How will the "molecular revolution" contribute to biological recording?

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Abstract

Soaring throughput, plummeting costs and increased sensitivity for assaying degraded or low concentration DNA are driving a revolution in the way we monitor biodiversity. Arguably the biggest "game-changer" is environmental DNA (eDNA) – which refers to free-floating DNA released by organisms into their environment. Rare or elusive species can be detected with greater sensitivity and accuracy using eDNA than by most conventional methods, and we have the capability to screen and describe whole communities as well as performing targeted monitoring of single species. In this paper I discuss the basic approaches for molecular monitoring of biodiversity, provide case studies to demonstrate the effectiveness of the techniques, and consider any challenges and limitations that could impact molecular biological recording. I argue that eDNA surveys offer exciting new opportunities to engage the public in biological recording and that molecular approaches will complement conventional surveys, giving us unprecedented insights into species distributions. Finally, with the number of eDNA studies increasing at a rapid pace, I argue that we need to rapidly establish ways for managing molecular records. Integrating molecular records into existing biological records databases would enhance our understanding of species distributions and may be something that the Biological Records Centre should be considering to mark its landmark anniversary.

Fifty years ago the Biological Records Centre (BRC) was at the pioneering heart of a revolution in the way we record biodiversity. Today, we are undergoing a revolution in the way we *describe* biodiversity. In recent years we have progressed from being able to identify individual organisms from specific DNA sequences or "barcodes" to analysing hundreds of thousands of DNA barcodes from environmental samples, enabling us to describe whole communities. This high throughput approach, known as "DNA metabarcoding", is particularly powerful for revealing cryptic biodiversity (Creer *et al.*, 2010; Bik *et al.*, 2012; Yu *et al.*, 2012).

In addition to this increase in throughput, another revolution is ongoing in the field of environmental DNA or "eDNA". Organisms release DNA into their environments, for example through faeces, moulting, mucous secretion, or releasing gametes. The sensitivity of DNA-based assays means that it is possible to detect tiny amounts of degraded eDNA present in the environment. This has great promise for biodiversity monitoring because it is non-invasive, and it has already been proven effective for monitoring rare and/or elusive species, particularly in freshwater environments. Indeed, eDNA monitoring in aquatic systems was identified as one of the 15 most important global conservation issues in a 2013 horizon scanning exercise (Sutherland *et al.*, 2013) and may be a "game changer" in biodiversity monitoring. The aim of this article is to evaluate how these molecular approaches are likely to contribute to biological recording. I briefly summarise the main approaches, present key case studies in eDNA monitoring of biodiversity, detection of invasive alien species (IAS) and detection of trophic interactions, demonstrate through an example how the public can be engaged in molecular biological recording, and discuss the burning issues relating specifically to eDNA analyses. Finally I evaluate some of the logistics of molecular biological

recording, such as whether it is cost-effective, and open a debate on how the data should be managed.

The approach

Why take a molecular approach for biological recording?

Between 10 and 50% of species from well-studied taxonomic groups (e.g. mammals, birds, amphibians, higher land plants) are threatened with extinction (Millenium Ecosystem Assessment, 2005) and current rates of species loss could be up to 1000 times higher than background rates (i.e. those before human influence, Millenium Ecosystem Assessment, 2005; Pimm *et al.,* 2014). Despite good knowledge of certain taxonomic groups, it is thought that 15% more plant species and the great majority of animals are yet to be described (Pimm *et al.,* 2014). We have a poorer understanding of the status of organisms in aquatic compared to terrestrial environments (Millenium Ecosystem Assessment, 2005), but freshwater ecosystems seem to be particularly vulnerable (McLellan *et al.*, 2014). Indeed, populations of freshwater species declined by an average of 76% between 1970 and 2010 – almost double the rate of decline for populations of terrestrial species (McLellan *et al.*, 2014).

Reliable cost-effective methods for large-scale screening of biodiversity are essential if we are to slow this rate of species loss. Traditional methods, based for example, on trapping, netting, acoustic or observational surveys can be costly, time consuming, and sadly are often inefficient. Perhaps the greatest limitations of traditional surveys are 1) the difficulties associated with recording rare and/or elusive species, and 2) dealing with taxonomically similar species, juvenile life stages or cryptic biodiversity. Methods based on DNA barcoding are promising to complement traditional approaches and help

overcome these limitations. This is becoming more feasible due to the rapidly decreasing costs associated with DNA sequencing and technological improvements for assaying degraded or low concentration DNA. Another benefit of molecular methods is that they are easily auditable, since samples can be split and analysed in independent laboratories (Ji *et al.*, 2013). However, DNA-based methods are not without their own limitations. It is impossible, for example, to infer age structure of populations from DNA data, and there are other challenges, which I discuss below.

DNA barcoding or metabarcoding?

The technology and application of DNA barcoding and metabarcoding in ecology has been reviewed extensively elsewhere (see for example Valentini, Pompanon, & Taberlet, 2009; Creer *et al.*, 2010; Taberlet *et al.*, 2012; Bohmann *et al.*, 2014) therefore just a brief introduction is provided here. In "traditional" DNA barcoding, a short gene segment (500-800 bp) is amplified by polymerase chain reaction (PCR) using widely conserved primers, and sequenced (using Sanger sequencing) from DNA extracted from a single individual. The DNA sequence or "barcode" should be species specific, allowing taxonomic identification by comparison with a public DNA data bank such as the International Barcode of Life (iBOL) Consortium's Barcode Library (Barcode of Life Data Systems, www.boldsystems.org). The most commonly used DNA barcodes are the mitochondrial cytochrome c oxidase subunit I gene (*COI)* for animals (Hebert, Ratnasingham, & deWaard, 2003), the chloroplast ribulose biphosphate carboxylase gene (*rbcL)* gene for plants (Hollingsworth & Andra Clark, 2009), and the ribosomal internal transcribed spacer (*ITS*) for fungi (Schoch *et al.*, 2012). Mitochondrial or chloroplast genes generally make attractive molecular markers because of their uniparental inheritance, high mutation rates and the fact that they are found in multiple

