EnviroDNA Aquatic Native Vertebrate eDNA Method

DOCUMENT DETAILS

Method Name: EnviroDNA Aquatic Native Vertebrate eDNA Method

Method Reference #: AfN-METHOD-F-05

Relevant Environmental Asset Class: Fauna

Confidence Levels: Level 1, 2, or 3

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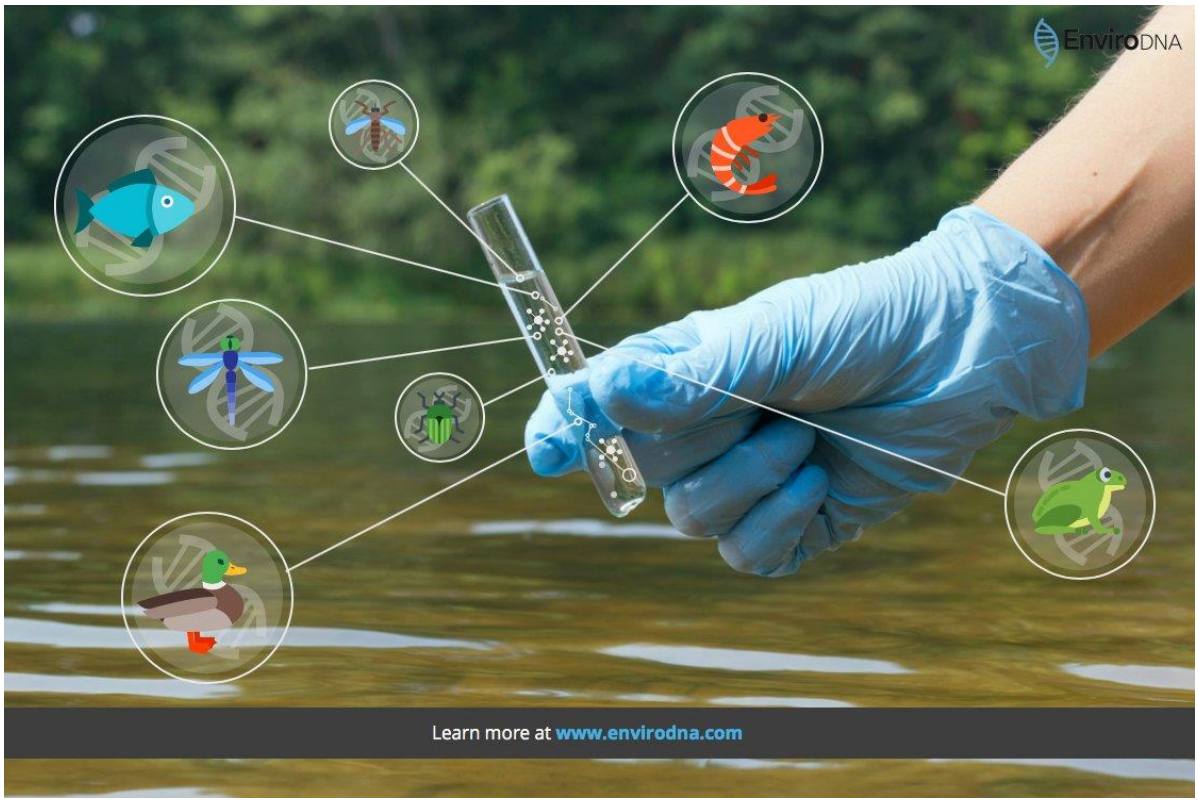
1. INTRODUCTION

About EnviroDNA



EnviroDNA is the first company in Australia offering a dedicated eDNA service to clients. The company was born out of Cesar Australia, an organisation that, for the last 10 years, has combined a passion for sustainability with cutting edge science and technology in wildlife conservation and Australia's agricultural sector. EnviroDNA has developed innovative technology and approaches that enable the detection of species using eDNA. eDNA offers an unprecedented opportunity for monitoring native and invasive species, as well as for biodiversity assessments. For more information about EnviroDNA, see: https://www.envirodna.com/about/company. EnviroDNA have developed this Method to outline their approach to developing an Environmental Account using eDNA. If you are considering using this Method as part of your Environmental Account, please contact EnviroDNA at info@envirodna.com to discuss your project and get an estimate of costs involved.

Accounting for Nature Disclaimer:
This Method describes the EnviroDNA proprietary approach to developing an Environmental Account using eDNA. While this is the first Method approved by AfN that utilises on eDNA technology, there are other possible approaches and applications of eDNA available. As such, any Proponent or Organisation may also develop their own fit-for-purpose Methods (either proprietary or not) that utilises eDNA.

Aim and Background of this Method

This Method has been developed by EnviroDNA to assess the condition of aquatic vertebrates by measuring species richness over time, and is applicable Australia-wide at the property, catchment or regional scale. This Method uses environmental DNA (eDNA) analysis to detect species, including birds, fish, amphibians, reptiles, and mammals (see Appendix A for more information about eDNA metabarcoding. Other species groups are also amenable to eDNA detection but require different molecular resources). Aquatic vertebrates have declined dramatically since human population expansion, and they are of increasing public interest both for their intrinsic value as unique species, and for their central importance to tourism and primary industries. This method can be used in conjunction with the AfN's Regional Waterway Condition Method to gain a deeper understanding of the condition of river systems. In addition, while this Method can provide a broad account of aquatic vertebrate fauna diversity, it can also be used to understand the presence/absence and distribution of iconic species (focal species) in a region.

