (fungal) taxonomy uses ITS2 whilst algal taxonomy is currently using RbcL gene. Micro-eukaryotes from freshwater, terrestrial, and marine systems represent the ‘dark’ (or hidden) diversity and are poorly represented in any of the databases. Together with microbes these primary producers represent the foundations for our ecosystem but are severely underrepresented.

Recent announcements of the endeavour to sequence the complete genomes of all eukaryotic life under the guise of the EarthBiogenome project (https://www.earthbiogenome.org) with the UK contribution from the welcome trust via the ‘Darwin Tree of life’ (https://www.sanger.ac.uk/science/programmes/tree-of-life), promises to provide comprehensive data for all taxonomic groups and will thereby transform our ability to design and develop new DNA-based tools.

‘SMART’ Effects-Based Monitoring

There is an increasing push towards ‘effects-based monitoring’ where an explicit link can be articulated between the environmental perturbation and the metric being assessed. With appropriate systems level understanding, this approach can be designed to assess both ‘exposure’ and ‘impact’, evaluating if the system is responding to the challenge and if that response impacts function. These ‘effects’ can be assessed at the level of the ecosystem, community or the individual. Key to evaluating effect is to establish Differential Species Sensitivity (DSS), determining both the vulnerability of the species as well as their contribution to ecosystem processes. Predicting DSS require assessment of exposure (influenced by behaviour and physiology), toxicokinetics (influenced by traits affecting Absorption, Distribution, Metabolism, or Excretion); toxicodynamics (influenced by traits affecting the Mode and Mechanism of action) and ecosystem processes effects (e.g. traits associated with food web and ecosystem process interactions) for the constituent organisms within an ecosystem under investigation.

To illustrate how DNA tools may be used to underpin ‘effects-based monitoring’ at multiple organisational levels we will consider how they may be employed to evaluate the release of the toxic heavy metal cadmium into a fresh-water ecosystem. Metagenomic analysis of sediments can be interrogated to determine the frequency of the cadA and cadB gene systems that underpin bacterial cadmium tolerance to evaluate exposure, whilst metatranscriptomic analysis may reveal if cadmium is impacting on expression of genes involved with nitrogen cycling, representing a key ecosystem service provided by the bacterial community. A metabarcoding approach can be utilised to profile the community structure of the primary producers (diatoms) where ‘exposure’ can be evaluated by the loss of metal sensitive species whilst ‘impact’ measured by evaluating the productivity of the primary producer community – a sensitive species may be lost without a resulting impact to the overall producer community whilst loss of productivity would result in an ecosystem level impact. At an individual organism level ‘exposure’ of fish within the water system will result in the induction of the metal binding protein metallothionein whilst impact can be assessed through the induction of the DNA repair processes associated with the genotoxic activity of cadmium which would represent a long-term damage to the fish population. This example is incorporated to act as a ‘simple’ scenario, however, our knowledge of genes involved in the nitrogen cycling in bacterial is far from complete. Nitrogen sensitive primary producers can become tolerant to cadmium if exposed over a long period and an organism’s physiology (estrogenic and moult cycles) can have a significant impact on metal physiology and therefore responses to heavy metals can illustrate the high degree of biological understanding required when exploiting these tools.

Key to designing DNA-based assays to address the challenge of ‘effects-based monitoring’ is ensuring that contextualised ‘**S**pecific **M**easurable **A**chievable **R**elevant and **T**ime-constrained’ (**SMART**) metrics are defined. The assessment must be ‘**S**pecific’ in relation to the nature of the effect being measured – generic or non-specific outcomes such as ‘a healthy environment’ will not support for quantifiable outcomes. The criteria must be **M**easurable and the quantification **A**chievable within reasonable bounds – defining an unrequired level of precision or scope will erroneously exclude possible

valuable tools. Requiring exact species numbers when relative biomass will support the same outcome is unhelpful. 'Effects' need to be context specific – functional parameters will relate to specific services or functions of a specific ecosystem. In some aquatic systems 'fish stock levels' are relevant whilst in others carbon turn-over is paramount. Finally, defining the **T**ime-constraints associated with monitoring and management is essential. Environmental 'effects' directly impacting human health, such as beach water quality, need fast or immediate response whilst routine surveillance of environmental quality can be reported on monthly. In evaluating DNA-based assays these SMART principles should be applied.

Ecosystem process

Biogeochemical cycling

Bacterial communities, both free living and organism associated microbiomes, are responsible for nutrient cycling within our ecosystems whether through carbon degradation or nitrogen fixation/nitrification these biogeochemical cycles are key for maintenance of a healthy ecosystem. Changes in bacterial community structure will influence the efficiency of these processes, the robustness, and productivity of the ecosystem. Metagenomics and metatranscriptomics can be exploited to profile the potential and activity of these communities, however, translating a DNA or RNA based profiles into predictive models for biogeochemical pathways is extremely challenging. Furthermore, determining the 'tipping point' for change where specific alterations in community structure result in an 'effect' that translates into impact is difficult. The crucial role played by the Rhizosphere in nutrient cycling is well establish with omics tools being used to unlock the interdependency of bacteria, plants, fungi and soil invertebrates (and their microbiomes) contributing to these processes. Translating these research methodologies into 'routine' assays that can be used to measure nutrient cycling capacity of the soil has the potential to provide effect indicators relating to these processes. At present extracting high-level outcomes from full metagenomic and metatranscriptomic analysis that would deliver quantifiable metrics for functional parameters associated with nutrient cycling are extremely expensive and analysis requires application of complex information pipelines. International programs such as the 'earth microbiome' project (http://www.earthmicrobiome.org/) have provided open access to both robust protocols and shared data whilst accessible data resources (EBImetagenomics, MG-RAST, JGIs-IMG (img.jgi.doe.gov) and Camera (camera.calit2.net)) and associated analytical tools provide powerful resources for comparative analysis. These tools provide the ability to analyse the abundance and activity of specific functional pathways from metagenomes and metatranscriptome data. Large systematic temporal and spatial application will allow us to directly link the presence and activity of these biochemical processes with the higher-level sustainability and robustness of the environments from which they are been isolated.

