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eDNA: Review of applicability for monitoring and detecting biotic populations of the Murray-Darling Basin

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Contents

List of Tables

Executive Summary

This review examines the current suitability and feasibility of environmental DNA (eDNA) based survey methods in supporting biological monitoring applications in the Murray-Darling Basin (MDB). Potential applications of eDNA based methods range from those focussed on single-species detection (e.g. rare species, invasive species) to surveys designed to quantify community structure (i.e. multi-species detection). The latter, may serve to replace or complement sampling protocols traditionally employed in biomonitoring programs to assess ecosystem health (e.g. AusRivAS). eDNA based methods have numerous potential advantages over traditional survey techniques, including:

- requiring lower cost and effort
- being non-destructive to the organism
- entailing less labour intensive and safer sampling methods
- being able to target rare or cryptic organisms that are not routinely detected.

While the principles of eDNA based monitoring are well established, there are numerous logistical and technical issues that must be addressed to ensure that monitoring results are reliable in the context of specific applications. These include:

- linking taxonomic and genetic information
- developing appropriate genetic markers
- better understanding and reducing rates of detection error
- understanding persistence and dispersion patterns of DNA after shedding from the host organism and how this is affected by the surrounding environment (e.g. pH, turbidity, temperature, ultra-violet light and contaminants)
- and how to move beyond simple presence/absence interpretation toward quantitative or semi-quantitative abundance/biomass estimates.

These technical challenges are compounded where applications span a broad range of environments or highly variable environmental conditions. Varied levels of progress have been made thus far in addressing each of these challenges.

Despite these logistical and in some cases unresolved technical issues, eDNA based research and monitoring shows great promise. These approaches are currently being used in a number of case studies within the Murray-Darling Basin but are yet to be more formally adopted by larger-scale programs. Currently, the most widely used application is in the detection of single species, for example, to determine the distribution of animals considered of national significance (e.g. Platypus), species of conservation significance (e.g. Alpine stonefly), invasive species (e.g. Red fin) and cryptic species (e.g. Alpine Stonefly) or morphologically similar but genetically diverse (e.g. Carp gudgeons). Monitoring for multiple species in a targeted way has also been successfully implemented in cases where there are a relatively small number of known “target” species, (e.g. simultaneous detection of multiple fish species). However, the use of eDNA techniques to characterise community composition (e.g. of macroinvertebrates assemblages employed in biomonitoring) remains more challenging.

There are multiple opportunities to adopt eDNA based sampling methods in species detection programs in the MDB. However, eDNA is not a panacea; there are many questions where other information will be required, for example, to quantify abundances, life-stages, age classes, population size structure, and organism health.

If eDNA based methods are to be more rigorously adopted in place of existing sampling programs, we recommend the following activities:

- Ongoing research to address specific methodological issues (section 4; section 6, priority 1).

- Transition from and complement existing programs by running existing sampling in parallel with eDNA based sampling methods until any unforeseen issues have been resolved.
- Establish centralised and accessible reference databases linking genetic and taxonomic information (section 6, priority 1).
- Developing a best practice framework incorporating appropriate Quality Assurance and Quality Control (QAQC) for all aspects of eDNA based methods, covering sample collection, lab protocols, data collation and use of reference databases (section 6, priority 2).
- Developing eDNA standard methods that are comparable, repeatable and widely applicable monitoring frameworks (section 6, priority 2).

Support capacity building across research organisations (universities, government departments, CSIRO, NGOs) through the establishment of data storage, data facilitation, connectivity, coordination and integration across monitoring and evaluation programs.

1 Introduction

Detecting and monitoring the distribution of species and associated composition of ecological communities within particular habitats is at the heart of many natural resource monitoring programs. Historically, most programs have relied on the determination of individual species distributions through physical capture or observation within particular habitats, for example, through visual surveys (in the case of plants and some animals), or through active or passive trapping or capture (in the case of many animals, and in some cases plant and animal propagules).

However, species detection is often imperfect; many habitats are hard to sample effectively, and many species are rare or cryptic, making their presence or absence difficult to determine without considerable cost. As a result, a range of indirect survey methods have been devised to increase the accuracy of species detection. Examples include; call surveys to detect amphibians and birds, fur and scat surveys as a means of tracking mammalian biota, and remotely triggered image capture to detect rare, nocturnal and shy animals. Among these indirect approaches, genetic based methods, and more particularly, environmental DNA (eDNA), are emerging as a potentially valuable tool for detecting species presence within particular habitats.

eDNA is genetic material that has been shed from an organism (e.g. cells, faeces, urine and saliva) which is then extracted from an environmental medium (e.g. water, air, soil, sediment) (Diaz-Ferguson & Moyer 2014, Rees *et al.* 2014). eDNA sampling is not a targeted sampling technique for the monitoring of a certain group (i.e. kick netting for macroinvertebrates, fyke netting for fish) but rather a generalised sampling of an environmental medium that collects all genetic material within a sample of that medium. The DNA is then extracted from the sample with targeted genetic markers used to focus on individual species or groups of organisms (e.g. fish, arthropods, mammals). eDNA sampling can take various forms, for example, DNA fragments may be collected from an environmental medium or may be extracted from a bulk biological sample of unknown composition (e.g. animal gut or scat, or animals are collected en masse in a kick or net).

Extracting genetic material to characterise microbial and fungal community diversity within aquatic environments has occurred since the 1980s (Diaz-Ferguson & Moyer 2014), but largely without consideration of actual taxonomic identity of component species. Advances in genetics and associated technology are changing the approach of using eDNA sampling for broad scale monitoring and research, and is becoming an increasingly more suitable and feasible option (Diaz-Ferguson & Moyer 2014, Goldberg, Strickler & Pilliod 2015, Shaw, Weyrich & Cooper 2017, Rees *et al.* 2014). The use of eDNA in monitoring and evaluation programs requires careful consideration of the question(s) being asked, the spatial extent, the temporal scale and the taxonomic resolution of the species or taxonomic group (Rees *et al.* 2014, Thomsen & Willerslev 2015).