Aquatic environments pose particular challenges for monitoring and, given biodiversity is declining at an unprecedented rate worldwide, there is an urgent need for methods that will help managers make more informed decisions (Dudgeon et al. 2006; B. Pusey et al. 2011; Thomsen et al. 2012). Traditional aquatic fauna monitoring methods cannot provide this information at the scale required. Identification of DNA shed by organisms into their environment provides a new method for detecting species with the potential to transform biodiversity monitoring. Relative to traditional survey techniques (such as electrofishing), eDNA is cheaper and more broadly deployable, enabling implementation at large temporal and spatial scale; provides higher sensitivity to detect rare or cryptic species; is non-invasive, and therefore safer for target species as well as field operators; and can, in principle, access the full spectrum of aquatic and terrestrial biodiversity that underpins ecosystems (see Appendix B).

While water sampling for eDNA analysis is very simple, survey design and appropriate handling and analysis of eDNA samples requires computational, statistical, and laboratory expertise. EnviroDNA will adhere to reproducible best practices in laboratory work, including segregation of activities in dedicated cleanrooms and appropriate use of negative and positive controls, and in sequencing, analysis, and reporting.

Table 1. Summary of the Method

Purpose	The purpose of this method is to measure the species richness of aquatic vertebrate fauna in freshwater ecosystems.		
Application	Australia-wide		
Scale	Applicable from property to regional scale.		
Scope	Monitoring freshwater aquatic vertebrate species richness through time		
Target Audience	Farmers, land managers and project managers are the intended audience for this method. This method will require the advice of EnviroDNA to assist with sampling design and sample collection considerations during the planning phase, and processing of environmental samples.		
Decisions to inform	This method can be used to inform decisions about where to focus conservation and ongoing monitoring efforts.		
Confidence Level/s	The confidence level for this method is dependent on sampling intensity. The minimum number of total sites for each confidence level is outlined below. As such, a Level 1 account is only feasible for very large properties, projects, or regional assessments, and a Level 3 account is more suited to smaller areas.		
	Level 1 175+ sites 35+ Assessment Units	Level 2 50+ sites 10+ Assessment Units	Level 3 5+ sites 1+ Assessment Units

Justification of Confidence Level

The confidence estimates outlined above are based on a power analysis simulation model (details available upon request) and provide guidance for proponents on the scale of sampling required to achieve a given confidence level. **If project resources allow, then an account specific power analysis is highly recommended to accurately determine sampling intensity and confidence, based on pre-existing or preliminary survey data.**

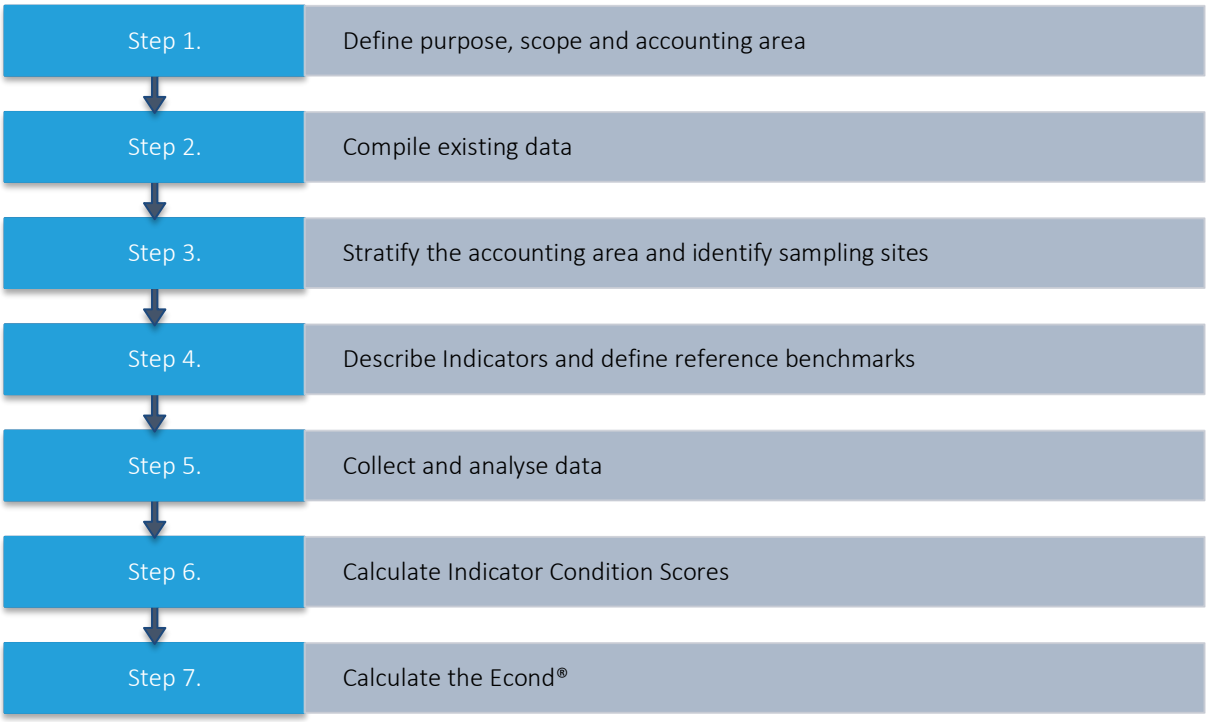
What an Environmental Account Looks Like

The *Accounting for Nature®* Framework requires accounts to be comprised of three key components for them to be certified:

1. An **Environmental Account Summary** – a public document that summarises the results of the environmental account in a form that is readily communicated to the public.
2. An **Information Statement** – describes in detail the method used and the actions taken to address each of the seven steps under the framework including rationale behind asset selection, choice of indicators, Method used, analysis and management of data and calculation of the Econd®.
3. The **Environmental Account** – a database (such as an Excel file) that contains all the data described in Asset Tables, Data Tables, and Balance Sheets.
4. An **Audit Report** or **Self Verification Report** that verifies the Account was prepared in accordance with the approved Methods, the *AfN Standard* and *AfN Audit rules*.
    - An Audit Report is completed by an AfN Accredited Auditor and is required if you are seeking to have your account "Certified" (Tier 1); OR
    - A Self-verification Report contains the results of your self-verification assessment and AfN's Technical Assessment and is required if you are seeking to have your account "Self-verified" (Tier 2).