Keystone species for trophic cascades

Risk assessors are challenged to perform an assessment that protects 95% of species in an ecosystem. By protecting most of the community structure, it is hoped to also conserve

processes and, thereby the ecosystem services supported. This approach assumes a level of functional redundancy that buffers against the reliance on individual species but ignores that some species are critical for ecosystem processes or of high conservation value. The fact that some organisms form keystone species in trophic cascades and food weds cannot be overlook when developing DNA-based tools to support 'effects-based monitoring'. The development of ecologically informed weighting within analytical workflow is essential. Ideally, species abundance and occupancy data need to be incorporated into predictive ecological models to evaluate ecosystem robustness. This is a highly dynamic area of research with encouraging outputs showing that ecosystem modelling approaches can exploit the occupancy and semi-quantitative abundance data provided by eDNA metabarcoding approaches robustly to data artefacts common in eDNA data (McClenaghan *et al.* 2020). These approaches rely heavily on extensive knowledge of the species interdependencies within an ecosystem and long-term data associated with a particular ecosystem which brings into focus the need to link DNA-based data sets with the classically acquired data.

Toxicokinetics: Absorption, Distribution, Metabolism, or Excretion (ADME).

ADME remains a core tenet of ecotoxicology when considering potential chemical effects on individual constituents of an ecosystem as well as possible bioaccumulation or biomagnification. Key to measuring these parameters are analytical techniques that track chemicals and their metabolites through an ecosystem. Developments in chromatography and mass spectroscopy now enable multi-analyte measurements at extreme sensitivity, which allows us to empirically trace chemicals through the environment. However, prediction of species toxicokinetics and the implications for their DDS extrapolated from there DNA is an area of key research. Kinetic traits important to consider encompass uptake receptors, transport molecules, metabolic pathways and excretion systems. Metabolism plays a key contribution to toxicokinetics with enzymes associated with Phase 1 processes having well define structure-function relationships derived as part of pharmacogenomic modelling of drug and pesticide metabolism. This provides an opportunity for prediction of metabolic potential based on the comparative sequence and expression levels. Given the expanding phylogenetic representation (see (https://www.earthbiogenome.org), there is a potential for *in silico* based prediction of sensitivity based on changes in toxicokinetics (EFSA 2018). However, it should be noted that there is a dearth of information relating to invertebrate drug metabolism (Han *et al.* 2017) and this will substantially restrict the power of these predictions.

Toxicodynamics: Linking chemical exposure to effect.

It is not surprising that approaches focused on identifying mechanistic links between chemical exposure and organism impacts have been initially embraced by the preventative risk assessment community which employs a discrete suite of ecotoxicology species to assess chemical risk (REACH and Agrochemical regulation). These assessments focus on laboratory testing of a restricted set of test species under defined protocols. This consistency has allowed the required mechanistic understanding to be developed so that responses to key chemical classes can be linked to specific physiological and biochemical responses. The best developed program is the US-EPA ToxCast which has overseen the

development of >69 high throughput bioassays targeting endpoints associated with cell stress, endocrine disruption, growth/development, immunity, lipid metabolism, general metabolism and xenobiotic metabolism. Recent field deployment of these assays to assess impacts of mixture pollution on wild fish populations within 38 streams in the US demonstrated that only a restricted sub-set of 11 biological endpoint gave significant responses within wild cohorts (Blackwell *et al.* 2019). Extensive chemical analysis of the streams considering >700 analytes was also used to evaluate the pollutant pressure within the streams in question. This study both identifies the most sensitive markers as well as suggesting the need for broader bioassay coverage to assess potential adverse impacts (Berninger *et al.* 2019).

Essential to these developments is the concept of an Adverse Outcome Pathway (AOP) (Villeneuve *et al.* 2020) which mechanistically links exposure to effects at a higher levels of biological organisation, ultimately resulting in impacts on the health of an individual or population. In its ideal form a well-developed AOP will precisely characterise the links between a Molecular Initiating Event (MIE) and undesirable outcome, such as reduced reproduction or a terminal pathology (e.g. tumour formation or liver damage). An AOP links a MIE with any given adverse outcome via a linear pathway of connected Key Events (KEs) that have been causally related using experimental evidence (i.e. it is known that an upstream KE is necessary for the next KE to occur). This reductionist approach looks to remove the 'black-box' between exposure and effect. It is exceptionally rare for all components in a pathway to be fully characterised, even in model organisms, but the AOPs concept provides a valuable framework on which to develop novel bioassays where responses can be placed within the trajectory between exposure and outcome.

Transcripts (and miRNAs) provide ideal targets to broaden bioassay utility both in relation to functional information provided as well as increasing breadth of species where they can be deployed. Analysis of the global impact on gene expression using 'transcriptomic' profiling (microarray or RNAseq) provides a valuable tool to explore the impact of environmental challenges. Reduction in cost of sequencing and informatics developments allow modestly straightforward analysis of transcriptomes from any species, although interpretation relies on functional interpretation based on homology mapping of differential expression genes onto model organisms – a processes that highlighted the lack in depth of our detailed understanding of invertebrate biology except in one arthropod (*D. melanogaster*) and one nematode (*C. elegans*). However, interpretation of holistic data can be challenging especially for data that is derived from 'field' populations where responses reflect both physiological status as well as **ALL** environmental conditions not limited to specific chemicals of interest. Despite these restrictions evaluating changes in specific transcripts with established links to the mechanism of action via the MIE of a specific chemical or chemical class as well as those KE that are linked to adverse outcomes provide extremely valuable tools. Whether investigating non-target impact of pesticides or impacts associated with legacy metal ions, transcripts can be selected encoding proteins with established links to exposure and impact.

A major challenge in developing these targeted bioassays has been the genetic knowledge base associated with the complete eukaryotic biodiversity of our ecosystems.