While there are many studies examining the applicability of eDNA sampling and methods, no actual long-term monitoring programs have transitioned to solely using this approach. This is due to a combination of factors, including technical limitations, knowledge gaps, lack of genetic reference libraries/databases and the transition between monitoring programs without compromising the legacy of existing datasets. Many of these factors can be overcome, but only with additional short-term costs. Complementing rather than replacing monitoring and research programs and so providing multiple lines of evidence will be the best approach. The current application of eDNA sampling and methods is more common in certain mediums (i.e. freshwater) and to certain organisms (i.e. fish, amphibians) but this does not necessarily restrict the technique to these groups or sampling environments. Although it does indicate that the development of eDNA sampling and methods in monitoring and evaluation programs in these areas has the potential to be more readily implemented in the short term.

This review focuses on the potential applications and advantages of eDNA sampling in monitoring and evaluation programs and the applicability of these methods in the Murray-Darling Basin. The

review outlines the technical and logistical requirements needed to enhance eDNA sampling and methods plus the steps required to improve the capacity and delivery of eDNA based programs.

2 Potential applications

2.1 Background

Species distributions are fundamental to many natural resource management programs and are the basis for understanding a variety of questions including long-term ecosystem "health", community responses to ecological changes, and conservation impacts and issues. On-going monitoring programs occur through the Murray-Darling Basin on a range of organisms including vegetation, fish, invertebrates, birds and turtles and, on ecosystems in general for water quality and wetland health using a variety of traditional approaches (Table 1).

Table 1: Traditional approaches to these monitoring programs are specific to organisms.

Organism	Sampling techniques
Fish	fyke netting, electrofishing, bait trapping
Waterbirds	spotting transects, breeding occurrence
Vegetation	transects, condition assessments
Invertebrates	artificial substrates, aquatic netting (e.g. AusRivAS), drift netting
Amphibians	audio traps, nocturnal surveys, tadpole surveys
Turtles	cathedral traps, fyke netting, visual observation

These approaches are used to answer a range of questions on the ecology and biology of species and of ecosystems in general. The potential applications of eDNA based methods range from those focussed on single-species detection (e.g. rare species, invasive species) to surveys designed to quantify community structure (i.e. community detection), which may serve to replace or complement sampling protocols traditionally employed in biomonitoring programs to assess ecosystem health (e.g. AusRivAS).

The successful use of eDNA methods has been shown in a variety of environmental mediums such as terrestrial sediment, aquatic sediment, freshwater, marine and soils (Thomsen & Willerslev 2015). Currently, the most common application of eDNA is in presence/absence monitoring for single-species with the potential suitability for incorporation into some community-based monitoring and evaluation programs. In Australia, freshwater systems have had eDNA methods applied most frequently however the potential to apply eDNA methods to other environmental mediums is certainly feasible and can be applied.

2.2 Single species detection

eDNA has been implemented successfully for single-species detection predominantly in freshwater systems. In Australia, studies on fish species and platypus are leading the way in the applicability of eDNA techniques (Bylemans *et al.* 2018, Lugg *et al.* 2018).

Single species detection relies on the development of genetic markers from previously sourced genetic information. The requirement for prior knowledge of a targeted species is fundamental to eDNA single species detection methods. Unless genetic information is available from a database, targeted surveys using traditional sampling methods are undertaken to extract DNA directly from the organism. Once this DNA had been sequenced unique markers for that species can be developed that then enable environmental samples to be collected and sequenced to assess the presence of

that species in the system. This detection method is extremely powerful for the detection of threatened species, invasive species and other species of interest.

Threatened species

The need to detect species is paramount to the ability to conserve a species. Knowing the presence and absence of a species from an area is important information in establishing species management and recovery plans, and protection programs. Especially for species that are cryptic – that is difficult to locate and/or require a large amount of effort to locate a specimen. The potential for eDNA methods to be used in citizen science projects for species of conservation interest is also a possibility and has been shown to be beneficial in a program in the UK on newts (Biggs *et al.* 2015). Additionally, eDNA techniques offer a non-destructive sampling method for threatened species which is important to limit the impact that researchers have on endangered and vulnerable species.

Invasive species

Similarly, eDNA can be used to assess the spread of invasive species by targeting the presence of a known or high-risk invasive species in a system. Especially in freshwater systems where aquarium species pose a high risk of establishment. The ability to detect and monitor for potential invasives in both artificial (e.g. aquaculture facilities, aquaria) and natural systems (e.g. post possible release/escape) may play an important role in biosecurity and biocontrol. Examples include Oriental weatherloach and the Smooth newt. Currently eDNA methods have been tested alongside traditional approaches in South Australia to detect invasive fish species in waterways with positive results (Hinlo *et al.* 2017).

Cryptic species

Cryptic species are those that are difficult to detect, either due to their behaviour (e.g. burrowing), camouflage (e.g. colouration), or morphological similarity to other species (e.g. species complexes). For example, Australian species like the EPBC listed freshwater Alpine Stonefly, *Thaumatoperla alpina*, can be difficult to collect in freshwater sampling as they have a burrowing behaviour as larvae, and are not often picked up in aquatic macroinvertebrate sampling. It is likely therefore that our knowledge of the true distribution of this species would be greatly improved using eDNA sampling techniques, which would likely be both more efficient and cost-effective. A pilot study on this species is currently being conducted by the Centre for Freshwater Ecosystems, La Trobe University.