Upon certification of the account, the Environmental Account Summary and Information Statement will be published on the AfN Environmental Account Certification Registry.

Overview of Process



Note – Expert Assistance

While proponents may wish to be involved in some of the stages of account preparation, the technical expertise of EnviroDNA will be required for several of the steps outlined above. EnviroDNA provides expert assistance for proponents in sampling design, sample processing and data analysis. We can also provide training in data collection methods. The initial scoping of the project (**Step 1)** is best undertaken by project proponents, but it would be useful to involve experts from EnviroDNA from the outset. **Steps 2-4** require expertise in GIS and freshwater ecology and experimental design, and the expertise of an aquatic ecologist with knowledge of the distribution of aquatic species in the environmental account area. Data collection (**Step 5**) can be undertaken by proponents following training by EnviroDNA. The processing of samples (**Step 5**) requires EnviroDNA's specialist lab. The calculation of condition scores (**Step 6**) and the Econd® (**Step 7**) can be undertaken by proponents (or an AfN Accredited Expert) or EnviroDNA by following the instructions provided in this methodology.

2. CREATING THE ENVIRONMENTAL ACCOUNT

Step 1. Define purpose, scope, and accounting area

The preliminary step to developing an Environmental Account is to **describe** the Environmental Account through defining its intended **purpose**, **scope** and **accounting area.**

Table 2. Overview of defining the purpose, scope and accounting area.

Purpose:	Describe the specific purpose of the account.
Scope:	Describe the scope of the account. This method is suitable for: - *Snapshot* – a one-off assessment of aquatic vertebrate species richness - *Change over time* – an ongoing assessment of the change in species richness through time
Accounting Area:	Describe the accounting area (include location and size details). Provide a map of the accounting area that shows location and size information. Defining the purpose, scope, and scale of an eDNA study is a critical planning step which in part determines optimal sampling design, and hence the power and sensitivity of detection of aquatic vertebrate species. EnviroDNA should be consulted to ensure project aims can be met within practical constraints. *NB. The accounting area must stay the same for the lifespan of the account. If the accounting area changes (such as a new area to be added, or an area to be removed), then a new account must be developed, or the account, 're-set' and started again with the new accounting area.*

Output of Step 1

- A description of the accounting area including **location** and **size**
- A table describing the **purpose** and **scope** of the account
- A **map** showing the accounting area

Step 2. Compile existing data

Prior knowledge

To provide an initial snapshot of biodiversity at the broadest taxonomic level considered in this Method, sites representative of the full range of habitats should be included. These data can then guide future sampling and experimental design in a formal modelling framework, to maximise species coverage, or prioritise species and habitats of exceptional interest. Existing data can be integrated at the planning stage to guide sampling design, these data can include:

- Hydrology (Australian Hydrological Geospatial Fabric [Geofabric]),
- Riparian habitat types (National Vegetation Information System Major Vegetation Groups [NVIS MVG],
- Australian River Assessment System [AUSRIVAS] macroinvertebrate predictive models,
- Interim Biogeographic Regionalisation for Australia [IBRA] subregions), and
- Biodiversity (Atlas of Living Australia [ALA], reports from local managers).

The quality and availability of relevant public information varies geographically at present. At catchment and regional scale, local managers and affiliated experts are likely to be best placed to identify relevant GIS data.

Output of Step 2

- A map, table and description of all major assets (waterways and aquatic environments) in the assessment area.
- Optionally, map layers capturing relevant environmental variables: Major Vegetation Groups bordering assets, hydrological connectivity and flow, and existing data on known aquatic vertebrates in the assessment area.

Step 3. Stratify the accounting area and identify sampling sites

EnviroDNA should be consulted during the initial sampling design phase to ensure that sites are established in a way that will represent the range of potential habitat types and which reflect the ecology of the species that are expected to occur in that locality.

Assessment units

The first stage of sampling design involves stratifying the account area into assessment units based on management consideration and hydrological and/or ecological characteristics.

Table 3. Overview of how to generate Assessment Units

Regional scale account (areas in the hundred thousands and millions of Ha)	For regional scale accounts, assessment units will be defined by existing catchment/sub-catchment structures (e.g. based on Geofabric) and further subdivided based on management considerations, habitat types, or other ecological characteristics.
Property or project scale account (areas in the hundreds or thousands of Ha)	For smaller project or property scale accounts, the account area may be stratified into assessment units defined by habitat types (e.g., NVIS MVG). Smaller, or relatively homogeneous, properties may contain only one assessment unit - the entire property.

Sampling design

Once assessment units have been defined and mapped, a sampling program should be designed based on the criteria outlined in Table 1 below. Sampling sites are allocated to waterways within each assessment unit randomly using a GIS sampling tool but constrained by the site spacing criteria and practical considerations.

Table 4. Overview of how to select and establish sampling sites within each assessment unit

Assessment units	**Level 1** At least 35 assessment units	**Level 2** At least 10 assessment units	**Level 3** At least 1 assessment unit
Number of sites	At least 5 sites per assessment unit		
Site spacing	Sites should be located no closer than 1 km apart to ensure independence between samples. Sites should be located no further than 100 km apart to ensure that accounts covering large areas (e.g. > 250,000 ha, thousands of kilometres of waterway) are adequately sampled. (**Note**. This means that a Level 3 Account in a large area would require more than 5 sample sites and likely more than 1 assessment unit).		