For an effect assay to be developed we need to know the DNA sequence encoding our target from the organism of relevance from the ecosystem under consideration. Although, the growing number of eukaryotic genomes (32K Feb 2020 - https://gold.jgi.doe.gov/) is impressive, the phylogenetic representation is very bias toward Chordata (vertebrates) – which does not match the species used for ecosystem assessment. Currently the Earthbiogenome project and UK ‘Darwin Tree of Life’ project are looking to address this discrepancy on a local and global level.

Analysis: Delivering for the business of the environment

Operational implementation/constraints: TIME – from dynamic surveillance to routine monitoring. The operational demands and the technological solutions.

The modality of biomonitoring currently reflects cost, expertise and analytical processes with many physical variables being measured in real time. Whilst chemical samples are processes routinely (weekly or monthly), biological/ecological assessment is performed sporadically. Even in scenarios where data return is linked directly to public health (such as beach water testing), the time required to assess the microbial samples significantly limits the dynamics of reporting the data. DNA-based tools have the potential to change this dynamic in three key areas; i) reduction of cost – accounting for sampling time, processing and analysis is more cost effective when performing targeted qPCR or metabarcoding of eDNA samples, ii) expertise – eDNA samples can be taken by a non-specialist and processed in bulk; and iii) technological developments have the potential to enable acquisition of data in real-time.

Currently ecosystem (microscopic community diatom or invertebrate kick samples) or microbiological sampling requires significant expertise to process (culture or sort samples) samples, combined with taxonomic expertise to identify the constituent organisms (microbes or macro invertebrates). In contrast, eDNA metabarcoding can be automated to a central location providing species list to local expert ecologist and can also inform occupancy or biomass-based models. Filter preservation protocols make it straightforward to integrate sampling into routine processes whilst streamed high-throughput workflows will both reduce cost and turn-around time. Sampling innovations include programable fix filtration samplers (Stern et al. 2015) that actively sample large volumes over extended periods giving rise to the potential for improved representation of a water body being sampled and a more-rapid turn-around for sampling personal.

A pioneering project aimed to generate a DNA-based equivalent of the Trophic Diatom Index (TDI) used to calculate an Ecological Quality Ratio (EQR) servicing a Water Framework Directive (WFD) requirement was developed by the Environment Agency. The key challenges faced by the project are common to these approaches and include i) Developing an appropriate reference database; ii) establishing robust sample collection and storage, iii) identifying and optimising appropriate DNA barcode; and, iv) converting the DNA-based taxonomic quantification into an equivalent EQR metric. This was successfully achieved and a successful UK wide side-by-side study was performed against the established light microscopy procedure. The resultant NGS procedure generated a ‘eTDI’ with good correlation to the light microscopy method. Comparative analysis with established methods is an important starting point when thinking about integrating DNA-based methods into ecological assessments.

Developments leading to lab-on-a-chip based devices that combine microfluidic based sample processing, LAMP with lateral flow / electrical detection or technologies such as

Nanopore sequencing has the potential to deliver ‘field-base’ single organism detection or community-based profiles. The components that underly these innovations are already present in the marketplace requiring the market demand to drive novel device/platform development.

Environmental protection against environmental evaluation: Delivering tools for prevention whilst supporting routine monitoring.

Whilst routine monitoring and surveillance will continue to be an essential component for environmental management, the ability to predict where environmental issues will occur and act to prevent or minimise impact should be paramount. Global challenges, such as climate change, combined with local circumstances associated with pollution, use of agrochemical or biological agents (disease or alien invasion) need to be modelled to evaluate long-term environmental sustainability. This modelling can substantially benefit from both the monitoring data provided by DNA, in addition to the understanding of ecosystems provided when they are considered through a genomics lens. Predictions of sensitive keystone species, synergistic interactions between the pollutant mixtures and between chemicals and disease vectors need to be considered and action taken proactively to minimise impact. The number of global disease vectors exemplified by fungal pathogens effecting amphibians (such as Chytridiomycosis) and plants (such as *Phytophthora infestans*) illustrates the need to understand how biological vectors interact with physical and chemical channellings. European Food Standards Agency (EFSA) has developed a white paper unifying the environmental, food and human health approaches to risk assessment embracing the utility of ‘omic tools to assist in addressing issues associated with multiple or mixtures of challenges.

Developing *in silico* platforms that can support ‘virtual’ ecotoxicology predictions leveraging the genomics outputs from the Earthbiogenome Project and Darwin Tree of Life initiative, provides exciting potentials for the future. Already programs such ECOdrug and seqAPASS have shown that with limited knowledge we can predict the differential action of drugs on different organisms. The ability to extend this to consider the full diversity of an ecosystem is a tantalising future horizon.

Addressing emergent threats through new technologies: From Nanopore to Nanostring.

Current deployment of DNA-based environmental tools directly employs generic research workflows and tools. The commercial drivers underlying the area of clinical diagnostics has delivered significant innovations in the area of DNA-based detection and quantification. Each element of a DNA assay has multiple possible innovative solutions from use of LAMP to PCR based microfluidic chips for amplification to Bioluminescent Assay in Real-Time (BART) and chemiluminescent detection; the possible options for developing DNA assay are substantial. Measures of multiple targets (multi-analyte measurements) without amplification using approaches such as Nanostring or ‘chip’ based technologies are compatible, but the environmental DNA marketplace currently is too limited to justify companies developing their specific platforms against this emerging

marketplace. Many of these technologies would be compatible with field deployment, providing a great advantage for immediate diagnostics but perhaps the most exciting are lateral flow or electronic detection. DNA lateral flow devices would bring the 'pregnancy test' style dip sticks to the DNA assay marketplace – although these would only provide a 'present / not present endpoint', would need to be combined with an amplification technology, they would be accessible to non-specialist and would need to be field ready. Similarly, cheap and disposable screen-printed carbon electrodes can be used to create DNA-based biosensors (Shoaie and Forouzandeh 2018; Huang *et al.* 2019), again, a technology that will deliver rapid semi-quantitative data compatible with field deployment.