copies in a cell, coupled with the fact that it is relatively straightforward to design conserved PCR primers. However neither *COI* nor *rbcL* are perfect. Some taxonomic groups, such as nematodes, are notoriously problematic to resolve using *COI,* and also exhibit extensive rearrangements in their mitochondrial genes (Powers, 2004). Therefore the locus of choice for nematodes (and other meiofauna) is usually the 18S (small subunit or "SSU") rRNA gene, which is a nuclear gene present in typically 50-100 copies (Floyd *et al.*, 2002; Powers, 2004, although note that this region greatly underestimates diversity in some meiofaunal groups, Tang *et al.*, 2012). Use of *COI* for barcoding a broad range of taxa has recently been criticised since the region does not have sufficiently conserved regions for primer design (Deagle *et al.*, 2014). Some applications, for example identification of museum specimens or environmental samples (see below), require shorter fragments for analyses than the standard barcodes because of DNA degradation problems. For this reason, a considerable amount of effort has gone into developing "mini-barcodes" based on the minimum amount of sequence required for species identification. Mini-barcodes are typically a 90-250 bp long portion of the primary barcoding genes (e.g. for *COI* Hajibabaei *et al.,* 2006; Meusnier *et al.,* 2008 and *rbcL* Little, 2014).

Arguably the great disadvantage of DNA barcoding is that analysing single organisms using traditional DNA sequencing methods is expensive and inefficient. Rather than focussing on single organisms, DNA metabarcoding characterises species assemblages either from a homogenised "soup" of whole organisms (for example obtained from pitfall traps or other mass trapping methods, e.g. Yu *et al.*, 2012) or from environmental DNA (see below). DNA from the whole community is PCR amplified using similar markers to those for standard barcoding/mini-barcoding, sequenced on a Next

Generation Sequencing ("NGS") platform (for example an Illumina HiSeq or MiSeq sequencing system) and analysed using bioinformatics pipelines. The throughput of next generation sequencers has increased a million fold since the turn of the century, while costs have plummeted (e.g. Glenn, 2011). Metabarcoding is revolutionising our understanding of the diversity of microscopic eukaryotes (Bik *et al.*, 2012) in environments that are traditionally difficult to study such as soil (Porazinska & Giblin-Davis, 2009), other sediments (Creer *et al.*, 2010), and the deep sea (Fonseca *et al.*, 2010). This approach is considered the leading technological advance for biodiversity measurement (Ji *et al.*, 2013) and could lead to a shift in the focus of biodiversity monitoring away from reliance on indicator species, which are not always appropriate surrogates for the health of whole communities (e.g. Cushman *et al.*, 2010).

A crucial question associated with metabarcoding studies is whether the method accurately reflects the true diversity both qualitatively and quantitatively. The validity of metabarcoding has been demonstrated by testing against artificially assembled samples of known composition (e.g. Hiiesalu *et al.*, 2012; Yu *et al.*, 2012) and several studies have demonstrated that metabarcoding generates reliable qualitative estimates of diversity (Fonseca *et al.*, 2010; Hiiesalu *et al.*, 2012; Yu *et al.*, 2012; Yoccoz *et al.*, 2012; Ji *et al.*, 2013). For example, Yu *et al.*, (2012) individually sequenced DNA barcodes from over 1300 insects collected in malaise traps at three sites in China. DNA from the 547 individuals representing different Operational Taxonomic Units ("OTUs") was then pooled into several mixtures, analogous to ecological communities with known composition, and mass sequenced by metabarcoding. A total of 598 OTUs were recovered during the bioinformatics steps from >130,000 DNA sequences. The number of OTUs is greater than the number identified from individual sequencing

perhaps because of the ability of metabarcoding to detect prey in predators' gut contents and parasite DNA in hosts (see section "*Detecting records within records: molecular detection of trophic interactions"*). Encouragingly, there was high correlation between sequencing methods in their estimates of unweighted alpha and beta diversity (i.e. diversity within and between samples respectively, based on presence-absence data rather than abundance), demonstrating that metabarcoding accurately recovers these important indices.

It could be argued that validating metabarcoding based on artificially constructed species assemblages does not guarantee the method will translate to real world situations. This is an important argument if biodiversity assessments based on metabarcoding are to be used to inform policy-making. To address this issue, Ji *et al.*, (2013) compared metabarcoding data sets against three large-scale standard biodiversity data sets, comprising over 55,000 morphologically identified indicator specimens, from China, Malaysia and the U.K. that were collected for answering policy questions. Encouragingly the data from metabarcoding and standard datasets were highly consistent, returned correlated diversity estimates, and produced the same conclusions for policy making (Ji *et al.*, 2013). While these studies offer encouragement for the use of metabarcoding alongside standard methods for biodiversity assessment, one of the remaining major challenges is whether the methods are quantitative, which I discuss further in the section on "*Challenges and limitations*" at the end of this review.

Environmental DNA (eDNA)

In 2003, a pioneering study obtained environmental DNA from Pleistocene animal and plant communities in the Siberian permafrost, as well as from more recent (600 to 3000

year old) cave and coastal sediments in New Zealand (Willerslev *et al.*, 2003). Since then, over 1000 papers have been published on eDNA, and the technology has been used to reconstruct both past and present flora and fauna from soil and other sediments (Sønstebø Gielly, & Brysting, 2010; Andersen *et al.,* 2012; Jørgensen *et al.,* 2012; Yoccoz *et al.,* 2012; Pedersen *et al.,* 2013), for non-invasive tracking of animals (for example from their faeces, see Beja-Pereira *et al.,* 2009 for a review, or footprints in snow Dalén *et al.,* 2007), for detecting genetically modified pollen in air (Folloni *et al.* 2011) and detecting prey species in predator gut contents (discussed under "*Detecting records within records: molecular detection of trophic interactions"* below). However perhaps the greatest potential of eDNA technology is for monitoring current biodiversity in aquatic environments (Table 1) and we only began to realise this potential quite recently. The field took a leap forward in 2008 with the application of eDNA to detection of invasive American bullfrogs, *Rana catesbeiana,* in French wetlands (Ficetola *et al.*, 2008, discussed further below). Since then, eDNA has been used to detect a number of rare, elusive or invasive species (Table 1), and the approach is shifting from targeting specific species to describing whole communities using metabarcoding. Government agencies across the world are realising the potential of eDNA to contribute towards biodiversity monitoring and early detection of invasive species, and are investing in the approach. However, we still have many challenges ahead before eDNA data can be used to inform policy and decision making.