Practical considerations	Sites should be initially allocated using GIS software, constrained to areas with practical access, allowing for repeat surveys. Water should be safely accessible, as best as can be judged from satellite and topological data, though the exact location of sites can be adjusted in the field based on local access and flow considerations.
Timing	Surveys should occur at least once per year, in either spring or autumn. Repeat sampling in subsequent years is to be undertaken in the same season as previous sampling.
Site establishment	eDNA sampling is non-invasive and does not require establishment of permanent equipment.

Output of Step 3

- A map and table showing the **stratification** of the accounting area and identifying assessment units
- A map and table with central coordinates of each **sample site** within the accounting area.

Step 4. Describe environmental indicators and determine reference benchmarks

Indicators

This Method measures species richness of aquatic vertebrate species, with data aggregated within each assessment unit and then across the entire project area. Species lists are generated for each site, and then aggregated across sites for a species richness estimate for each assessment unit. Most simply, all native aquatic vertebrate species can be considered, but invasive, or rare and threatened species can also be targeted through the sampling design and consideration of habitat requirements.

Species richness	- Two eDNA samples are collected at each site and data combined to produce a species list for each site. See Appendix C – Sampling Procedure. - A species list for each assessment unit is then produced by aggregating the data from each of the sites within that unit (at least 5 sites). - A condition score for species richness is calculated as the total number of species observed as a percentage of the number of species expected to occur in that assessment unit based on the pre-1750 expected species list for that unit. - This provides a simple yet robust measure of the condition of each assessment unit.

Reference Benchmarks

The following steps determine the reference benchmark for each assessment unit. It is recommended that this exercise be undertaken spatially using a GIS system - so that each assessment unit within the account can be queried.

1. Develop an *expected species list* for each assessment unit from any available and collated data sources and in consultation with experts. This list should describe all native species expected within the assessment unit (species generally assumed to occur pre-1750, including extinct species) and should consider the presence of suitable habitat within proximity of site.
2. This list may also need to be revisited if eDNA detects native species that were not previously expected to occur in the assessment area. Consideration should be given to the EnviroDNA reference library, which can be extended if tissue can be obtained for priority species that are not yet represented.
3. Calculate *expected species richness*: the number of species expected to be present in each assessment unit.

Output of Step 4

- A table describing the **environmental indicators** to be measured in the account
- A table that includes the **Reference Benchmark value** for each assessment unit in the account area

Step 5. Collect and analyse data

Conducting sampling

Depending on project organisation, scale and duration, sampling can be conducted either by a dispersed team of management groups, field workers or citizen scientists using single-site sampling kits, or by a small team of specialists using dedicated water filtration equipment (Thomas et al. 2018). Guidance is provided in Appendix C on using EnviroDNA's single-site water filtration kit.

Photo: Smith-Root Inc.

DNA sequencing and data analysis

DNA extraction, amplification by polymerase chain reaction (PCR), sequencing library preparation, sequencing, and data analysis are conducted by EnviroDNA. For broad monitoring of aquatic vertebrates, a widely used VERTEBRATE amplicon enables species of fish, amphibians, birds and mammals to be identified (McColl-Gausden et al. 2021). In general, EnviroDNA's current VERTEBRATE amplicon reference database has good coverage for native and non-native fish, but there are gaps that need to be filled for amphibians and birds, which is an ongoing process. A notable disadvantage of this amplicon is that common species can sometimes prevent rare species from being identified if DNA concentrations differ by many orders of magnitude. If detection of rare species is a priority, then more specific amplicons may be better suited, and these can be run alongside the VERTEBRATE amplicon to maximise coverage. For more detail on the eDNA analysis refer to Appendix D.

Data storage

All eDNA samples collected should be considered an ongoing resource and should therefore be stored appropriately after initial eDNA analyses (storing purified DNA at temperatures at or below -20C). A single water sample typically provides enough DNA for up to 10 independent analyses. These samples represent an "eDNA Biobank" which can be drawn on to screen for other species not considered key assets at present, or as reference samples for before and after studies (e.g., to determine impacts of catastrophic events such as bushfire or flood). EnviroDNA will be able to securely store DNA, and raw and processed sequence data, and assist in data visualisation and interpretation.

Output of Step 5

- A **data table** containing raw data for each environmental indicator for each sample (e.g., a spreadsheet containing the number and type of species detected at each site and assessment unit).
- Optional: A **folder** (stored on a cloud computing resource) containing all raw sequence data and sample information.
- Optional: A **cryopreserved eDNA bank** of excess samples, stored for future use.
- Optional: An **online database** containing processed data in a user-friendly format for visualisation and analysis.

Step 6. Calculate Indicator Condition Scores

Species Richness Indicator Condition Scores (ICS) are calculated for each assessment unit using aggregated site data.

1. *Expected Richness* is the number of expected native species per assessment unit (described in Step 4).
2. *Observed Richness* is the number of observed native species per assessment unit detected by eDNA analysis and aggregated across each site within the assessment unit.
3. ICS are calculated from *Observed Richness* as a percentage of *Expected Richness* (i.e., *Observed Richness* / *Expected Richness* x 100) for each assessment unit.

Output of Step 6

- A **Data Table** (e.g. a spreadsheet) containing counts of native species detected at each assessment unit and percentages of expected species (Indicator Condition Scores)

Worked Example 1 – Aggregating species observation data for an assessment unit

Table 1 on the next page contains a worked example that demonstrates how data is aggregated from site surveys to the assessment unit.