For genetically cryptic species (i.e. species complexes), the use of eDNA may enhance studies that are not able to morphologically distinguish between species that co-occur and/or have overlapping ranges. Carp gudgeons (*Hypseleotris* spp.) provide a good example; there are eight described species, but at least three of these are almost indistinguishable in the field based on taxonomic features.

2.3 Multi-species detection

Multi-species detection can be thought of in two ways. The first is essentially the parallel testing for multiple individual target species by screening environmental samples with specifically designed primer sets. The second is less targeted and involves characterising the diversity of eDNA within a sample, with no (or fewer) a priori assumptions about the genetic material (and hence species) that may be detected within a sample. Currently there are multiple technical considerations (e.g. PCR bias in section 4) that force a trade off in the ability to successfully detect individual target species from a sample, and the ability to characterise genetic diversity within that same sample. As a result, a decision must be made prior to laboratory processing of samples as to the goals of a particular study to balance what is needed to be targeted to address the question being asked.

Simultaneous targeting of multiple species

Examples of where this approach may be useful is when there are a relatively small number of target species of interest with clearly established taxonomy and genetic markers. Relevant examples might include testing for particular species of fish, for example, those of interest from a conservation (e.g. Murray cod, Golden perch, Silver perch) or bio-control perspective (e.g. Oriental weatherloach, Carp, Red fin).

Biological diversity and/or community composition

Characterising the diversity and composition of plant and animal communities is a common objective of scientific surveys. In some instances, such surveys may be purely exploratory, for example, in assessing biological diversity in poorly studied regions or may be motivated by a requirement to assess past, current or potential future changes in diversity associated with human impacts. For example, benthic macroinvertebrate communities have been widely used to assess the effects of pollution, land-use change, and flow alteration on stream health in Australia (e.g. Parsons, Thoms & Norris 2002). Such programs can also be costly, in part due to the time and costs associated with processing samples to enumerate and identify the species present. As such they are ideal potential candidates for adoption of eDNA based methods. Community composition studies have been undertaken through a variety of ecosystems both terrestrial and freshwater (Kuzmina, Braukmann & Zakharov 2018). Community studies on bacteria and fungi have used eDNA techniques for decades, now these techniques are being applied to fish assemblages, macroinvertebrate populations, vegetation assemblages both in freshwater and terrestrially, and marine communities. The applicability of eDNA techniques for assessing biological communities is highly dependent on the question being considered in monitoring programs. Extant terrestrial vegetation communities may be more easily assessed visually through transects rather than taking soil samples, depending on the vegetation knowledge of people undertaking the sampling. Whereas, vegetation seed bank studies may benefit from eDNA techniques as they may provide an association between a seed and a species that would not occur otherwise. However, while the applicability of community-based studies varies on the question being asked there are also limitations in knowledge depending on the environmental medium being sampled (e.g. freshwater sediments vs water column samples) (Thomsen & Willerslev 2015). Within some community-based studies comparisons to previous sampling methods have shown that eDNA often shows a higher sensitivity for species detection (Kuzmina, Braukmann & Zakharov 2018, Biggs *et al.* 2015, Macher *et al.* 2018).

Food webs

Another potential novel application of eDNA based approaches is in disentangling food webs, which play an important role in determining the flow of energy through ecosystems, and how energy flows may be altered by changes in species composition (e.g. through the introduction of invasive predator or loss of an important prey species from an ecosystem). Traditionally studying food-webs has involved dietary analyses, often by dissecting and examining gut contents (or scats) of important consumer organisms (e.g. fish or waterbirds). Thus far only a small number of studies have applied eDNA approaches to studying food webs (Cavallo *et al.* 2018). One of the advantages in relation to traditional gut-content analysis is that prey composition may be better discerned from masticated tissue that may not be distinguished through visual identification methods. There are however instances in which significant biases have occurred in the analysis of gut contents, possibly due to chemical denaturing of DNA within the gut (Xue *et al.* 2018).

3 Potential advantages

eDNA based methods have numerous potential advantages over traditional survey techniques, including; the potential to require lower cost and effort, being non-destructive to the organism, entailing less labour intensive and safer sampling methods, and being able to target organisms that are not routinely detected due to their rarity or cryptic nature.

3.1 Lower cost and effort

The cost benefit of eDNA based monitoring and evaluation is becoming more apparent each year as the cost of consumables and DNA sequencing decrease, laboratory and data processing become more stream-lined and success rates increase. Traditional monitoring and evaluation methods often depend on labour intensive methods either in the laboratory or identifications, particularly for high taxonomic resolution, or in the field for collections/assessments. McInerney and Rees (2018) conducted a study using eDNA and traditional methods assessing impacts on in-stream biota that showed that in strict dollar terms, the traditional approach was 6 times more expensive than the eDNA approach. Although, the cost benefit between approaches depends on several factors that may include the sensitivity required in the sampling, the ease of collection and the question being asked of the monitoring (Smart *et al.* 2016). Development of genetic markers or species-specific assays may further increase costs initially, however this is usually a once off cost. For long-term monitoring and evaluation programs establishing bioinformatics pipelines to increase efficiency would further reduce costs.

There are potential cost benefits based on increases in sampling scale, as the costs of larger sampling can further reduce the costs of eDNA methods as multiple samples can be handled simultaneously or multiple organisms can be looked at from the same sample depending on the question being addressed (Smart *et al.* 2016). For example, McInerney *et al*. (2016) were able to examine fungal, algal and macroinvertebrates in individual stream benthic samples. Although some questions being evaluated at a larger scale may not show a cost benefit.

3.2 Less labour and safer sampling

Sampling for eDNA requires little labour and usually incorporates lower OH&S risks, especially in freshwater systems where the use of boats, electrofishing backpacks, nets, and fast flow increase risks in the field. eDNA sampling can be considered as safer sampling, as samples are often collected from areas where there are lower risks and without high risk equipment. Field based sampling for eDNA is often less labour intensive with samples taken more quickly compared to traditional approaches, although eDNA samples must be processed after collection and this can happen either in the field or in the lab.