Perhaps the greatest advantages of this method are that it is non-invasive, and very sensitive, with eDNA detection rates generally outperforming conventional survey methods (see below for more details). eDNA can be analysed in several different ways depending on the environment under study and whether assays need to be targeted (i.e.

species specific), or describe whole communities (see Fig. 1 for a basic overview of the approach). Species-specific assays use standard or quantitative PCR (qPCR) with primers that only amplify the target species. qPCR has the advantages of much greater sensitivity, so that very low DNA concentrations can be detected, and by definition the ability to quantify the number of copies of target DNA that are present. This can then be translated into estimates of relative abundance (see "*Challenges and limitations*" below for further discussion).

Few metabarcoding studies have so far been carried out on eDNA, but this is set to rapidly change as there is huge appeal in describing whole communities from environmental samples. The first studies to blaze the trail for eDNA metabarcoding were carried out by the same group that recovered eDNA from the ancient animal and plant communities, described above. Andersen *et al.*, (2012) investigated whether metabarcoding could accurately recover vertebrate diversity from samples of soil collected from enclosures in safari parks, zoos and farms. DNA sampled from the soil surface accurately reflected taxonomic richness, and interestingly, relative biomass of the species present (Andersen *et al.*, 2012). Very few other studies have so far employed eDNA metabarcoding for biodiversity assessment (but see Thomsen *et al.*, 2012a; Kelly *et al.*, 2014), but the approach is being tested extensively at time of writing. The key question is whether this method can be used to generate sufficiently reliable estimates of species abundance as well as presence-absence (as discussed under "*Challenges and limitations*"). If it can, it will revolutionise the recording of biological diversity.

Case studies in molecular biological recording

Detection of invasive alien species

With new EU regulation on invasive alien species introduced in January 2015, there is recognition of a pressing need for more effective early warning systems for invasive species (Schulz & Vedova, 2014). One of the great promises of eDNA is that it can be used to detect rare species that can easily go unnoticed. This makes it a particularly promising tool as an early warning system for detecting IAS (or indeed for pathogens) before they establish, as well as for monitoring establishment and spread, particularly in aquatic environments (Darling & Mahon, 2011).

As mentioned above, the first application of eDNA for detection of invasive species was on American bullfrogs, *R. catesbeiana* in France (Ficetola *et al.*, 2008). American bullfrogs are native to eastern North America but were introduced worldwide during the 20th century. They are recognised as one of the world's 100 worst invasive species (DAISIE, http://www.europe-aliens.org; Dejean *et al.*, 2012) and have been linked to the decline of native amphibians via competition, predation and spread of disease (Ficetola *et al.*, 2008 and references therein). In France there are three established populations and two are subject to control methods. An assay was developed based on standard PCR using species-specific primers (Ficetola *et al.*, 2008) and later the sensitivity of the method was compared to traditional methods based on auditory nocturnal and visual diurnal encounter rates carried out at the same time as eDNA sampling (Dejean *et al.*, 2012). American bullfrog eDNA was detected in 38 ponds out of 49 sampled, compared to only seven sites for the traditional methods, suggesting that their distribution had been previously underestimated using traditional methods (Dejean *et al.*, 2012). Encouragingly, positive eDNA results were obtained for all 7 sites where bullfrogs were detected using traditional methods (Dejean *et al.*, 2012). One potential reason for the discrepancy between methods is the ability of eDNA to detect

bullfrogs at very low densities and at any life stage. Since these pioneering bullfrog studies, species specific assays have been developed and successfully deployed to detect other invasive species including fish, reptiles, crustaceans, molluscs, and echinoderms as well as fungal pathogens (Table 1), and the number is rapidly increasing.

It is widely known that prevention is the most effective form of management for IAS (Leung *et al.*, 2002; Hulme, 2009). Perhaps one of the greatest opportunities for eDNA methods is in routine surveillance of invasion pathways in order to detect IAS before they enter the environment. Invasive species can enter aquatic systems via a number of pathways (Hulme, 2009; Roy *et al.*, 2014). Ship ballast water is a particularly important source, and discharge standards are in place in some countries (e.g. the USA) to prevent IAS release (Frazier *et al.*, 2013). eDNA methods are likely to be particularly useful for identifying larval stages of marine invertebrates in ballast, where morphological identification is unreliable and impractical. Single-species PCR assays have been developed for detection of the northern Pacific seastar, *Asterias amurensis* in mixed plankton or ballast water samples (Deagle *et al.,* 2003). However assays still typically need to be carried out in a lab, which is too time-consuming for effective ballast screening. There is an urgent need for on-board screening of ship's ballast before port entry to provide sufficient time to implement control measures (Mahon *et al.*, 2011). A leap in this technology came in 2011, when a portable microfluidic detection platform was developed for ballast water screening (Mahon *et al.*, 2011). The system combines DNA extraction and standard PCR with a carbon nanotube microfluidic detection chip to identify target species (Mahon *et al.*, 2011). Chip-based assays were successfully designed and tested for three ballast-transported IAS; quagga mussel, *Dreissena*

rostriformis bugensis, Chinese mitten crab, *Eriocheir sinensis,* and golden mussel *Limnoperna fortuneii* (Mahon et al., 2011).

Targeted assays such as these are appropriate for active surveillance of priority species, but they have an obvious drawback: they miss non-target IAS that may be present in the sample. Passive surveillance of IAS pathways using metabarcoding is therefore of great interest and may provide a substantial benefit for IAS management. The ability of metabarcoding to detect very rare species in complex samples such as mixed plankton or ballast has been demonstrated by artificial spiking of indicator species with known biomass (Zhan *et al.*, 2013). Metabarcoding assays for screening ballast are currently in development and undergoing field trials, but Mahon, Nathan, & Jerde, (2014) tested the utility of this approach for passive detection of IAS in the bait trade pathway of the North American Great Lakes Basin. Surprisingly, the study detected seven non-bait species in a total of six bait shops. Of greatest concern was the detection, and subsequent confirmation of invasive white perch, *Morone americana,* in bait shops in three states across the Great Lakes region (Mahon *et al.*, 2014). Since *M. americana* was not expected to be present in the sample, this perfectly illustrates the greater power of metabarcoding for detecting non-target species.