Despite these innovative detection systems all current processes use relative laborious filtration and DNA extraction protocols. Many extraction processes have been automated using standard liquid handling and magnetic bead technologies. There are endeavours to miniaturise these processes into a lab-on-a-chip format for the field which would substantially enhance automation of the processing (Rérolle *et al.* 2018). Key innovations associated with acoustic based filtration may also provide novel methods to extractive the biological material from water samples (Liu *et al.* 2019).

Currently the majority of metabarcoding and single species sequencing approaches employ amplification as an essential starting point. This methodology does innately incorporate bias associated with both primer sequences and the amplification processes. Research papers using 'skimming' or bait capture approaches are becoming more common (Liu *et al.* 2015; Gauthier *et al.* 2019) and are starting to illustrate some significant advantages over standard barcode amplification especially for eukaryotic assemblages.

The rate of development of NGS technologies does not show any indication of stopping, with incremental developments associated with utility, such as Illumina's iSeq100 and Nanopores Flongal & MinION Mk1C offerings will increase the democratisation of NGS approaches to every laboratory. However, there are major innovations on the horizon including nanowires and solid state nanopores that both promise an order of magnitude change in sequence generation and reduction in cost.

From Nucleic acids to Numbers: Converting Measurements to Metrics

Transparent data processing is essential when employing any nucleic acid derived measurements since the DNA is derived from the organism of interest but doesn't directly represent the organism being observed. Therefore, when measurements are reported they incorporate environmental sampling meta-data. For eDNA this may be the hydrodynamic information relating to the water body, the quantity filtered etc. It is essential that these metadata for the data represent meaningful environmental metrics. Significant work has been done in identifying the minimal information needed to support biosamples (https://www.ebi.ac.uk/biosamples/) including the development of methodologies for describing sampling location (Griffin *et al.* 2017; OSGeo 2020) and appropriate temporal and spatial information. It may be interesting to consider the valuable innovations in the earth observations field that have occurred from adopting the data cube

concept from data sciences – an approach that may enable better inoperability between various DNA-based measurements.

Single target analysis with qPCR and dPCR is a relatively straightforward required standard process to follow, though incorporating and reporting appropriate control is essential. The Laboratory of Government Chemistry has a long-standing program validating the protocols and standards associated with the precise and accurate quantification of nucleic acids by dPCR and qPCR for biomedical assays (Whale *et al.* 2017). This program provides a template for the standards and validation procedures that would be appropriate when expanding DNA-based environmental testing.

Processing metabarcoding data is conceptually very easy as it is based on identifying representative sequences and then counting their occurrence. However, the wide variety of algorithms available for each step of the informatic processing pipeline means that subtle changes in processing can deliver significantly different results. This is exacerbated since the analysis is often performed within a command-line based Linux environment by an informatics specialist or through an automated pipeline, so that the person interpreting that data may not be aware of the assumptions incorporated within the processing. It is therefore essential that the numbers generated from the nucleic acids are accompanied by the transparent work-flow description providing the programs (and versions) used for the processing. Ideally, the creation of virtual machines or containers that replicate informatic pipelines should also be made freely available to ensure reproducibility of the analysis. Although there are some commercial and freeware products providing graphical interface for metabarcoding, none are developed specifically for environmental management. Software engineer development of a GUI interface that could exploit new processes within a modular environment and its design for the non-specialist would be extremely valuable.

Once the numbers have been generated, they need to be converted into metrics that can be employed to support environmental management. Currently, diversity outputs represented by Operation Taxonomic Units (OTUs) or Amplicon Sequence Variant (ASV) can be used to derive community metrics such as Shannon indices but converting these to metrics linked to environmental quality can be opaque. The molecular diatom assay (Kelly et al. 2020) exploited a purposely derived reference set allowing the OTUs to be converted to their nearest taxonomic neighbour. Together with a DNA-abundance conversion factor, this enable the generation of the Ecological Quality Ratio (EQR) equivalent to that generated by microscopic derived Trophic Diatom Index (TDI). Although this process allowed the alignment of the DNA data to historical data sets, it did not exploit the full richness of the DNA dataset. With appropriate reference data this can be converted into standard taxonomic representation, however, this ignores significant within-species diversity information represented in the data. There are dozens of ecological diversity indices to establish ecosystem health and it is clear that substantive work is required to establish them as appropriate DNA-based metrics (Daly *et al.* 2018).

Intriguingly, although metagenomic and metatranscriptomic data is more informatically challenging – generating the numbers from the sequences – the interpretation is modestly straightforward. If the metric required is frequency or occurrence of AMR genes (Kavvas *et al.* 2018) or biogeochemical pathways (Nelson *et al.* 2016) these can be extracted

relatively straightforwardly. Although genes contributing to these pathways continue to be discovered and classified, the systems to mine this data can be programmed to dynamically update this information.

DNA-based methods can support better environmental management through the concept of an *in silico* portal representing a ‘digital twin’ of the ecosystems being evaluated. This allows us to integrate all aspects of the environments being evaluated, from land-use (past and present), chemical measurements, through to multi-layered diversity information. Combining diversity data with virtual estimations of potential sensitivity, incorporated with modelled uncertainty estimates (based on data such as comparative genomics), would then allow us to identify vulnerable species. This virtualisation of risk assessment embracing new measurement technologies together with digital platforms for analysis has the potential to transform our ability to management environmental change.

Conclusions

Our fundamental knowledge is being enhanced through the application of genomics, providing both the genetic knowledgebase to understand living systems but also elegant and powerful tools to explore ecosystems. Ancient DNA (aDNA), environmental DNA (eDNA) and invertebrate-derived intestinal DNA (iDNA) are just a few of the research innovation that are enabling us to explore a changing world through past and present biodiversity, to food web and ecological networks. These research tools can be harnessed to provide rapid and robust monitoring tools whilst also informing predictive models that may better allow for environmental protection.