3.3 Non-destructive and low impact on organisms

Sampling for eDNA has less disturbance on an organism as eDNA sampling focuses on the presence of the organism in the medium being sampled by targeting genetic traces left in the environment. Once genetic data has been collected from an organism and is available on a genetic reference library, the need to collect the physical organism decreases when assessing presence/absence. However, some questions asked within monitoring and evaluation programs may still require an organism to be handled (e.g. abundance, sex or age).

3.4 Picks up difficult to detect organisms

Using eDNA methods has the ability to detect organisms that may be difficult to detect due to their rarity, cryptic behaviour or general low abundance. eDNA sampling has the advantage that it can

detect all life stages which may not be possible or may be difficult with a traditional survey method. For example, a water sample can reveal the presence of an organism that would ordinarily be very difficult to find by its scarcity at the targeted level (e.g. adult fish, late instar macroinvertebrates). An increase in detection rates for difficult to detect organisms can occur in either single or multiple species eDNA studies with many of the detected taxa making up a very small part of the overall community.

4 Technical/logistical requirements

While the principles of eDNA based monitoring are well established, there are numerous technical and logistical issues that must be addressed to ensure that monitoring results are reliable in the context of specific applications. The below highlighted issues are not an exhaustive list but the most common that need to be considered for eDNA applications. Many of these issues require further investigation to be able to resolve while others can be reduced by incorporating best-practice methods and providing a framework of standards and building capacity through training and information sharing.

4.1 Better understanding and reducing rates of contamination and errors

Contamination and errors are an issue for eDNA analysis and can occur from the collection stage to the sequencing stage.

Sampling

Sample contamination is of particular importance when dealing with eDNA samples as DNA is easily transported and can be amplified from a single fragment. Equipment for collecting needs to be sterile and this can often be achieved through washing with a dilute bleach solution. The risk of contamination from collectors as well as contaminants from non-target environments needs to be understood and can be reduced through training and/or technology in order to minimise this risk.

Laboratory

Contamination of samples can further occur in the laboratory through human error, poor laboratory practices, and through the presence of residual DNA (e.g. on benchtops or in the air). To reduce the potential for contamination within the laboratory, following best practice for lab standards and hygiene is important as well as having a sterile room or space (e.g. a laminar flow cabinet) for genetic work to decrease the chances of residual DNA contaminating unprocessed samples.

Primers

Reproducibility across samples and studies will require careful consideration of choices of primer sets. On one hand using the same sets of primers might ensure the same spread of taxa are targeted. However, not all primer sets will be suitable for all geographic locations and it may be that primer sets tailored to regions are more comparable than applying a strict set of primers, this will be especially true when the objective is to assess biodiversity. Indexing primers, attached to sequences so that they can be traced back to samples, are known to jump between samples, creating false positive results. This can be somewhat mitigated through careful selection of indexing primers and through replicate sampling.

PCR

PCR bias occurs where amplification of target genes is uneven among species. This results in species that are present in equal proportions in a sample being represented by widely different numbers of

sequences, suggesting that one species contributes more to a sample than another (Hansen *et al.* 1998). In extreme cases PCR bias can result in false negative results where one species completely outcompetes another. When whole communities are being sequenced this competition among priming sites is intensified. One way to account for PCR bias is to develop correction factors, taking into account how each species amplifies in the presence of other species. Calculating correction factors is a big task when the number of target species is large but has been shown to work (Thomas *et al.* 2016). Indexing primers can also introduce further primer bias.

An alternative method to reduce PCR bias is not to perform PCR at all but rather sequence the raw DNA in a sample, called "shotgun sequencing". This requires breaking the DNA up into small regions, sequencing each of these and then "stitching" the genomes back together in silico (Venter *et al.* 2004). Unfortunately, this method can result in a much greater portion of non-target DNA being amplified and thus less chance of detecting the target organisms.

Analyses

There are many ways to filter and analyse eDNA data and new software is being developed at a rapid rate. How data are treated can have significant effects on results and interpretation of results. For this reason, standardised pipelines will be required in order to compare results among studies. For instance, eDNA data sets invariably contain many reads of organisms in low quantities and distinguishing true rare taxa from contamination or sequencing errors is difficult and often subjective. Often some threshold is applied under which taxa, with representation less than the threshold in read numbers, are omitted. Standard methods for determining such thresholds are currently lacking. Similarly, no standard method exists for assigning taxonomies to sequences. A commonly used approach is to accept species level identification on sequences that have a 97% match to a known species and to disregard all other data. This renders a large portion of the data unusable. Alternatively, some researchers use further arbitrary thresholds to assign taxonomies at higher levels, for example, >95% for genus and >90% for family level associations. In each case, assignment is somewhat subjective, and thresholds will change depending on the gene fragment used. Ultimately, having a comprehensive database of reference DNA barcodes and knowledge of genetic diversity within species is needed to overcome this issue.

Sequencing

Further variation that is perhaps unavoidable will occur between sequencing runs. Optimally all samples that are analysed together should be sequenced together, as this will ensure all samples are sequenced in similar proportions. For example, a sample that has been sequenced by itself will have more reads, and thus more chance to detect taxa, than a sample sequenced alongside many other samples, as in the latter case each sample receives a portion of the total sequencing read depth whereas in the former the sample receives the whole read depth. Variation in the number of samples and amount of genetic material among sequencing runs will mean that samples sequenced on one run may have more chance of being completely sequenced than on another run. This will have implications for comparing samples over time, where for instance each year a new sequencing run is conducted with only the samples collected during that year. Standardising sample numbers and concentrations, as well as sequencing standardised mock communities may reduce this confounding.