Monitoring biodiversity for conservation

One of the great challenges in conservation is to effectively monitor rare and/or elusive species without causing disturbance. eDNA methods are particularly promising for monitoring of rare or elusive species due to their sensitivity and non-invasive nature. Thomsen *et al.*, (2012b) evaluated the use of eDNA for targeted monitoring of rare freshwater species from diverse taxonomic groups (amphibians: common spadefoot

toad, *Pelobates fuscus,* great crested newt, *Triturus cristatus;* fish: European weather loach, *Misgurnus fossilis;* mammals: Eurasian otter, *Lutra lutra;* crustaceans: tadpole shrimp, *Lepidurus apus;* and insects: large white-faced darter, *Leucorrhinia pectoralis*). eDNA detection rates in natural freshwater ponds, where the species were known to be present, were 100% for *P. fuscus, M. fossilis* and *L. apus,* and above 80% for *T. cristatus* and *L. pectoralis* (Thomsen et al., 2012b). Although detection was more difficult in larger freshwater systems, detection rates for the two species investigated (*L. lutra* and *M. fossilis*) were still comparable to, and may provide a valuable complement to, traditional methods (Thomsen *et al.*, 2012b). A small-scale metabarcoding study, using conserved vertebrate primers and carried out on the same pond water samples, detected all species of fish and amphibians previously recorded from the ponds, as well as several birds and a mammal (Thomsen *et al.*, 2012b). This illustrates the potential for the monitoring of entire communities for biodiversity using metabarcoding, potentially shifting the focus away from a small number of indicator species.

Monitoring biodiversity in the marine environment is obviously more challenging due to the scales involved. Despite additional challenges to eDNA studies from marine water chemistry and the dilution effects of strong tides and currents, eDNA has already shown promise for monitoring marine mammals (Foote *et al.*, 2012) and fish (Thomsen *et al.*, 2012a). Foote *et al.*, (2012) evaluated whether eDNA from harbour porpoises, *Phocena phocena,* could be detected in a 4 million litre sea pen holding four porpoises, and in natural field sites in the Baltic Sea. Porpoise eDNA was consistently detected in 15 ml samples from the sea pen, despite daily flushing of the pen by tidal water movements in the harbour basin. However eDNA detection was less consistent in the

natural sites, and was less successful than acoustic surveys. The study did however detect a non-target species; long-finned pilot whale, *Globicephala melas,* which is rare in the Baltic. One acknowledged limitation of this study was the small volume of water collected in the natural sites (3 x 50 ml at each acoustic monitoring site), suggesting that much larger volumes of water might be required to adequately sample marine eDNA. More encouragingly though, a metabarcoding study, carried out in the Sound of Elsinore, Denmark, recovered eDNA from 15 marine fish species from just three ½-litre samples of seawater (Thomsen *et al.*, 2012a). eDNA outperformed eight out of nine conventional survey methods in terms of number of species detected (Thomsen *et al.*, 2012a). Common species and one rare vagrant (European pilchard, *Sardina pilchardus*) were detected, demonstrating the potential of eDNA for detecting rare species that are often missed by conventional methods. An extension of the technique for monitoring elusive species is discussed below.

So far, the majority of studies have focussed on monitoring of animals, particularly in aquatic environments. There is no doubt that more effort needs to be put into monitoring of plants in both aquatic and terrestrial environments. In one excellent study, eDNA was extracted from soil taken from meadow and heathland, and plant community diversity inferred using metabarcoding (Yoccoz *et al.,* 2012). eDNA-based diversity estimates were then compared to those from conventional above-ground surveys, and the estimates were highly consistent (Yoccoz *et al.,* 2012). The same study also demonstrated that that species diversity can be recovered using eDNA even in more challenging tropical environments.

Detecting records within records: molecular detection of trophic interactions.

Although "eDNA" in its strictest sense refers to free DNA in the environment, the term is often used more loosely to include prey DNA in the gut or faeces of predators, plant DNA in the guts of herbivores, parasite DNA in the host (or vice versa), or other interactions that leave behind traces of DNA. In an imaginative use of the technology, haematophagus leeches were used as a screening tool for monitoring mammalian biodiversity (Schnell *et al.*, 2012). Remarkably, over 80% of leeches sampled in the Central Annamite region of Vietnam tested positive for mammalian DNA, and two of the species detected (Truong Son muntjac, *Muntiacus truongsonensis* and Annamite striped rabbit, *Nesolagus timminsi*) were only recently described and had not been confirmed in the study area despite extensive surveying, including over 2000 nights of camera trapping for *N. timminsi* (Schnell *et al.*, 2012). Surveying elusive species with leeches or other haematophagous species (e.g. mosquitoes and ticks) is inexpensive compared to camera trapping and other conventional methods, and could be promising for mammal biodiversity screening in certain situations. However, as discussed below, the field of "molecular detection of trophic interactions" extends far beyond single species interactions and biodiversity screening.

Molecular tools have been used to study diet for over ten years (e.g. Symondson, 2002), but interest in the field has soared recently thanks to improvements in technology for high-throughput assaying of degraded DNA, in combination with analyses of ecological networks from thousands of species' interactions (Roy & Lawson Handley, 2012; Clare, 2014; Symondson & Harwood, 2014). Indeed, this emerging field recently motivated a special issue of the journal *Molecular Ecology* (Volume 23, Issue 15, August 2014), and is already providing unprecedented insight into complex ecological interactions, which are almost impossible to study using conventional methods (see