The challenge often is not technological but stems from the specificity of the question being posed. Ecosystems are complex and the definition of 'Environmental Health' problematic. If we pose a specific question – is an endangered or alien species present, there are very robust DNA-tools to address this question. However, if the question is more general, such as 'is this ecosystem healthy', then one must define explicitly 'health' prior to being able to identify a tool that can address the question. The debate associated with what defines a healthy environment is independent of the validity of tools used for its evaluation. If you define 'healthy' by degree of ecosystem diversity at a microbial level, then an appropriate DNA-based method can be identified to quantify this metric. The more precise the question the easier it is to identify an appropriate tool. If your question relates to the abundance of anti-microbial resistance (AMR) genes, then a metagenomic methodology can be used to address this specific question, with the ability to define sensitivity limits and associated assay parameters. If preservation of 'ecosystem function' is the requirement, then we must define the elements of 'function' that need to be preserved prior to selecting an appropriate approach. Comparisons can be of value to frame defined questions, such as the impact of point source pollution in a river or comparison of two fields treated under different agrochemical regimes, providing specificity to the question and leads to unequivocal answers. For example, the CEH country-side survey helps by identifying paired or equivalent sites where specific metrics can be compared. The greater the complexity of the question the more challenging it is to identify a specific tool – not because of tool limitation but because of the way the question is being posed. Therefore, we need to break down the larger question of 'ecosystem health' into more explicit tractable metrics – SMART metrics for 'ecosystem health'. For each metric a specific DNA-based workflow can be identified. Then we need to optimise, validate, develop quantifiable metrics and implement these workflows. Identifying assays that can address multiple questions and provide an efficiency saving should prioritise the development of these approaches. By rethinking how we 'phrase' questions associated with environmental health and embracing new technologies we attain a 'green future' for our children to inherit.

References

AGGA, G. E., Arthur, T. M., Durso, L. M., Harhay, D. M. And Schmidt, J. W. (2015). Antimicrobial-resistant bacterial populations and antimicrobial resistance genes obtained from environments impacted by livestock and municipal waste. In: *PLoS One*. Vol. 10

AMARASINGHE, S. L., Xueyi, S. S., Dong, X., Zappia, L., Ritchie, M. E. And Gouil, Q. (2020). Opportunities and challenges in long-read sequencing data analysis. *Genome Biology* 21(1):1-16

ARBORBIOSCIENCES. (2020a). Mybaits Expert - Plant - Angiosperms-353. Available at: Https://Arborbiosci.Com/Genomics/Targeted-Sequencing/Mybaits/Mybaits-Expert/Mybaits-Expert-Angiosperms-353/[Accessed: 30/03/20]

ARBORBIOSCIENCES. (2020b). Mybaits Expert - Plant - Compositae-1061 Arborbiosciences. Available Aa: Https://Arborbiosci.Com/Genomics/Targeted-Sequencing/Mybaits/Mybaits-Expert/Mybaits-Expert-Compositae-1061/ [Accessed: 30/03/20]

ARBORBIOSCIENCES. (2020c). Mybaits Expert Whole Genome Enrichment (WGE). Arborbiosciences. Available At: Https://Arborbiosci.Com/Genomics/Targeted-Sequencing/Mybaits/Mybaits-Expert/Mybaits-Expert-Wge-Whole-Genome-Enrichment/ [Accessed: 30/03/20]

ARDUI, S., Ameur, A., Vermeesch, J. R. And Hestand, M. S. (2018). Single Molecule Real-Time (SMRT) sequencing comes of age: Applications and utilities for medical diagnostics. *Nucleic Acids Research* 46(5):2159-2168

BADOTTI, F., Silva De Oliveira, F., Garcia, C. F., Vaz, A. B. M., Fonseca, P. L. C., Nahum, L. A., . . . Góes-Neto, A. (2017). Effectiveness of ITS and sub-regions as DNA barcode markers for the identification of Basidiomycota (Fungi). *BMC Microbiology* 17(1):1-12

BERNINGER, J. P., Demarini, D. M., Warren, S. H., Simmons, J. E., Wilson, V. S., Conley, J. M., . . . Bradley, P. M. (2019). Predictive analysis using chemical-gene interaction networks consistent with observed endocrine activity and mutagenicity of U.S. treams. *Environtal Science and Technology* 53(15):8611-8620

BGI. (2020). BGI Next generation sequencing platforms and specifications. Https://Www.Bgi.Com/Global/Resources/Sequencing-Platforms/: [Accessed: 20/02/20].

BIGGS, J., Ewald, N., Valentini, A., Gaborioud, C., Griffiths, R. A., Foster, J., . . . Dunn, F. (2014). Analytical and methodological development for improved surveillance of the Great Crested Newt. Appendix 5. Technical advice note for field and laboratory ampling of Great Crested Newt (*Triturus Cristatus*) environmental DNA.

BLACKWELL, B. R., Ankley, G. T., Bradley, P. M., Houck, K. A., Makarov, S. S., Medvedev, A. V., . . . Villeneuve, D. L. (2019). Potential toxicity of complex mixtures in surface waters from a nationwide survey of United States streams: Identifying in vitro

bioactivities and causative chemicals. *Environmental Science and Technology* 53(2):973-983

BOWERS, R. M., Kyrpides, N. C. And Woyke, T. (2017). Minimum Information about a single amplified genome (MISAG) and a metagenome-assembled genome (MIMAG) of bacteria and archaea. *Nature Biotechnology* 35(8):725-731

CORSE, E., Meglecz, E., Archambaud, G., Ardisson, M., Martin, J. F., Tougard, C., . . . Dubut, V. (2017). A from-benchtop-to-desktop workflow for validating HTS data and For taxonomic identification in diet metabarcoding studies. *Molecular Ecological Resources* 17(6):E146-E159

COUNCIL, N. E. R. (2020). Definition of Technology Readiness Levels. In: UKRI Ed. Https://Nerc.Ukri.Org/Research/Funded/Programmes/Technologypoc/Tech-Readiness-Levels/