4.2 Understanding persistence and dispersion patterns of DNA after shedding from the host organism

The persistence of eDNA in the environment needs to be considered and currently there is insufficient information on how eDNA persists in or is dispersed within different environments. Studies have shown various eDNA persistence times ranging from hours to decades to thousands of

years, dependent on the environment (i.e. marine ~7 days, freshwater ~2-4 weeks, terrestrial sediments ~ up to 400,000 years, Thomsen & Willerslev 2015). However, this will vary depending on a variety of variables such as UV exposure, pH, temperature, and water flow (see next section).

The variable persistence of DNA in the environment raises issues regarding where DNA originates from. DNA in the environment can be dispersed throughout a landscape (i.e. blown in from elsewhere) or transported (carried in on other organisms, including in the stomachs of other organisms). In freshwater ecosystems DNA can be transported downstream, with distances assumedly variable depending on the strength of stream flow.

There is currently little information about how far eDNA travels; although much more literature is available on freshwater than terrestrial transport. Transport of eDNA may affect estimates of local community compositions; however, conversely it may provide a better picture of catchment scale processes.

eDNA can come from a variety of sources. For a freshwater system it could be an animal drinking at a stream or faeces deposited by birds. Another source of DNA in environmental samples are bacterial genomes that generally contain a large portion of DNA and can swamp out other larger taxa. Where larger taxa are the focus of a study, for instance for aquatic bio-monitoring, bulk-sample collection (i.e. the collection of the animals themselves for genetic extraction) may be a better option than an environmental sample and will likely better reflect traditional survey approaches (Macher *et al.* 2018). However, optimising survey designs and developing site occupancy models can improve inferences around detection (Lugg *et al.* 2018).

4.3 Effects of the surrounding environment

Understanding effects of the surrounding environment (e.g. pH, turbidity, temperature, ultra-violet light and contaminants) on DNA is important as it may cause variations and fluctuations in results. In terrestrial ecosystems DNA longevity depends on temperature and UV exposure and on how recently or often the DNA is wetted. DNA binds to silica in soils and, if these soils remain dry, can last decades. As soils become wetted bacteria increase in number and consume DNA. Higher temperatures, acidity and UV exposure break DNA chains with exposure fragmenting DNA until it becomes too small to be amplified. In aquatic systems turbidity acts to increase persistence by providing silica binding substrate, lowering UV exposure and lowering temperature. However, acidic rivers will degrade eDNA more quickly than a neutral or alkaline river.

4.4 Appropriate markers

Many global genetic databases rely on the DNA barcoding principle (Hebert, Ratnasingham & deWaard 2003) where a targeted gene fragment (usually part of the mitochondrial COI gene) is used as a universal marker to determine species. Focus on the COI barcode fragment can be useful for eDNA studies but can be also be a limitation particularly in broad scale community studies (Thomsen & Willerslev 2015). The COI barcode is also not suitable for all applications, for instance the mitochondrial CYTB region is often used as the universal marker for fish and for plants the chloroplast markers Trnl, rbcl and matK.

Further when choosing a targeted gene region for community-based studies, the primer pair required needs to be considered. Current genetic analysis methods vary from PCRs to high throughput sequencing (HTS) technology, these options have various limitations and/or restrictions. HTS does not facilitate the amplification of large gene fragments >500bp such as the COI barcode fragment (658 bp). For this reason, smaller gene regions around 400 bp are targeted that provide greater certainty around consensus sequences in results but reduces the information content of samples. The universal primers used to amplify the chosen gene fragment can also vary in their applicability to target organisms. For instance, the widely used universal arthropod primers (Folmer

et al. 1994) perform well at amplifying insect barcodes but will not amplify flatworm barcodes. Moreover, one primer set may perform better than another at reliably amplifying a greater portion of taxa (Elbrecht & Leese 2017) or of amplifying taxa from a particular region. In order to maximise detection success and the taxonomic breadth covered, multiple primers for multiple gene fragments may be required (Carew *et al.* 2018; Stat *et al.* 2017).

Single species detection requires prior genetic information of the species that enables specific marker design to ensure that the target species is detected, and non-target species aren't (Ficetola *et al.* 2008). Without developing species specific markers, it is likely that false positive results will occur.

4.5 How to move beyond simple presence/absence interpretation toward quantitative or semi-quantitative abundance/biomass estimates

Many studies require an estimate of biomass or abundance of taxa and the biases explained above will affect the ability to do this using eDNA. The issue of estimating abundance or biomass from eDNA remains contentious and the literature currently shows conflicting results with suggestion that confounding variables need to be taken into account. Some studies have found positive correlations between eDNA concentrations and abundance and biomass (Takahara *et al.* 2012, Doi *et al.* 2015 and 2017; Thomsen *et al.* 2012, Pilliod *et al.* 2013). These studies generally focus on single species, often fish, and conclude that eDNA provides an assessment of relative abundance rather than quantitative measures. Other studies have found confounding issues regarding habitat (Hinlo *et al.* 2018), collection methods (Lacoursière-Roussel, Rosabal & Bernatchez 2016b), or life histories (Maruyama *et al.* 2014), or correlations with biomass but not but not abundance (Lacoursière-Roussel *et al.* 2016a). It is possible that estimates of biomass and abundance from eDNA may be achievable but models that take into account DNA production, transport and decay are likely to be required (Barnes & Turner 2016) and likely to only be applicable to large well understood organisms in the near future.