Clare, 2014 for an excellent recent review). Molecular dietary analyses have already been performed on a range of animals including mammals (Australian fur seals, *Arctocephalus pusillus,* Deagle, Kirkwood, & Jarman, 2009; snow leopards, *Panthera uncia,* Shehzad *et al.,* 2012; bison, *Bison bonasus,* Kowalczyk *et al.,* 2011; Asian musk shrew, *Suncus murinus,* Brown DS *et al.*, 2014), birds (little penguins, *Eudyptula minor,* Deagle *et al.,* 2010; Cory's shearwaters, *Calonectris diomedea,* Alonso *et al.*, 2014), reptiles (slow worms, *Anguis fragilis,* Brown, Jarman, & Symondson, 2012; smooth snake, *Coronella austriaca,* Brown, Ebenezer, & Symondson, 2014), fish (e.g. predation of endangered Acigöl carp, *Aphanius transgrediens,* by invasive mosquitofish, *Gambusia affinis,* Keskin 2014), terrestrial invertebrates (centipedes, *Lithobius* spp. Eitzinger *et al.*, 2014; spiders, Araneae: Orbicularae, Chapman *et al.*, 2013; Welch *et al.*, 2014; ladybird beetles, *Harmonia axyridis,* Brown PMJ *et al.*, 2014), and marine invertebrates (rock lobsters, *Jasus edwardsii,* Redd *et al.,* 2014). By far the greatest effort so far has been invested in the molecular investigation of diet in insectivorous bats (Clare *et al.*, 2009, 2011, 2014a; Clare, Symondson, & Fenton, 2014b; Razgour, Clare, & Zeale, 2011; Zeale, Butlin, & Barker, 2011; Burgar *et al.*, 2014; Sedlock, Krüger, & Clare, 2014; Alberdi *et al.*, 2012; Bohmann *et al.*, 2011). Conventional methods for studying bat diet are fraught with challenges for obvious reasons: bats are nocturnal, highly generalist (even species considered specialists consume many closely related prey species, Clare *et al.*, 2011), cryptic, they feed on the wing and are fast, agile flyers (Clare *et al.*, 2009, 2011, 2014a,b; Razgour *et al.*, 2011; Zeale *et al.*, 2011; Burgar *et al.*, 2014; Sedlock *et al.*, 2014). The challenges are exacerbated in bats from biodiversity hotspots, since prey items are highly speciose and largely undescribed (Bohmann *et al.*, 2011; Burgar *et al.*, 2014). However even when prey species are uncharacterized, DNA sequences can still be phylogenetically grouped

into “molecular operational taxonomic units” or “MOTUs” (Floyd *et al.*, 2002) and used to compare diets within and between species. Key insights from these studies include: incredibly high diversity of prey even outside biodiversity hotspots (e.g. ~600 prey species in Canadian little brown bats, *Myotis lucifugus,* (Clare *et al.*, 2014a), significant local variability in diet (*M. lucifugus,* Canada, Clare *et al.,* 2014a), strong seasonal, and annual variation in diet (big brown bats, *Eptesicus fuscus,* America, Clare *et al.,* 2014b), and resource partitioning (several species of Jamaican insectivorous bats, Emrich *et al.*, 2014) or niche differentiation (European pond bat, *Myotis dasycneme* and Daubenton’s bat, *M. daubentonii*, Krüger *et al.*, 2014) in sympatric species.

Involving the public in molecular biological recording

One of the most important aspects of biological recording is engaging the public in nature, science and conservation. An important question then is how can we do this with molecular recording? Does the technical nature of molecular work mean it is off limits to amateur volunteers? A recent eDNA study on great crested newts (GCN), *Triturus cristatus,* led by the Freshwater Habitats Trust (FHT) and carried out in the U.K., emphatically demonstrated that the answer to this question is “no” (Biggs *et al.*, 2014; 2015). In the first part of this study, a targeted qPCR assay was developed and intensive surveys carried out on 35 ponds during the GCN breeding season, to compare eDNA detection rates to traditional survey methods. Remarkably, detection rates were 99.3% for eDNA; substantially higher than for bottle trapping (76%), torch counting (75%) or egg searching (44%), but similar to torch counting and bottle trapping combined (95%, Biggs *et al.,* 2014). Secondly, 80 volunteers were recruited to survey 239 ponds across England, where GCN had been recorded breeding in the previous year. An additional 30 sites were surveyed firstly from ponds within the GCN range

but with no prior record of newts, and secondly outside the GCN range, in order to check for false positives. Volunteers were provided with written instructions and standardised sampling kits (including sterile gloves, a pipette, plastic bag, sampling ladle and six sterile 50 ml tubes containing absolute ethanol for sample preservation) and asked to collect 30 ml water samples at twenty locations around the pond margin, without entering the water. Volunteers then combined samples from a single pond in a plastic bag, homogenised the sample, transferred six 15 ml subsamples to individual 50 ml tubes containing ethanol, and posted the samples to FHT headquarters in Oxford, U.K. Standard Habitat Suitability Index (HIS) score sheets were also completed by volunteers at the same time as sample collection. GCN eDNA was detected in 91.2% of sites, indicating a small number of false negatives (8.8%), but encouragingly there was no evidence of false positives from the samples taken either from ponds within the range but without newts, or outside the GCN range. Twenty-six sites were also resurveyed by a professional in order to assess variability among surveyors. The same eDNA results were obtained in all but two of the volunteer and professional surveys, and very low eDNA concentration was responsible for the discrepancy between surveys at these two sites (Biggs *et al.*, 2014).

These encouraging results demonstrate that large-scale collection of eDNA samples by volunteers is both feasible and effective and provides benefit to both the research and volunteer communities. The GCN study demonstrates the enormous contribution of volunteers to research, since large-scale surveys over short time periods (e.g. coinciding with an organism's breeding season) would be far more logistically challenging with only a small research team. From the volunteer's perspective, monitoring of GCNs and other protected species is off limits to many potential recorders since specialist licenses

are needed. With eDNA surveys, volunteers can become involved without the need for licenses. However a drawback is that collecting water samples is less rewarding than counting real organisms! As with all methods of biological recording, professional volunteer support and prompt feedback of results is essential to ensure volunteers feel justifiably valued for their contributions. As discussed below, a combination of both conventional and eDNA methods is likely to prove the most powerful approach for biological recording, and engaging the public in both types of survey would be the ultimate outcome.

Challenges, limitations and important considerations associated with molecular biological recording

Quality control and validation

Validation is always an important step in biological recording. Observational records from the general public are typically validated by expert verification of photographs that are submitted with the record to online recording schemes. eDNA methods require very high quality control standards and validation, particularly when the technology is applied for IAS detection and biodiversity monitoring for management purposes, as there can be serious financial implications of false positives (Darling & Mahon, 2011; Rees *et al.*, 2014; Bohmann *et al.*, 2014). It is therefore critical to adopt standardised quality control measures such as inclusion of sample and PCR blanks, and to carry out replication of sampling and PCR (for more advice on these issues, see recent papers by Rees *et al.*, 2014; Bohmann *et al.*, 2014 and Ficetola *et al.*, 2014). For legally sensitive work, it could be necessary to adopt similar procedures to those used for ancient DNA, which requires specialised containment facilities and rigorous sterile techniques to

avoid contamination (Yu *et al.*, 2012). Of course this will also require specialist training and increase costs.