CRITCHLOW-WATTON, N., Dobbie, K. E., Bell, R., Campbell, S. D. G., Hinze, D., Motion, A., . . . Towers, W. (2014). Scotland's state of the environment report. Scotland's Environment Web.: https://www.environment.gov.scot/our-environment/state-of-the-environment/2014-state-of-the-environment-report/

DALY, A. J., Baetens, J. M. And Baets, B. D. (2018). Ecological diversity: measuring the unmeasurable. *Mathematics* 6(7):119

Defra (Department for Environment food and Rural Affairs). (2018). A Green Future: Our 25 Year Plan To Improve The Environment. HM Government https://www.gov.uk/government/publications/25-year-environment-plan

EFSA, P. (2018). Scientific Opinion on the state of the art of Toxicokinetic/Toxicodynamic (TKTD) effect models for regulatory risk assessment of pesticides for aquatic rganisms. *EFSA Journal* 16(8):5377

EMBL-EBI, M. (2020). Mgnify submit, analyse, discover and compare microbiome data. Available At: Https://Www.Ebi.Ac.Uk/Metagenomics/ [Accessed: 30/03/20]

FAIRCLOTH, B. C. (2017). Identifying conserved genomic elements and designing universal bait sets to enrich them. *Methods in Ecology and Evolution* 8:1103-1112

FELDGARDEN, M., Brover, V., Haft, D. H., Prasad, A. B., Slotta, D. J., Tolstoy, I., . . . Klimke, W. (2019). Validating the amrfinder tool and resistance gene database by using antimicrobial resistance genotype-phenotype correlations in a collection of isolates. *Antimicrobial Agents and Chemotherapy* 63(11)

FICETOLA, G. F., Miaud, C., Pompanon, F. And Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biological Letters* 4(4)

GASC, C., Constantin, A., Jaziri, F. And Peyret, P. (2017). Ocappi-Db: An oligonucleotide probedDatabase for pathogen identification through hybridization capture. Database 2017 (Baw172)

GAUTHIER, M., Konecny-Dupré, L., Nguyen, A., Elbrecht, V., Datry, T., Douady, C. And Lefébure, T. (2019). Enhancing DNA metabarcoding performance and applicability with bait capture enrichment and DNA from conservative ethanol. *Molecular Ecology Resources* 20(1):79-96

GRAY, M. A., Pratte, Z. A. And Kellogg, C. A. (2013). Comparison of DNA preservation methods for environmental bacterial community samples. *FEMS Microbiology Ecology* 83:468-477

GRIFFIN, P. C., Khadake, J., Lemay, K. S., Lewis, S. E., Orchard, S., Pask, A., . . . Schneider, M. V. (2017). Best practice data life cycle approaches for the life sciences. F1000Research 6

HAN, J., Won, E. J., Kang, H. M., Lee, M. C., Jeong, C. B., Kim, H. S., . . . Lee, J. S. (2017). Marine copepod cytochrome P450 genes and their applications for molecular ecotoxicological studies in response to oil pollution. Marine Pollution Bulletin 124(2):953-961

HARRIS, S., Morris, C., Morris, D., Cormican, M. And Cummins, E. (2014). Antimicrobial eesistant *Escherichia Coli* in the municipal wastewater system: Effect of hospital effluent and environmental fate. Science of the Total *Environment* 468-469:1078-1085

HOANG, M. T. V., Irinyi, L., Chen, S. C. A., Sorrell, T. C., Group, T. I. B. O. M. F. W. And Meyer, W. (2019). Dual DNA barcoding for the molecular identification of the agents of invasive fungal infections. *Frontiers in Microbiology* 10(1647)

HONG, J. And Gresham, D. (2017). Incorporation of unique molecular identifiers in Truseq adapters improves the accuracy of quantitative sequencing. *Biotechniques* 63(5):221-226

HORNUNG, B. V. H., Zwittink, R. D. And Kuijper, E. J. (2019). Issues and current standards of controls in microbiome research. *FEMS Microbiology Ecology* 95(5)

HUANG, B., Ji, L., Liang, B., Cao, Q., Tu, T. And Ye, X. (2019). A simple and low-cost screen printed electrode for hepatocellular carcinoma methylation detection. *Analyst* 144(10):3282-3288

HYNES, H. B. N. (1961). The invertebrate fauna of a welsh mountain stream. *Arch. Hydrobiology* 57:344-388

ILLUMINA. (2016). Nextera DNA library prep reference guide. San Diego, California: Illumina

ILLUMINA. (2020). Sequencing platforms: compare NGS platform applications. Available At: Https://Emea.Illumina.Com/Systems/Sequencing-Platforms.Html [Accessed: 20/02/20]

JAUSET-RIBIO, M., Svobodova, M., Mairal, T., Mcneil, C., Keegan, N., Saeed, A., . . . O'Sullivan, C. K. (2016). Ultrasensitive, rapid and inexpensive detection of DNA using paper based lateral flow assay. *Scientific Reports* 6(1):1-10

JEANNIARD DU DOT, T., Thomas, A. C., Cherel, Y. And Trites, A. W. (2017). Combining hard-part and DNA analyses in scats with Biologging and Ssable isotopes can reveal different diet compositions and feeding strategies within a population. *Marine Ecology Progress Series* 584

JIAO, X., Zheng, X., Ma, L., Kutty, G., Gogineni, E., Sun, Q., . . . Huang, D. W. A benchmark study on error assessment and quality control of CCS reads derived from The Pacbio RS. *Journal of Data Mining in Genomics and Proteomics* 4(3)

JOHNSON, J. S., Spakowicz, D. J., Hong, B.-Y., Petersen, L. M., Demkowicz, P. D., Chen, L., . . . Weinstock, G. M. (2019). Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nature Communications* 10(1):1-11

KAVVAS, E. S., Catoiu, E., Mih, N., Yurkovich, J. T., Seif, Y., Dillon, N., . . . Palsson, B. O. (2018). Machine learning and structural analysis of *Mycobacterium tuberculosis* pan-genome identifies genetic signatures of antibiotic resistance. *Nature Communications* 9:4306