- In this example, the assessment unit is a forested upper-catchment reach in a conservation area. It is a relatively small stretch of river (30km), so five sites are established, spaced 5km apart as per the sampling design instructions in Table 4.
- At each of the five sites, two eDNA samples are taken to maximise the likelihood of detecting all species present.
- The samples are sent to EnviroDNA and analysed in the lab. A species list is produced for each of the five sites (see Table 1).
- An aggregate species list is produced for the assessment unit and *Observed Richness* is calculated (i.e. the sum of observed species).
- A list of *expected species* native to the assessment unit is produced, by reviewing biodiversity databases (e.g. Atlas of Living Australia), published research and from a discussion with a local freshwater ecologist.
- An *Indicator Condition Score* for the assessment unit is calculated as the percentage of observed to expected species (introduced species and native species outside their natural range are excluded).

Table 5. Worked Example One – Aggregating Observation Data from the Site to the Assessment Unit

REFERENCE		OBSERVATION DATA					Assessment Unit ICS		
Pre-1750 species list	Expected richness	Site 1	Site 2	Site 3	Site 4	Site 4	Aggregated Species List	Observed species richness	Indicator Condition Score
Anguilla australis	18	*Anguilla australis*	*Anguilla reinhardtii*	*Anguilla australis*	*Anguilla australis*	*Anguilla australis*	*Anguilla australis*	16	88.9
Anguilla reinhardtii		*Anguilla reinhardtii*	*Carassius auratus*	*Anguilla reinhardtii*	*Anguilla reinhardtii*	*Anguilla reinhardtii*	*Anguilla reinhardtii*		
Carassius auratus		*Carassius auratus*	*Galaxias brevipinnis*	*Carassius auratus*	*Carassius auratus*	*Carassius auratus*	*Carassius auratus*		
Galaxias brevipinnis		*Galaxias brevipinnis*	*Galaxias maculatus*	*Galaxias brevipinnis*	*Anguilla australis*	*Galaxias brevipinnis*	*Galaxias brevipinnis*		
Galaxias maculatus		*Misgurnus anguillicaudatus*	*Galaxias ornatus*	*Prototroctes maraena*	*Limnodynastes peronii*	*Galaxias maculatus*	*Galaxias maculatus*		
Galaxias ornatus		*Nannoperca australis*	*Misgurnus anguillicaudatus*	*Limnodynastes peronii*	*Litoria raniformis*	*Galaxias ornatus*	*Galaxias ornatus*		
Misgurnus anguillicaudatus		*Prototroctes maraena*	*Nannoperca australis*	*Litoria raniformis*	*Hydromys chrysogaster*	*Ornithorhynchus anatinus*	*Misgurnus anguillicaudatus*		
Nannoperca australis		*Limnodynastes peronii*	*Perca fluviatilis*	*Hydromys chrysogaster*	*Ornithorhynchus anatinus*		*Nannoperca australis*		
Perca fluviatilis		*Limnodynastes tasmaniensis*	*Percalates novemaculeata*	*Ornithorhynchus anatinus*			*Perca fluviatilis*		
Percalates novemaculeata		*Litoria ewingii*	*Prototroctes maraena*				*Percalates novemaculeata*		
Prototroctes maraena		*Hydromys chrysogaster*	*Limnodynastes peronii*				*Prototroctes maraena*		
Limnodynastes peronii		*Ornithorhynchus anatinus*	*Limnodynastes tasmaniensis*				*Limnodynastes peronii*		
Limnodynastes tasmaniensis			*Litoria ewingii*				*Limnodynastes tasmaniensis*		
Litoria ewingii			*Hydromys chrysogaster*				*Litoria ewingii*		
Litoria lesueurii							*Hydromys chrysogaster*		
Litoria raniformis							*Ornithorhynchus anatinus*		
Hydromys chrysogaster									
Ornithorhynchus anatinus									

Step 7. Calculate the Econd®

The Econd® is an index between 0 and 100, where 100 describes the 'ideal' or 'undisturbed' reference condition of an environmental asset, and 0 indicates the asset is completely degraded. For this method, the Econd® for native aquatic vertebrate richness is calculated as the length-weighted average of assessment units. If there is only one assessment unit, then the Econd® calculation will be the same as the Indicator Condition Score (see Step 4 on the preceding pages).

Worked Example 2 – Aggregating indicator condition scores for each assessment unit to produce a sub-catchment area Econd® for aquatic vertebrates

Table 2 below shows how the final Econd® for aquatic vertebrates is calculated for the account area.

The same hypothetical project is used for this example. In total, there are three assessment units:

- a forested upper-catchment reach in a conservation area (from example 1),
- a lowland reach used for intensive grazing, and
- a lowland reach where riparian management programs have been implemented.

The final Econd® for the property is calculated as the waterway length weighted average of each assessment unit. While assessment unit one is in the best condition, its relatively small extent means that it contributes less to the overall Econd® for the property.

Table 6. Aggregating data from assessment units to calculate an Econd® for a sub-catchment area.

Assessment Unit	Expected Richness	Observed Richness	Unit ICS	Assessment Unit Length	Length Weight	Area Weighted Score (ICS x Length Weight)	Econd®
1	18	16	88.9	30 km	0.18	16.0	49.1 *= sum of length weighted scores*
2	22	7	37.8	115 km	0.69	26.1	
3	22	9	54.1	21 km	0.13	7.0	

Output of Step 7

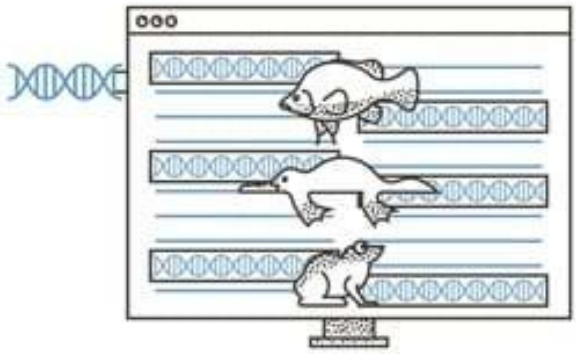
- A **data table** (e.g. a spreadsheet) containing all the raw data for each indicator for each sample, including the calculations for the ICS and Econd®.
- A **summary table** showing the Econd® scores.