Dynamics of eDNA in the environment

A particularly important question is whether presence of eDNA equates to presence of living organisms. To answer this, we need to understand the dynamics of eDNA, which determines its detectability, in different environments. Detectability of eDNA is governed by how much DNA is released into the environment (a product of the amount of DNA shed by individuals, density of animals, and their residence time in the environment, Pilliod *et al.*, 2014) and how long eDNA persists in the environment (a product of the rate of DNA degradation - influenced by environmental chemistry, microbial composition, UV exposure, and temperature - and DNA transport – influenced by water flow rates, currents and wave action etc., Barnes *et al.*, 2014). Little hard data exists on the DNA shedding rates of organisms (but see Pilliod *et al.*, 2013 for an exception) but knowledge of DNA persistence in different environments is increasing. The consensus from several studies is that rate of DNA degradation in water varies widely between different environments, but DNA rarely persists for more than two weeks (Rees *et al.*, 2014; Barnes *et al.*, 2014). For example, in freshwater mesocosm experiments, amphibian DNA concentration rapidly declined until it could no longer be detected two weeks after removal of animals (Thomsen *et al.*, 2012b). This suggests rapid degradation of eDNA in freshwater, even in benign, controlled conditions (Dejean *et al.*, 2011; Thomsen *et al.*, 2012b). In an experiment to investigate DNA degradation in seawater, Thomsen *et al.*, (2012a) found that DNA fragments degrade beyond detectability within 0.9-6.7 days. This high rate of DNA degradation provides confidence that a positive result reflects real occurrence. Interpretation of

results from soil and other sediments is more complicated as eDNA can persist over long time scales (e.g. Willerslev *et al.*, 2003) and data therefore reflects both current and previous biota. For example, an analysis of plant eDNA in temperate, modern agricultural sites demonstrated that crops cultivated 40-50 years ago could still be detected (Yoccoz *et al.,* 2012). Interestingly, this study also found a positive relationship between the number of DNA sequences in the soil and the years since crop abandonment (Yoccoz *et al.,* 2012), indicating eDNA is degraded at a steady rate. This information is helpful for modelling the dynamics of eDNA in sediments and for accurately interpreting results.

A second concern, which is often raised, is how applicable eDNA methods are for surveying in lotic environments or large water bodies – including the sea. eDNA surveys have already successfully been carried out on several stream or river-living species using eDNA (Table 1) and it is clear that eDNA methods are more challenging in fast flowing rivers, large lakes and the marine environment than in ponds and small streams. However this is also true for conventional surveying methods. As with conventional methods, probability of detection will vary with species, organism density, stream size, flow rate and season (Goldberg *et al.*, 2011). A key issue with eDNA studies in flowing water though is how far DNA can be transported before it is degraded beyond levels of detection. Few studies have yet investigated the actual dynamics of eDNA in flowing water, and this is definitely an area that warrants further research. In one study though, transport of eDNA from two lake-dwelling invertebrates, *Daphnia longispina* and *Unio tumidus*, was detected in a river up to ~12 and 9 km (respectively) downstream of the lake (Deiner & Altermatt, 2014). This indicates that

eDNA can persist over relatively large distances in river systems, and that there may be species-specific transport distances for eDNA (Deiner & Altermatt, 2014).

The studies mentioned here have demonstrated that eDNA surveys perform well, even in challenging environments, but the dynamics of eDNA in different environments are complex. It is clear from this that researchers need to carefully consider the ecology, behaviour, and to an extent the physiology, of their target organisms, as well as the flow dynamics and chemistry of their environment to fully understand the results of eDNA surveys.

Are the methods quantitative?

Perhaps the most important question currently occupying the eDNA community – particularly those keen on metabarcoding approaches – is whether the methods can go beyond describing presence/absence and reliably be used for estimating abundance or biomass (Rees *et al.*, 2014; Bohmann *et al.*, 2014). Organism abundance and/or biomass is critical information for those who need to monitor and manage biodiversity or commercially exploited stocks. Obtaining quantitative estimates from eDNA is challenging because of the large number of factors that influence DNA dynamics in the environment, discussed in the previous section. Several approaches for estimating abundance have been explored including: measuring DNA concentration using qPCR (Takahara *et al.*, 2012; Thomsen *et al.*, 2012b; Pilliod *et al.*, 2013) or digital droplet PCR (ddPCR, Nathan *et al.*, 2014), counting the number of sequences per OTU in metabarcoding (Yu *et al.*, 2012; Kelly *et al.*, 2014), avoiding PCR (Zhou *et al.*, 2013) and site occupancy modelling (Pilliod *et al.*, 2013; Schmidt *et al.*, 2013).

Encouragingly, methods based on qPCR have consistently demonstrated a positive (but non-linear) relationship between animal density or biomass and eDNA concentration (e.g. Takahara *et al.*, 2012; Thomsen *et al.*, 2012b; Pilliod *et al.*, 2013). The drawback of this method though is that qPCR is time consuming and costly because it focuses on one target species at a time. ddPCR is a relatively new method, which also focuses on target species but could potentially provide a much more cost-effective, but equally accurate means of estimating eDNA concentration (Nathan *et al.*, 2014). ddPCR essentially involves random partitioning of target DNA into several thousand individual droplets which are then amplified by PCR. Random sampling means that droplets can contain target or background DNA or both. The fraction of positive droplets is then used to calculate the concentration of target DNA (see Nathan *et al.*, 2014 for more information).