KEBSCHULL, J. M. And Zador, A. M. (2015). Sources of PCR-induced distortions in high-throughput sequencing data sets. *Nucleic Acids Research* 43(21):E143

KELLY, M.G., Juggins, S., Mann, D.G., Sato, S., Glover, R., Boonham, N., Sapp, M., Lewis, E., Hany, U., Kille, P., Jones, T. Walsh, K. (2020) Development of a novel metric for evaluating diatom assemblages in rivers using DNA metabarcoding. *Ecological Indicators* 118: https://doi.org/10.1016/j.ecolind.2020.106725

KING, R. A., Read, D. S., Traugott, M. And Symondson, W. O. C. (2008). Invited Review: Molecular analysis of predation: a review of best practice for DNA-based approaches. *Molecular Ecology* 17(4):947-963

KIVIOJA, T., Vaharautio, A., Karlsson, K., Bonke, M., Enge, M., Linnarsson, S. And Taipale, J. (2011). Counting absolute numbers of molecules using unique molecular identifiers. *Nature Methods* 9(1):72-74

KOONIN, E. V., Makarova, K. S. And Aravind, L. (2003). Horizontal gene transfer in prokaryotes: Quantification And Classification1. Http://Dx.Doi.Org/10.1146/Annurev.Micro.55.1.709 55(2001):709-742

KREHENWINKEL, H., Pomerantz, A., Henderson, J. B., Kennedy, S. R., Lim, J. Y., Swamy, V., . . . Gillespie, R. G. (2020). Nanopore sequencing of long ribosomal DNA amplicons enables portable and simple biodiversity assessments with high phylogenetic resolution across broad taxonomic scale. *Gigascience* 8(5)

LAWSON-HANDLEY, L., Lunt, D., Shum, P., Winfiled, I., Read, D. And Hanfling, B. (2017). A review of recent advances in genetic methods to identify improvements in CAMERAS partners monitoring activities. Hull: Scottish Government

LEVY, S. E. And Myers, R. M. (2016). Advancements in next-generation sequencing. *Annual Reviews* 17:95-115

LEXOGEN. (2019). Lexogen I5 6 Nt unique dual indexing add on kit (5001-5096). Https://Www.Lexogen.Com/Store/I5-Unique-Dual-Indexing-Add-On-Kit/: Lexogen Gmbh. Available At: [Accessed: 20/02/20]

LI, C., Chng, K. R., Boey, E. J. H., Ng, A. H. Q., Wilm, A. And Nagarajan, N. (2020). INC-Seq: accurate single molecule reads usingnNanopore sequencing. *Gigascience* 5(1).

LIU, G., He, F., Li, X., Xhao, H., Zhang, Y., Li, Z. And Yang, Z. (2019). Multi-level separation of particles using acoustic radiation force and hydraulic force n a microfluidic chip. *Microfluidics and Nanofluidics* 23(23)

LIU, S., Wang, X., Xie, L., Tan, M., Li, Z., Su, X., . . . Zhou, X. (2015). Mitochondrial capture enriches mito-DNA 100 fold, enabling PCR-free mitogenomics biodiversity analysis. *Molecular Ecology Resources* 16(2):470-479

MCCLENAGHAN, B., Compson, Z. G. And Hajibabaei, M. (2020). Validating metabarcoding-based biodiversity assessments with multi-species occupancy models: A case study using coastal marine eDNA. *PLoS One* https://doi.org/10.1371/journal.pone.0224119

MCMILLAN, E. A., Gupta, S. K., Williams, L. E., Jové, T., Hiott, L. M., Woodley, T. A., . . . Frye, J. G. (2019). Antimicrobial resistance genes, cassettes, and plasmids present in *Salmonella enterica* associated with United States food animals. *Frontiers in Microbiology* 10(PMC6479191)

MURRAY, D. C., Coghlan, M. L. And Bunce, M. (2015). From benchtop to desktop: important considerations when designing amplicon sequencing workflows. *PLoS One* 10(4):E0124671

NELSON, M. B., Martiny, A. C. And Martiny, J. B. H. (2016). Global biogeography of microbial nitrogen-cycling traits in soil. *Proceedings of the National Acadademy of Sciences USA* 113 (29):8033-8040

NICHOLLS, S. M., Quick, J. C., Tang, S. And Loman, N. J. (2019). Ultra-deep, long-read nanopore sequencing of mock microbial community standards. *Gigascience* 8(5)

OSGEO. (2020). Geonode. Available At: Https://Www.Osgeo.Org/Projects/Geonode/ [Accessed: 30/03/20]

PAUL, N., Shum, J. And Le, T. (2010). Hot start PCR. *Methods in Molecular Biology* 630:301-31

PETROVA, O. E., Garcia-Alcalde, F., Zampaloni, C. And Sauer, K. (2017). Comparative evaluation of rRNA depletion procedures for the improved analysis of bacterial biofilm and mixed pathogen culture transcriptomes. *Scientific Reports* 7:41114

POCOCK, M. J., Evans, D. M. And Memmott, J. (2012). The robustness and restoration of a network of ecological networks. *Science* 335(6071):973-977

ROY, S., Wei, S. X., Ying, J. L. Z., Safavieh, M. And Ahmed, M. U. (2016). A novel, sensitive and label-free loop-mediated isothermal amplification detection method for nucleic acids using luminophore dyes. *Biosensors and Bioelectronics* 86:346-352

RÉROLLE, V., Achterberg, E., Ribas-Ribas, M., Kitidis, V., Brown, I., Bakker, D., . . . Mowlem, M. (2018). High resolution pH measurements using Lab-on-chip sensor in surface waters of northwest European shelf seas. *Sensors* 18(8):2622

SHi, J., Yan, Y., Links, M. G., Li, L., Dillon, J. R., Horsch, M. And Kusalik, A. (2019a). Antimicrobial resistance genetic factor identification from whole-genome sequence data using deep feature selection. *BMC Bioinformatics* 20(Suppl 15):535