3. COMPILE ENVIRONMENTAL ACCOUNT AND SUBMIT FOR CERTIFICATION

Your AFN accredited expert can guide you through this process. For your account to be certified, it must be independently audited or self-assessed and must comply with the *Accounting for Nature® Standard*, which outlines the criteria that must be satisfied. An environmental account may incorporate multiple Environmental Assets (e.g. terrestrial mammals, freshwater and native vegetation), and always needs to include the following information:

- Environmental Account summary;
- Information Statement,
- Environmental Account; and;
- Audit Report or Self-Verification Report (supported by an AfN Technical Assessment).

The data collection and analysis procedures outlined in steps five to seven should be repeated at regular intervals (a minimum of every five years or where Base Year recalculation is required, as specified under the *Accounting for Nature®* Framework) to establish a trend over time. Ideally, surveys should occur annually, even if accounts are only certified once every five years.



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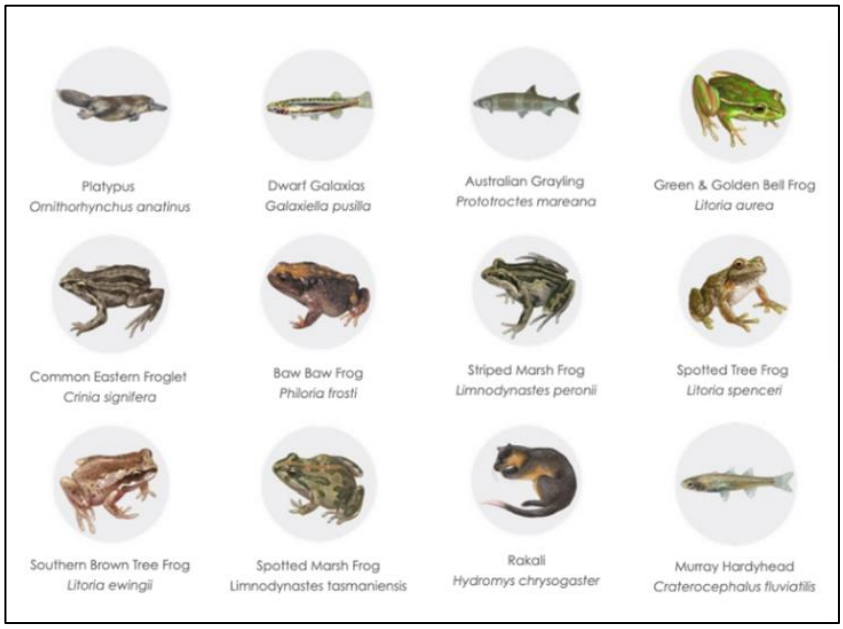
APPENDIX A – ABOUT METABARCODING

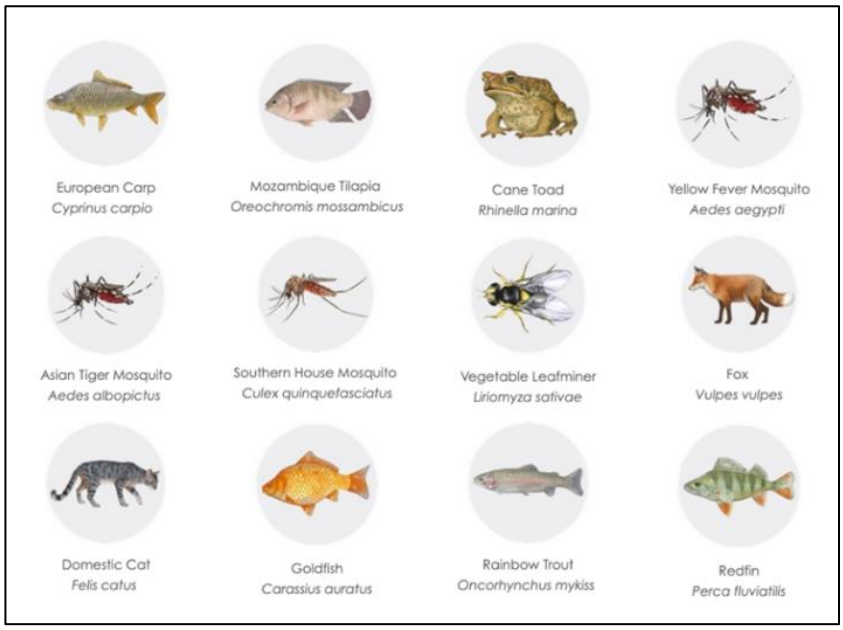
Environmental DNA was first used in the 1980s to study bacterial communities in marine sediments (Ogram, Sayler, and Barkay 1987). Detection of vertebrates from eDNA in water was shown in 2008 (Ficetola et al. 2008). Since then, eDNA has been used broadly to detect threatened (Biggs et al. 2015; Rooyen et al. 2021), native (Lugg et al. 2018) and invasive species (Smart et al. 2015; R. Tingley et al. 2019; Jerde et al. 2011), monitor entire assemblages of biodiversity (Deiner et al. 2016; Valentini et al. 2016; Port et al. 2016), estimate abundance and population size (Sigsgaard et al. 2016; Tillotson et al. 2018), and even population-based metrics such as spawning activity (Bylemans et al. 2017) and population genetic structure (Sigsgaard et al. 2016). New applications continue to emerge as technologies and methods improve. eDNA is now in routine use to address needs in water quality monitoring, environmental impact assessment, and to map, monitor, and manage disease vectors, target species, and, increasingly, biodiversity (Rourke et al. 2021; Lee, Liao, and Hsu 2021; Andruszkiewicz et al. 2017; Sepulveda et al. 2020, 2021; Feist and Lance 2021; Jarman, Berry, and Bunce 2018; Ji et al. 2021; Coleman et al. 2021).