In metabarcoding studies, in principle, the number of sequences per OTU could be taken as an estimator of species biomass. Unfortunately though, it seems that this relationship is not a simple one. In the study by (Yu *et al.*, 2012) mentioned under *"DNA barcoding or metabarcoding?"* the method accurately recovered unweighted alpha and beta diversity indices, but the number of sequence reads did not correlate with abundance. More recently, a metabarcoding experiment was carried out at Monterey Bay Aquarium in a large (4.5 million litre) tank with known species composition, to evaluate whether species biomass could be accurately recovered (Kelly *et al.*, 2014). Although the *rank* abundances of eDNA and biomass were perfectly correlated, a complex, non-linear relationship was found between the *proportion* of eDNA sequences recovered and the proportion of biomass in the tank (Kelly *et al.*, 2014). Together, these studies indicate that relating the number of sequences per OTU

to biomass is complex, perhaps because there are several opportunities for bias during the sampling, extraction, PCR, and bioinformatics stages (Yu *et al.*, 2012). Further work is needed to investigate the efficacy of metabarcoding for recovering abundance/biomass estimates in natural or semi-natural environments with known species composition. One possibility could be to use metabarcoding for describing species composition and qPCR or ddPCR for estimating abundance/biomass of species within the sample. However a combined approach would obviously be more costly and time consuming. Another prospect, which is generating considerable excitement, is that of PCR-free metabarcoding. PCR can introduce taxonomic biases, false negatives and positives, which can strongly influence abundance estimates (Zhou *et al.*, 2013). In the PCR-free approach, whole communities are sequenced following mitochondrial enrichment, and the individual barcodes are then retrieved during the bioinformatics steps (Zhou *et al.*, 2013). Although the method needs to be refined, one encouraging study demonstrated correlation between number of sequences and total biomass in bulk arthropod samples, suggesting this could provide a solution for estimating abundance (Zhou *et al.*, 2013).

Finally, statistical modelling offers another opportunity to improve quantitative estimates. Estimating abundance of animal populations via conventional methods is notoriously difficult unless individuals can be individually identified. One route around this problem is to estimate site occupancy – i.e. the proportion of an area occupied by a species (MacKenzie *et al.*, 2002). Although this is obviously different to species abundance, the two measures are positively correlated (MacKenzie & Nichols, 2004), so site occupancy can be considered a proxy for abundance. Several recent papers have advocated the use of site occupancy modelling (SOM) for eDNA surveys (Pilliod *et al.*,

2013; Schmidt *et al.*, 2013; Ficetola *et al.*, 2014). SOMs account for imperfect detection (an inherent feature of eDNA studies) and can be used to obtain more reliable estimates of species prevalence, estimate detection probability, determine the number of samples and site visits needed to obtain a high probability of detection (Kéry & Schmidt, 2008; Pilliod *et al.*, 2013; Schmidt *et al.*, 2013; Ficetola *et al.*, 2014) and test for the effects of site characteristics, such as animal density, on detection probability (MacKenzie *et al.*, 2002; Kéry & Schmidt, 2008; Pilliod *et al.*, 2013; Schmidt *et al.*, 2013; Ficetola *et al.*, 2014). Incorporating site occupancy modelling as routine into eDNA surveys could therefore lead to major improvements in the method.

Is it cost effective?

The costs associated with molecular analyses are plummeting, but how do eDNA surveys compare to conventional methods? On the whole they seem to be cheaper and less time consuming, with higher catch per unit effort than traditional methods (Jerde *et al.*, 2011; Dejean *et al.*, 2012). For example, it was estimated that eDNA surveys for American bullfrogs were 2.5 times cheaper and 2.5 times less time consuming than traditional surveys (Dejean *et al.*, 2012). For great crested newts, the estimated savings are even greater, with eDNA surveys potentially costing 6-10 times less than torch counting and bottle trapping (Biggs *et al.*, 2014). Nevertheless the cost of undertaking eDNA surveys is far from trivial, especially when a large number of replicates are required for validation purposes.

Will it replace traditional methods?

A key take home message from this review is that eDNA sampling should never be undertaken naively. It is just as important to understand the ecology of the study

organism and its environment as it is with conventional surveying. eDNA surveys should be designed on a case-by-case basis with the target organisms and environment in mind. eDNA methods cannot determine the age class of organisms, which is an important component of biodiversity monitoring and there are therefore clear advantages in combining both methods. The most effective approach will be to use them in combination.

Conclusion: the next phase in the life of the BRC?

To conclude, I have hopefully demonstrated that the molecular revolution *will* contribute to biological recording and that it will complement traditional surveys and offer new and exciting opportunities for engaging the public in citizen science. A final important consideration is how we should manage the data generated from molecular surveys. Should molecular data be integrated into current biological records databases or maintained separately? Although the International Barcode of Life Data Systems portal (http://www.boldsystems.org/) has over 3.5 million georeferenced DNA sequences, its purpose is quite distinct from biological records databases and it was never intended as a tool for mapping species distributions. The Atlas of Living Australia (ALA, http://www.ala.org.au/), which is the Australian node of the Global Biodiversity Information Facility (GBIF http://www.gbif.org/), is an incredible resource that incorporates biological records, mapping functions, taxonomic information, and species descriptions (even including vocalisations of certain animals). The ALA portal also links with the GenBank search engine for retrieving DNA sequences from individual species. However this is still quite different to treating the molecular data as a biological record. To my knowledge this has yet to be attempted. Integrating molecular records into existing national biological records databases would increase

our understanding of species distributions and facilitate direct comparisons of the methods. This will not be a trivial enterprise, but is perhaps something that the Biological Records Centre (and their equivalent organisations outside the U.K.) should be pioneering to take biological recording into its next phase.

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Figure legend

Fig. 1. A basic overview of the steps taken in eDNA barcoding or metabarcoding studies.

[1]Replicate samples should always be taken. General guidelines advise a minimum of three samples from a small pond, but this should be adapted to the specific environment under study. The number of samples required to obtain a high detection probability can be estimated using site occupancy modelling, as discussed in the text (see section *"Are the methods quantitative?"*). Numerous protocols exist for DNA capture and extraction. See Rees *et al.*, 2014 for advice.

Table 1: Examples of targeted aquatic eDNA case studies by taxa. Species are ordered alphabetically by scientific name, within taxon group.