SHI, W., Qi, H., Sun, Q., Fan, G., Liu, S., Wang, J., . . . Ma, J. (2019b). Gcmeta: A global catalogue of metagenomics platform to support the archiving, standardization and analysis of microbiome data. In: *Nucleic Acids Research*. Vol. 47. Pp. D637-648

SHIRATO, K., Semba, S., El-Kafrawy, S. A., Hassan, A. M., Tolah, A. M., Takayama, I., . . . Azhar, A. I. (2018). Development of fluorescent reverse transcription loop-mediated isothermal amplification (RT-LAMP) using quenching probes for the detection of the Middle East respiratory syndrome coronavirus. *Journal of Virological Methods* 258:41-48

SHOAIE, N. And Forouzandeh, M. (2018). Voltammetric determination of the *Escherichia coli* DNA using a screen-printed carbon electrode modified with polyaniline and gold nanoparticles. *Microchimica Acta* 185(217)

SHOKRALLA, S., Gibson, J. F., King, I., Baird, D. J., Janzen, D. H., Hallwachs, W. And Hajibabaei, M. (2016). Environmental DNA barcode sequence capture: Targeted, PCR-free sequence capture for biodiversity analysis from bulk environmental samples. Biorxiv **doi:** https://doi.org/10.1101/087437

SIEGENTHALER, A., Wangensteen, O. S., Soto, A. Z., Benvenuto, C., Corrigan, L. And Mariani, S. (2019). Metabarcoding of shrimp stomach content: Harnessing a natural sampler for fish biodiversity monitoring. *Molecular Ecology Resources* 19(1):206-220

SINGER, A. (2017). Antimicrobial resistance: More than just overusing antibiotics. *The Pharmaceutical Journal* 298(7898)

SM, C.-R., D, A. L.-B. And L, D. (2020). Removal of polycyclic aromatic hydrocarbons from soil amended with biosolid or vermicompost in the presence of earthworms (Eisenia Fetida). *Soil Biology & Biochemistry*. 40(7):1954-1959

SMITH, S., Momber, G., Bates, R., Garwood, P., Fitch, S., Pallen, M., . . . Allaby, R. G. (2015). Sedimentary DNA from a submerged site reveals wheat in the British Isles 8000 years ago. *Science* 347(6225):998-1001

STERN, R. F., Picard, K. T., Hamilton, K. M., Walne, A., Tarran, G. A., Mills, M., McQuatters-Gollop, A. And Edwards, M. (2015). Novel lineage patterns from an automated water sampler to probe marine microbial biodiversity with ships of opportunity. *Progress in Oceanography* 137(B):409-420

SYMONDSON, W. O. (2002). Molecular identification of prey in predator diets. *Molecular Ecology* 11(4):627-641

TEDERSOO, L., Ramirez, K. S., Nilsson, R. H., Kaljuvee, A., Kõljalg, U. And Abarenkov, K. (2015). Standardizing metadata and taxonomic identification in metabarcoding studies. *Gigascience*. Vol. 4

TITULAER, M., Melgonza-Castillo, A., Oanjabi, A. O., Sanchez-Flores, A., Martinez-Guerrero, J. H., Macias-Duarte, A. And Fernandez, J. A. (2017). Molecular analysis of stomach contents reveals important grass seeds in the winter diet of Baird's and Grasshopper sparrows, two declining grassland bird pecies. *PLoS One* 12(12):E0189695

VAN KESSEL, M. A., Speth, D. R., Albertsen, M., Nielsen, P. H., Op Den Camp, H. J., Kartal, B., . . . Lucker, S. (2015). Complete Nitrification By A Single Microorganism. Nature 528(7583):555-559

VILLENEUVE, D. L., Crump, D., Garcia-Reyero, N., Hecker, M., Hutchinson, T. H., Lalone, C. A., . . . Nepelska, M. (2020). Adverse outcomep athway (AOP) development I: Strategies and principles. *Toxicological Sciences* 142(2):312-320

VON BERG, K., Traugott, M., Symondson, W. O. And Scheu, S. (2008). The effects of temperature on detection of prey DNA in two species of carabid beetle. *Bulletin of Entomological Research* 98(3):263-269

WASEEM, H., Jameel, S., Ali, J., Saleem Ur Rehman, H., Tauseef, I., Farooq, U., . . . Ali, M. I. (2019). Contributions and challenges of high throughput qPCR for determining antimicrobial resistance in the environment: A critical review. *Molecules*. Vol. 24

WELSH GOVERNMENT. (2016). Environment (Wales) Act. In: Government, W. Ed

WHALE, A. S., Devonshire, A. S., Karlin-Neumann, G., Regan, J., Javier, L., Cowen, S., . . . Huggett, J. F. (2017). International interlaboratory digital PCR study demonstrating high reproducibility for the measurement of a rare sequence variant. *Analytical Chemistry* 89(3):1724-1733

WILKE, A., Bischof, J., Gerlach, W., Glass, E., Harrison, T., Keegan, K. P., . . . Meyer, F. (2016). The MG-RAST metagenomics database and portal in 2015. *Nucleic Acids Research* 44(D1):D590-594

WOYKE, T., Doud, F. R. And Schulz, R. (2017). The trajectory of microbial single-cell sequencing. *Nature Methods* 14(11):1045-1054

ZIPFEL, C. And Oldroyd, G. E. (2017). Plant signalling in symbiosis and immunity. *Nature* 543(7645):328-336

Would you like to find out more about us or your environment?

Then call us on

03708 506 506 (Monday to Friday, 8am to 6pm)

Email: enquiries@environment-agency.gov.uk

Or visit our website

www.gov.uk/environment-agency

incident hotline

0800 807060 **(24 hours)**

floodline

0345 988 1188 **(24 hours)**

Find out about call charges (https://www.gov.uk/call-charges)

Environment first

Are you viewing this onscreen? Please consider the environment and only print if absolutely necessary. If you are reading a paper copy, please don’t forget to reuse and recycle.