High-throughput DNA sequencing technologies enable all species from one or more target groups (e.g., fish, amphibians, or all vertebrates) to be identified (Taberlet et al. 2018; Pawlowski et al. 2021; Valentini et al. 2016; Ruppert, Kline, and Rahman 2019). This multi-species approach, termed metabarcoding, uses polymerase chain reaction (PCR) primers designed to bind to and amplify small regions of DNA that are conserved across all species in the target group (species barcodes, or amplicons). The ability to distinguish species relies on the presence of a curated reference library matching species to the sequences present in environmental samples, and to the presence of genetic variation in the chosen amplicon that uniquely identifies each species.

If the eDNA of a species is detected in a sample, how recently was the species there? The answer depends on the environment and to some extent on the species in question. In aquatic environments, eDNA in the water column breaks down in a matter of days or weeks (Valentini et al. 2016). Consequently, water samples generally provide up-to-date information about the presence of species, which is critical for conservation and biodiversity management. The distance over which eDNA can be detected also depends on the environment. In a flowing river or stream, eDNA can be transported downstream to areas where a

target species does not occur or diluted to undetectable levels at the point of origin (Pont et al. 2018). Research has shown that eDNA can be transported small distances or even kilometres away from its original source depending on flow, degradation, sedimentation (which can protect eDNA from degradation), resuspension, and other characteristics of waterways, as well as on species identity (Balasingham, Walter, and Heath 2017; Deiner and Altermatt 2014; Harrison, Sunday, and Rogers 2019). Flowing waterways are like conveyor belts of information for the broader landscape (Deiner et al. 2016), allowing species to be detected over larger distances. The many factors that can influence eDNA detection make the development of general rules for site distribution challenging, even for single species, however basic hydrological information such as flow rate and river dimensions are likely to be of use (Harrison, Sunday, and Rogers 2019). If required, finer scale information can still be obtained by sampling more densely along a waterway, using relative DNA concentrations to localise target species. The movement of eDNA in water makes it easier to collect concentrated samples by filtration, increasing the chance of detecting species. The amount of water that can be filtered depends on turbidity and the pore size of the filter used to capture eDNA, however the literature indicates that detection is generally robust across filter pore sizes in the size range of 0.22 uM to 5 uM for targeting aquatic vertebrates (Li et al. 2018; Harper et al. 2020; Coutant et al. 2021).



Importantly, while the advantages of eDNA for biodiversity monitoring are considerable, there is some information it cannot currently provide. eDNA cannot necessarily tell us the sex, size, developmental stage, or health of individuals, or specific habitat associations in waterways. Thus, environmental managers need to carefully consider the questions before deciding on the most appropriate monitoring technique. Currently, eDNA is most useful for determining the presence or absence of a species. While studies have suggested an association between organism abundance and eDNA concentration, there is considerable uncertainty in that relationship. This relationship also has not been estimated for the vast majority of Australian species and environments. Therefore, this Method uses simple, robust observations of site occupancy - the presence/absence of native species at a set of sites - to generate an Econd®.

APPENDIX B - COMPARING EDNA METABARCODING WITH ELECTROFISHING

Species detected in a Victoria catchment from 25 sites (required to be accessible for electrofishing) and three approaches: electrofishing and expert identification, and eDNA using two amplicons (the general vertebrate amplicon proposed in this Method, and a fish specific amplicon). Freshwater fish (target species here) are in bold. Adapted from (McColl-Gausden et al. 2021).

Electrofishing	eDNA (vertebrate)	eDNA (fishfish)
Anguilla australis	***Anguilla australis***	***Anguilla australis***
Carassius auratus	***Anguilla reinhardtii***	***Anguilla reinhardtii***
Cyprinus carpio	***Carassius auratus***	***Carassius auratus***
Gadopsis marmoratus	***Cyprinus carpio***	***Cyprinus carpio***
Galaxias brevipinnis	***Gadopsis marmoratus***	***Gadopsis marmoratus***
Galaxias maculatus	***Galaxias brevipinnis***	***Galaxias brevipinnis***
Galaxias ornatus	***Galaxias maculatus***	***Galaxias maculatus***
Gambusia holbrooki	***Galaxias ornatus***	***Galaxias ornatus***
Hypseleotris sp.	***Galaxias truttaceus***	***Galaxias truttaceus***
Misgurnus anguillicaudatus	***Gambusia holbrooki***	***Galaxiella pusilla***
Nannoperca australis	***Geotria australis***	***Gambusia holbrooki***
Perca fluviatilis	***Hypseleotris sp.***	***Hypseleotris sp.***
Philypnodon grandiceps	***Misgurnus anguillicaudatus***	***Maccullochella peelii***
Prototroctes maraena	***Nannoperca australis***	***Misgurnus anguillicaudatus***
Pseudaphritis urvillii	***Perca fluviatilis***	***Nannoperca australis***
Retropinna semoni	***Percalates novemaculeata***	***Perca fluviatilis***
Rutilus rutilus	***Philypnodon grandiceps***	***Percalates novemaculeata***
Salmo trutta	***Prototroctes maraena***	***Philypnodon grandiceps***
Tinca tinca	***Pseudaphritis urvillii***	***Prototroctes maraena***
Cherax destructor	***Retropinna semoni***	***Pseudaphritis urvillii***
Engaeus cunicularius	***Rutilus rutilus***	***Retropinna semoni***
Euastacus kershawi	***Salmo salar***	***Rutilus rutilus***
Euastacus yarraensis	***Salmo trutta***	***Salmo trutta***
Freshwater mussel	***Tandanus tandanus***	***Tinca tinca***
Hyridella drapeta	***Tinca tinca***	
Mordacia mordax	*Acanthiza lineata*	
Paratya australiensis	*Acanthiza pusilla*	
	Acridotheres tristis	
	Anas platyrhynchos	
	Ardea novaehollandiae	
	Bos taurus	
	Bubulcus ibis	
	Cacatua galerita	