4.6 Linking taxonomic and genetic data

The taxonomic knowledge of biodiversity globally and in Australia is patchy. However, some groups are considered well known such as fish, birds, and reptiles while others remain poorly known such as invertebrates. Having a good taxonomic understanding of a group enables genetic reference databases to be established to inform molecular based projects of species present, especially in community composition studies. These genetic reference databases (e.g. www.fishbol.org, 25% of species with barcodes, 89% of families; Becker, Hanner & Steinke 2011) form the backbone of community-based studies as they are used as a reference for eDNA collected during monitoring and evaluation programmes. Meanwhile other groups that are less taxonomically well-known have genetic databases that are being established to both increase taxonomic knowledge of Australia's biodiversity and assist in eDNA sampling (e.g. Aquatic Invertebrates of Australia (AIA), Carew *et al.* 2017).

However, the availability of reference data varies depending on geographic location and the data are often not vetted for incorrect identifications (Shackleton & Rees 2016). These issues can be minimised or mitigated by maintaining a local reference database of verified sequences. Without a genetic database, sequences will not be identified and will be listed as molecular operational taxonomic units (MOTUs), which has the potential to over or under estimate species diversity. For eDNA programmes that focus on single species detection the issue of a reference database is not as important as usually the taxonomy is already known, and genetic studies have been undertaken that enables markers to be readily designed.

5 Applications within the Murray-Darling Basin

The range of potential applications of eDNA methods is extensive and includes both the replacement of existing biomonitoring programs (e.g. case study 1) as well as potentially novel management questions (e.g. case studies 2 and 3). The feasibility and suitability to particular questions depends on a number of factors, including the organisms being targeted, the environmental medium used and a number of potential technical limitations. These technical limitations and the capacity, costs, and time to resolve them also differs among potential applications.

In the short term, it is recommended that eDNA based methods should continue to be trialled across a range of applications. However, we would also recommend that such trials be undertaken alongside existing or already established sampling programs as an opportunity to validate eDNA detection methods, and to resolve some of the technical issues.

Case study 1: eDNA techniques for community composition of aquatic macroinvertebrates

Please see full details published in reference: Macher *et al.* (2018)

Freshwater biological communities provide important ecosystem functions and services and are often used as the base for water quality monitoring. The assessment of freshwater biodiversity typically has focused on the collection of benthic macroinvertebrates and manual identification of species. However, genetic methods such as metabarcoding where extracted DNA is targeted by selected markers that are "universal" to a range of organisms are being increasingly used as they are faster and allow greater taxonomic range.

In this case study two genetic techniques were used:

- "bulk metabarcoding" where a sample of benthic macroinvertebrates is collected by kick netting and DNA then extracted from that sample
- "eDNA metabarcoding" where the DNA is collected from filtered water samples at the site.

The study compared metabarcoding results from bulk and eDNA samples from 19 streams in New Zealand. Targeted primers used were the universal "barcode" fragment cytochrome c oxidase I *COI* primers with high throughput sequencing on an Illumina MiSeq machine to generate genetic sample libraries. Results indicated that eDNA metabarcoding returned more species than the bulk metabarcoding, but the bulk metabarcoding returned a higher number of species for key aquatic bioindicator taxa (mayflies, stoneflies and caddisflies). In total 6,451 molecular operational taxonomic units (MOTUs) were recorded in the study, of these 77% were found only in eDNA samples, 10% only in bulk samples and 13% of MOTUs were recorded in samples from both techniques. Of the 77% of taxa that were recorded only in eDNA samples the majority were small species such as algae and zooplankton that would not have been retained in the kick netting approach.

The findings indicated that both techniques have their merits and hold great potential for application into monitoring and evaluation programs. One major difference between the techniques was the spatial scales covered, bulk metabarcoding captures the local scale whereas eDNA returns results from a much larger spatial scale potentially up to the sub-catchment scale. This is an important consideration when applying this method to monitoring programs. The authors suggest that eDNA sampling with universal primers is suitable for the assessment of whole-ecosystem taxonomic diversity, but that bulk-sample metabarcoding are more suitable for the targeting of specific benthic macroinvertebrates. Combining both methods may currently offer the best solution, as this would allow assessing the majority of a stream ecosystem's biodiversity.

What can eDNA be used for?

While eDNA methods may be both feasible and suitable for certain questions and monitoring there remains technical and logistical limitations to the delivery of some eDNA based techniques. Complementing existing projects with an eDNA component would address key knowledge gaps and enable the development of frameworks, protocols and QAQC processes.

Within freshwater ecosystems eDNA methods are suitable and feasible for a range of organisms (e.g. fish, turtles, platypus, macroinvertebrates) and, threatened and invasive species; particularly for single-species detection or for multiple targeted species. Community composition for microbial and fungal communities including biofilms, food webs and presence/absence studies for some groups of macroinvertebrates for which there are established genetic databases. Advances in other taxonomic groups are occurring rapidly. Until genetic databases are progressed however, the capacity to adopt eDNA based methods cannot be used to assess the composition of entire macroinvertebrate assemblages. As this work progresses, eDNA methods provide an opportunity to expand our knowledge of groups of organisms that may have been hampered by the limitations of more traditional 'morphological' species identification (refer case study 3). In this domain eDNA based monitoring holds great promise and will likely become feasible in the near future.

Case study 2: Detecting the invasion front of redfin perch, *Perca fluviatilis*, to inform the location of a containment barrier

Please see full details published in reference: Bylemans *et al.* (2016)

Blakney Creek, a small intermittent lotic system of the Upper Lachlan catchment, contains one of only three self-sustaining populations of southern pygmy perch, *Nannoperca australis*. Recently, this endangered species has been under additional pressure from the invasive redfin perch, *Perca fluviatilis*, which was first recorded in the creek in 2005. The continued upstream spread of redfin perch in this system is pushing the already fragmented southern pygmy perch populations to the brink of extinction. To protect the remnant southern pygmy perch population in Blakney Creek and adjoining Urumwalla Creek, a redfin perch exclusion barrier was planned to be installed in 2015. Determining the optimal location for the construction of this containment barrier relies on detailed knowledge of species distributions. Both conventional surveys and eDNA-based methods were employed to determine the upstream invasion front of redfin perch.