Taxon	Environment	Objective	Method	References
Amphibians				
Rocky Mountain tailed frogs (*Ascaphus montanus)*	Streams	Monitoring biodiversity	Standard and qPCR	Goldberg *et al.*, 2011; Pilliod *et al.*, 2013
Idaho giant salamanders (*Dicamptodon aterrimus)*	Streams	Monitoring biodiversity	Standard and qPCR	Goldberg *et al.*, 2011; Pilliod *et al.*, 2013
Spadefoot toad (*Pelobates fuscus)*	Ponds	Monitoring biodiversity	qPCR	Thomsen *et al.*, 2012

American bullfrog, (*Rana catesbeiana)*	Ponds	Detection of IAS	Standard PCR	Ficetola, Bonin, & Miaud, 2008; Dejean *et al.*, 2012
Great crested newt (*Triturus cristatus)*	Ponds	Monitoring biodiversity	qPCR	Thomsen *et al.*, 2012b; Biggs *et al.*, 2014
Fish				
Goldfish *(Carassius auratus)*	Bait shop tanks	Detection of IAS via bait trade pathway	Standard PCR	Nathan *et al.*, 2014
Prussian carp (*Carassius gibelio)*	River basin	Detection of IAS	Standard PCR	Keskin, 2014
North African catfish (*Clarias gariepinus)*	River basin	Detection of IAS	Standard PCR	Keskin, 2014
African jewelfish (*Hemichromis letourneuxi)*	Artifical ponds	Assess detection method in controlled lentic system[1].	qPCR	Moyer *et al.*, 2014

Silver carp (*Hypophthalmichthys molitrix)*	Chicago area waterway (large river and canal complex)	Detection of IAS	Standard PCR	Jerde *et al.,* 2011; Mahon *et al.,* 2013
Bighead carp (*H. nobilis)*	Chicago area waterway (large river and canal complex)	Detection of IAS	Standard PCR	Jerde *et al.,* 2011; Mahon *et al.,* 2013
Bluegill sunfish (*Lepomis macrochirus*)	Ponds	Detection of IAS	qPCR	Takahara *et al.,* 2013
European weather loach (*Misgurnus fossilis)*	Ponds and streams	Monitoring biodiversity	qPCR	Thomsen *et al.,* 2012b
Round goby *(Neogobius melanostomus)*	Bait shop tanks	Detection of IAS via bait trade pathway	Standard PCR	Nathan *et al.,* 2014
Nile tilapia (*Oreochromis niloticus)*	River basin	Detection of IAS	Standard PCR	Keskin, 2014

Tubenose goby *(Proterorhinus semilunaris)*	Bait shop tanks	Detection of IAS via bait trade pathway	Standard PCR	Nathan *et al.*, 2014
Topmouth gudgeon (*Pseudoasbora parva)*	River basin	Detection of IAS	Standard PCR	Keskin, 2014
Bull trout (*Salvelinus confluentus*)	Streams	Monitoring of species displaced by *S. fontinalis*, testing assay sensitivity and accuracy.	qPCR	Wilcox *et al.*, 2013
Brook trout (*Salvelinus fontinalis)*	Headwater streams	Detection of IAS and testing assay sensitivity and accuracy; investigating DNA dynamics in streams	qPCR	Wilcox *et al.*, 2013; Jane *et al.*, 2014
Eurasian rudd *(Scardinius erythrophtalmus)*	Bait shop tanks	Detection of IAS via bait trade pathway	Standard PCR	Nathan *et al.*, 2014
Reptiles				

Burmese python (*Python bivittatus*)	Large wetlands	Detection of IAS	Standard PCR	Piaggio *et al.*, 2014
Mammals				
Eurasian otter (*Lutra lutra)*	Streams and lakes	Monitoring biodiversity	qPCR	Thomsen *et al.*, 2012b
Harbour porpoise (*Phocena phocena)*	Seawater from a sea pen and open sea	Monitoring biodiversity	qPCR	Foote *et al.*, 2012
Crustaceans				
Water hog-louse (*Asellus aquaticus)*	Rivers and lakes	Detecting indicator species	Standard PCR	Mächler *et al.*, 2014
Northern River Crangonyctid *(Crangonyx pseudogracilis)*	Rivers and lakes	Detection of IAS	Standard PCR	Mächler *et al.*, 2014
Daphnia longispina	Rivers (downstream of lake where species present)	Investigating DNA dynamics in rivers	Standard PCR	Deiner & Altermatt, 2014

Chinese mitten crab, (*Eriocheir sinensis)*	Ships ballast	Detection of IAS	Microfluidic chip	Mahon *et al.*, 2011
River shrimp (*Gammarus pulex)*	River	Detecting indicator species	Standard PCR	Mächler *et al.*, 2014
Tadpole shrimp (*Lepidurus apus)*	Temporary pools	Monitoring biodiversity	qPCR	Thomsen *et al.*, 2012b
Red swamp crayfish (*Procambarus clarkii)*	Ponds	Detection of IAS	qPCR	Tréguier *et al.*, 2014
Molluscs				
Ancylus fluviatilis	Rivers and lakes	Detecting indicator species	Standard PCR	Mächler *et al.*, 2014
Zebra mussel (*Dreissena polymorpha*)	Lakes and rivers	Detection of IAS	Standard PCR	Lance & Carr 2012
Quagga mussel, (*Dreissena rostriformis bugensis)*	Ships ballast; lakes and rivers	Detection of IAS	Microfluidicchip; standard PCR	Mahon *et al.*, 2011; Lance & Carr 2012

Golden mussel, (*Limnoperna fortuneii)*	Ships ballast	Detection of IAS	Microfluidicchip	Mahon *et al.*, 2011
Unio tumidus	Rivers (downstream of lake where species present)	Investigating DNA dynamics in rivers	Standard PCR	Deiner & Altermatt, 2014
Echinoderms				
Northern Pacific seastar (*Asterias amurensis*)	Mixed plankton or ballast	Detection of IAS	Standard PCR	Deagle *et al.*, 2003
Insects				
Scarce olive riverfly (*Baetis buceratus)*	Rivers	Monitoring biodiversity: vulnerable	Standard PCR	Mächler *et al.*, 2014
White-faced darter (*Leucorrhinia pectoralis)*	Ponds	Monitoring biodiversity	qPCR	Thomsen *et al.*, 2012b
Tinodes waeneri	Rivers and lakes	Detecting indicator species		Mächler *et al.*, 2014

Fungal Pathogens				
Crayfish plague (*Aphanomyces astaci)*	Large lakes	Pathogen detection	qPCR	Strand *et al.*, 2014
Batrachochytrium dendrobatidis (amphibian fungal pathogen)	Pond water and sediments	Pathogen detection	qPCR	Kirshtein *et al.*, 2007; Hyman & Collins 2012.

[1] Note *H. letourneuxi* is an IAS in southern Florida.