Canis lupus
Capra hircus
Cereopsis novaehollandiae
Cervus elaphus
Chelodina longicollis
Chenonetta jubata
Columba livia
Corvus splendens
Crinia signifera
Cygnus atratus
Dama dama
Emydura subglobosa
Eolophus roseicapillus
Eopsaltria australis
Equus caballus
Eulamprus tympanum
Felis catus
Fulica atra
Gallinula chloropus
Gallus gallus
Geocrinia victoriana
Grallina cyanoleuca
Gymnorhina tibicen
Homo sapiens
Hydromys chrysogaster
Limnodynastes peronii
Limnodynastes tasmaniensis
Litoria ewingii
Litoria lesueurii
Litoria raniformis
Macropus giganteus
Manorina melanocephala
Menura novaehollandiae
Mus musculus
Neobatrachus sudelli
Nycticorax species
Ocyphaps lophotes
Ornithorhynchus anatinus
Oryctolagus cuniculus
Petauroides volans
Petaurus breviceps
Phaps chalcoptera
Platycercus eximius

Porphyrio porphyrio

Psephotus haematonotus

Pseudocheirus peregrinus

Rattus fuscipes

Rattus norvegicus

Rattus rattus

Rusa unicolor

Sericornis frontalis

Streptopelia chinensis

Sturnus vulgaris

Sus scrofa

Trichoglossus moluccans

Trichosurus vulpecula

Turdus merula

Turdus philomelos

Vombatus ursinus

Vulpes vulpes

Wallabia bicolor

APPENDIX C - USING ENVIRODNA'S FIELD SAMPLING KIT

EnviroDNA's single site, 2 sample water filtration kit includes:

- 1 pair of latex gloves
- 2 disc filter units with 1.2 micron pore PES (Polyethersulfone) membrane
- 1 disposable 60ml sampling syringe
- 1 zip lock pouch (BioPE Recyclable) with sample label
- 2 disposable 5ml syringes with sample preservative (10x TE buffer)
- 1 compostable mailer bag for returning to EnviroDNA

ample collection

1. Assess the site for safety and access. Select sampling locations where water is flowing and relatively clear, where possible (enter the water as a last resort).
2. Don latex gloves, open the sampling syringe and first filter unit (avoid touching open ends).
3. Draw up water into the syringe, adjust to 50ml.
4. Screw syringe onto filter, push water through filter, then remove filter.
5. Repeat steps 3-4 until 500ml has been filtered, or filter is clogged.
6. Fill the syringe with air and push this through the filter to expel any remaining water. Return the syringe to its original packing to be reused for the second sample.
7. Screw preservative syringe into filter and expel entire volume, leaving the syringe attached.
8. Return filter and preservative syringe to its labelled packet.
9. Repeat steps 3-8 for the second sample. If possible, take the second sample from a different location at the site (up to 100m apart) to increase representativity.
10. For each sample, record the total volume of water filtered, GPS coordinates, date, and a unique site code (generated during site selection) on the sample label.
11. Return the two labelled filter units to EnviroDNA in the mailer bag.

DNA on filters in preservative solution is stable for up to three weeks at ambient temperatures, but it is preferable that samples are sent for storage and processing to EnviroDNA in batches as they are taken.

APPENDIX D - DNA SEQUENCING AND DATA ANALYSIS

DNA extraction, PCR amplification, sequencing library preparation, sequencing, and data analysis are conducted by EnviroDNA. eDNA samples typically contain low levels of informative DNA, so maintaining rigorous quality control and quality assurance standards is critical to data integrity. EnviroDNA will adhere to best laboratory practices, including segregation of activities in dedicated cleanrooms, will ensure appropriate negative and positive controls are implemented throughout the laboratory workflow, and will apply best practices to sequencing, analysis, and reporting in a transparent manner.

Details of a typical workflow can be found in recent published work (McColl-Gausden et al. 2021). In brief, analysis of eDNA samples involves DNA extraction, amplification of target species barcodes and the addition of sample-specific indexes and sequences required for DNA sequencing, pooling of amplicons from many samples, and DNA sequencing to read out the information present in amplicons required to resolve individual species. Sequencing generates around 100,000 reads per sample (from 2 pooled amplifications), and DNA sequences are then clustered by similarity and aligned against annotated sequences in a reference database to provide a list of detected species for each sample.

For broad monitoring of aquatic vertebrates, a widely used VERTEBRATE amplicon spanning approximately 100 base-pairs of the 12S ribosomal RNA gene region on the mitochondrial genome enables species of fish, amphibians, birds and mammals to be identified (McColl-Gausden et al. 2021). Managers should be aware that the ability to amplify across such a large group can limit specificity for some closely related species (e.g., ducks in the genus Anas have overlapping sequences at this region and so cannot be resolved to the species level). Another disadvantage of this amplicon is that common species can sometimes prevent rare species from being identified if DNA concentrations differ by many orders of magnitude. If detection of rare species is a priority, then more specific amplicons may be better suited. Finally, the breadth of life accessible to eDNA sampling means that gaps in current reference databases may be identified (in general, the current VERTEBRATE amplicon reference database has good coverage for native and non-native fish, but significant gaps that need to be filled for amphibians and birds). Improvement of reference databases is an ongoing process that requires sampling tissues from missing species (e.g., from field or museum collections). Sequencing of tissue samples from local species to supplement the reference database will enhance outputs of any analyses.