Conventional fish monitoring was conducted at 4 sites along Blakney Creek and 4 sites along Urumwalla Creek using unbaited traps and backpack electrofishing or dip netting according to the features of the sampling site. Environmental DNA surveys were conducted at the same 8 sites by collecting 8 x 2-L samples of water per site, filtering water onto filter paper and extracting DNA. DNA was amplified in triplicate using a redfin perch-specific TaqMan® real-time PCR assay targeting a short fragment of the 12S rRNA gene region.

Conventional fish monitoring detected redfin perch in all but the most upstream sampling site within Blakney Creek and failed to detect the species at any sites in Urumwalla Creek. eDNA surveys detected redfin perch at all sites in Blakney Creek including the most upstream site and detected redfin perch at an additional three sites in Urumwalla Creek with only the most upstream sampling site appearing to be free of redfin perch.

eDNA surveys were able to achieve greater sensitivity to detect redfin perch than conventional monitoring, detecting redfin perch at an additional four locations. This placed the redfin perch invasion front a greater distance upstream along Blakney Creek and extending into Urumwalla Creek beyond the distribution inferred via conventional surveys. The results of this eDNA survey informed the placement of the containment barrier to ensure redfin perch were excluded from critical breeding grounds of the endangered southern pygmy perch, *Nannoperca australis*.

Generally, current applications of eDNA techniques for monitoring are not as feasible within the terrestrial landscape especially from sediments. The increase in persistence of DNA within sediments changes the temporal scale of the sample and may effect results in studies if not taken into consideration. However, some studies could be undertaken if the question is suitable, for example, food web studies based on scats from mammals. New studies (being undertaken at CSIRO) on insect pollinators and their interactions with flowers show the interesting scope of question and applications that eDNA based methods can addressed.

By supporting the development of techniques and the building of capacity through continued support of research, particularly on questions and organisms that show the most suitability; the improvement in understanding of key knowledge gaps will mean an increase in the applications of eDNA based methods.

Case study 3: Detection of aquatic pondweeds using an eDNA approach

Please see full details published in reference: Kuzmina, Braukmann & Zakharov (2018)

Pondweeds are important macrophytes in freshwater ecosystems that provide habitat and food for a variety of animals (fish, birds and macroinvertebrates). They can be used as bioindicators for the evaluation of water quality. However, the ability to morphologically identify species can be difficult as it often requires the plant to be fruiting.

By using a targeted eDNA metabarcoding approach for the detection of pondweeds there is the potential to overcome identification issues during ecological surveys. A genetic reference library was created from the collection of 30 pondweed species that included species of ecological importance and threatened species. Water samples were collected and extracted DNA was put through high-throughput sequencing strategy using markers designed in the ITS2 and *atpB-rbcL* gene regions.

The targeted approach to pondweed species composition in freshwater ecosystems revealed an underestimation of their diversity compared to previous ecological surveys that had been conducted. This result suggests that eDNA is an effective tool for monitoring plant diversity in aquatic habitats. However, the results demonstrated that in community studies using eDNA detection the results will depend on the completeness and accuracy of the genetic reference libraries used.

Citizen science

Another potential use for eDNA based methods is that of volunteer or citizen science-based programs. With simplified collection techniques, sampling protocols and a network of training or accredited organisations it is feasible to link with well-established citizen science organisations e.g. Waterwatch or Landcare. On-going assessment of the suitability of citizen science-based approaches will need to be undertaken but if frameworks, guidelines and QA/QC processes are established for eDNA methods then there is significant potential for application within the MDB (refer case study 4).

Case study 4: Citizen science and eDNA sampling

Please see full details published in reference: Biggs *et al.* (2015)

In the UK, a citizen science program is in place for a protected species the great crested newt (*Triturus cristatus*). The effort required for surveys to assess the presence or absence from a site can be considerable with usually 4-6 annual field trips undertaken using at least three survey methods on each occasion. Because of the large survey effort required there has been no established of a national monitoring program, due to the costs for both professionals and volunteers. In this study, the authors tested whether eDNA could form the basis for a citizen science-based monitoring program for the great crested newt by:

- comparing the effectiveness of eDNA monitoring to traditional survey techniques, and
- assessing the ability of volunteers to collect eDNA samples throughout the newt's UK range.

To assess this a single water sample was collected for eDNA analysis at each pond at the same time as the traditional surveys were done. The eDNA collection method included a simple sterile sampling kit that contained equipment needed for collection, gloves and preservation solutions. In the laboratory, DNA was then extracted from samples and amplified with targeted primers and run through a quantitative PCR (qPCR) to assess real time PCR reads for positive results of the presence of the targeted DNA.

The results showed that from the 35 ponds visited four times over the breeding season, eDNA detected newts on 139 out of 140 visits and was more effective overall than the individual traditional survey methods. Volunteers successfully collected eDNA samples throughout the UK with 219 of 239 sites (91.3%) correctly identified as supporting newts. The remaining 8.7% of sites returned false negatives, that were due to small newt populations at that location or difficulties in sample collection.

While this shows the potential for the opportunity of eDNA surveys to be undertaken by volunteers in citizen science programs there is still more testing required to assess the validity and effectiveness of results. Additionally, although eDNA and volunteer based programs are attractive in terms of cost, it needs to be remembered that volunteer based programs still require an element of centrally-funded professional co-ordination, especially for DNA analysis.

What will it not improve

Not all ecological or biological monitoring or evaluation programs ask questions that can be readily answered by incorporating eDNA methods. Some examples where eDNA methods will not be suitable to address the outcome of monitoring conducted in the MDB are:

- vegetation recruitment studies (e.g. woody trees in riparian areas)
- overall wetland health estimates where assessment is based on physical characteristics
- fish movement studies (e.g. tracking individual fish as per PIT tag monitoring through fish ladders).

A current example is the assessment of the health of lignum patches where observational data are collected for scores based on health of the stems, density and if recruitment is present while measured data variables include length and height of the clump. This information combines to provide an indication of the health of the clump which is important as the clumps are an important habitat for waterbird breeding activity. eDNA techniques would not provide the required information to establish this result.

6 Summary and next steps toward adopting eDNA

To improve capacity for delivery of eDNA programs, support and development opportunities need to be provided to institutions and organisations from funding bodies (refer Schmeller *et al.* 2017). As highlighted in this review there are several technical and logistical issues (see section 4) that need to be addressed to make eDNA a feasible option for application into future monitoring programs. Along with the technical issues it is important that a careful strategy for the development and implementation of best-practices methods, guidelines and the establishment of QAQC protocols is instigated to improve the capacity to deliver eDNA based programs. Two broad priorities with identified steps are presented below to improve the capacity to deliver eDNA programs.

6.1 Priority 1: Increase knowledge

An overarching requirement to improve the application of eDNA methods to monitoring and evaluation programs is to increase knowledge through research, particularly for knowledge gaps identified in this review. Currently, the two major hurdles to eDNA based monitoring are the technical limitations and the lack of reference databases that combine genetic and taxonomic information. Continued support through funding projects that address these knowledge gaps will advance the applicability of eDNA based monitoring programs within the MDB and more broadly Australia.

Key technical knowledge gaps

- Increasing the understanding of persistence and dispersion patterns of DNA in different environments and the effects this has on the interpretation of results, and the spatial and temporal coverage of the sample. This includes effects by the surrounding environment (e.g.pH, turbidity, temperature, contaminants and flow).
- Understanding and reducing rates of contamination and errors in sampling, laboratory and analyses.
- Developing ways to move beyond simple presence/absence interpretation toward quantitative or semi-quantitative abundance or biomass estimates.

Referenced databases

- Establishing centralised and accessible genetic reference databases that link taxonomy and genetic information. Beginning with potential groups that would be suitable for eDNA based programs.

6.2 Priority 2: Framework, guidelines, QAQC

eDNA has many potential pitfalls in both the field and laboratory. While samples can be easily attainable making sure contamination is limited, sample collection is carried out correctly and samples are stored appropriately will help minimise field errors. The development of standardised methods to eDNA approaches and the establishment of QAQC processes would ensure a robust delivery of eDNA programs.

- Operational guidelines for best-practice sampling. Standardising of common techniques that will achieve deliverable results efficiently, including QAQC.
- Training through workshops and competency assessments that increases the potential collectors from highly trained professionals to citizen science networks (e.g. Waterwatch).
- Laboratory guidelines – lab processes and protocols, including QAQC.

Ideally operational procedures for eDNA methods would be developed within an established regulatory framework (i.e. National Association of Testing Authorities, Australia - NATA). This would

ensure that best-practice and up-to-date processes and procedures were being maintained to a high standard. It would also establish a network of organisations and institutions that are identified as reliably and consistently conducting competent eDNA programs. The regulatory framework for eDNA would encapsulate – the collecting of specimens, laboratory procedures and analyses, the storing of specimens and data, and QAQC procedures. By using a possible NATA based system the accreditation would require annual checks, the continuation of training and maintenance of competency of an organisation.

6.3 Recommendations for the delivery of eDNA based biological monitoring programs in the Murray-Darling Basin

- To provide support for the undertaking of research and develop programs focusing on eDNA projects, especially those that address identified technical knowledge gaps including sampling, laboratory and analyses issues that are required to enhance eDNA methods;
- To provide support for a period of transition from existing programs by running existing sampling in parallel with eDNA based sampling methods until any unforeseen issues have been resolved;
- To foster the implementation of eDNA methods for programs that are now suitable and feasible such as the single species detection of targeted organisms;
- The development and establishment of centralised and accessible genetic reference databases linking genetic and taxonomic information;
- The development of a strategic framework and operational methods that will benefit and enhance the development of eDNA methods in future monitoring and evaluation programs that enables eDNA standard methods that are comparable, repeatable and widely applicable within monitoring frameworks;
- The establishment of protocols to ensure QAQC for eDNA methods as they are employed by less established groups;
- To provide support of capacity building across research organisations (universities, government departments, CSIRO, NGOs) to establish of data repositories, data facilitation, connectivity through coordination and integration across monitoring and evaluation programs.

7 Conclusion

Within the MDB there are currently several monitoring and evaluation programs being conducted on a wide variety of organisms and landscapes. The spatial, temporal and taxonomic range of monitoring programs undertaken in the MDB is large and incorporates a variety of sampling techniques which have a wide variation in costs to institutions, organisations and government bodies. The recent advances in the use of eDNA techniques for species detection and biological monitoring, combined with the general ease of collection makes eDNA an innovative and potentially cost-reducing way of conducting broad scale monitoring programs in the future.

The benefits of eDNA techniques can be widely seen and the potential application to programs in some instances is both suitable and feasible. However, there remain technical and logistical issues that need to be addressed as well as the formation of frameworks and guidelines to be established in order for eDNA techniques to become the base of monitoring and evaluation programs. This review shows that in most cases eDNA techniques in combination with traditional approaches currently offer the most potential. It enables knowledge gaps to be filled and genetic reference databases to be established but only with additional short-term costs. While the potential for use in the MDB is clear, a strategic approach to the deployment of eDNA techniques within projects is important in order to establish quality long term approaches to eDNA techniques in the monitoring and evaluation programs.

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