Use of eDNA in the assessment of groundwater

August 2024

Prepared by: Judith Webber and Louise Weaver

PREPARED FOR: Tasman District Council

CLIENT REPORT No: FW23032

REVIEWED BY: Annette Bolton

ACKNOWLEDGEMENTS

This review was funded by Envirolink (a small advice grant) under contract number 2323-TSDC168.

Manager		Peer reviewer		Author
				
Theo Sarris		Annette Bolton		Judith Webber
Water and Environment Group Manager		Science Leader		Scientist
Author				
				
Louise Weaver				
Technical Science Lead				

CONTENTS

LIST OF FIGURES

LIST OF TABLES

EXECUTIVE SUMMARY

Freshwater ecosystems are under increasing pressure due to anthropogenic activities such as agricultural practices, urbanisation, and industrial applications. In recent years, there has been an increase in studies investigating the impacts of these practices on the biological communities that live in freshwater ecosystems. While many studies have focused on the biological communities of surface freshwaters, little research has been conducted on groundwater communities due to the inaccessibility of aquifer ecosystems. However, wells drilled into an aquifer can provide a suitable sampling window, allowing for the collection of microorganisms and macrofauna. Macrofauna collected can be identified using traditional identification methods that use visual (morphological) surveys and taxonomic keys. Traditional methods cannot be used to identify microscopic organisms, macrofauna inhabiting the inaccessible aquifer voids and at immature life stages that lack external morphological features. Instead, environmental DNA (eDNA) methodologies have been proposed to identify these organisms by detecting the short DNA fragments left behind in environmental substrates, such as groundwater and sediments (Barnes and Turner, 2016, Sansom and Sassoubre, 2017).

Environmental DNA has many advantages, including identifying taxa without requiring taxonomical expertise, a skill that can be particularly scarce for underground organisms. Moreover, environmental DNA can target a broad set of taxa with only one sample and is a rapid, cost-effective and non-invasive method. Environmental DNA can also detect low-density species, has a large sample processing capacity, and offers a high-throughput system for detecting species' presence and, thus, biological diversity.

While environmental DNA can be a valuable tool in monitoring groundwater species, the current literature highlights that this methodology is still developing, and our understanding of optimally conducting and interpreting environmental DNA results is still progressing. The following report provides an overview of environmental DNA methodology and outlines the advantages and limitations of this method when used in groundwater research.

1 SCOPE

Environmental DNA (eDNA) is increasingly used to assess the biodiversity of organisms inhabiting groundwater ecosystems. However, as a developing methodology, there is often confusion surrounding the interpretation of eDNA results, leading to conflicts among stakeholders.

This report was commissioned to provide clarity and achieve the following:

1. Offer an overview of the use of eDNA in ecological assessments of groundwater.
2. Discuss the advantages and limitations of eDNA methodology.
3. Provide guidance to stakeholders on interpreting eDNA data and draw informed conclusions from the results of this review.

APPROACH

A literature search using the search engines Web of Science, Google Scholar, Google, Science Direct and PubMed and dates between 1980 (the era in which eDNA methods were developed and applied to detect microorganisms in environmental substrates) and 2024 was undertaken. Search terms included "environmental DNA" and "eDNA" with combinations of "traditional assessments", "biodiversity", "taxonomic", and keywords, "freshwater", "groundwater", "subterranean", "aquifers", "ecosystems", "biological", "function", "limitations", "advantages", "Aotearoa", and "New Zealand". The search was repeated using the same terms, omitting "Aotearoa" and "New Zealand" to capture international research. Each publication was read for its applicability to this review and referenced where relevant.

2 GROUNDWATER AND (E)NVIRONMENTAL DNA

Groundwater is a global resource providing essential human services such as drinking water and irrigation. Biological communities (consisting of microorganisms[1], meiofauna and macrofauna) living within groundwater help sustain its quality and health (Mermillod-Blondin et al., 2023). Microorganisms play essential roles in water purification through biogeochemical cycling and degradation of contaminants (Griebler and Avramov, 2015). Stygofauna i.e. microorganisms, macrofauna and meiofauna, enhance aquifer water transmission (Hose and Stumpp, 2019, Stumpp and Hose, 2017), undertake organic matter processing (Kinsey et al., 2007, Simon and Benfield, 2001) and contribute to the subterranean food web (Saccò et al., 2022a). Macrofauna, particularly amphipods, have been used as bioindicators in groundwater monitoring due to their sensitivity to certain anthropogenic pollutants (Koch et al., 2021; Redžović et al., 2023) and their perceived similarity to surface macrofauna. This has recently been brought into question as more recent research has indicated that stygofauna may have a much higher tolerance to contaminants than previously thought (e.g. Di Lorenzo et al., 2021, (Groote-Woortmann et al., 2024).

Anthropogenic pollutants originate from agricultural, forestry and urban activities. These pollutants can cause changes in the physical and chemical properties of groundwater, which may decrease the biodiversity of the biological communities (Chen et al., 2020, Mateos-Cárdenas et al., 2019, NPSFM, 2020, Pawlowski et al., 2018, Sha et al., 2023, Xiu et al., 2020). These changes in biodiversity can compromise ecosystem functions, resulting in a deterioration of groundwater health (Hancock, 2002, Hancock et al., 2005).

Ecosystems with high biodiversity generally have greater stability, are healthier, and are more resilient than ecosystems with low biodiversity. In recent years, there has been an increasing demand to monitor biodiversity and species abundance within freshwater communities. While there has been a large increase in biodiversity monitoring for surface water environments, biological monitoring in groundwater ecosystems is limited due to their inaccessibility (Korbel et al., 2017, Steube et al., 2009). As a result, the biodiversity of groundwater ecosystems is understudied (Couton et al., 2023a).

Groundwater ecosystems can, however, be accessed through wells drilled into the aquifer from which microorganisms, meio- and macrofauna can be collected using pumps and filtering methods (Hahn, 2006, Thulin and Hahn, 2008, Weaver et al., 2021). Macrofauna can be identified by traditional methods using external morphological features and taxonomic keys. While commonly used in surface water biodiversity assessments, these traditional methods are limited in groundwater environments as they require specimens with intact anatomical features (these are frequently damaged during groundwater pumping) (Hahn, 2006). Additionally, traditional methods are time-consuming, require specialised expertise to identify species correctly, are biased toward larger, more abundant taxa, have difficulty identifying juvenile stages that may lack defining morphology and cannot be used to identify microscopic organisms. As such, there has been a drive to adopt alternative methodologies for identifying organisms inhabiting groundwater ecosystems.

Over the last few decades, eDNA and sequencing techniques have been increasingly used to identify species in the environment, including freshwater and, more recently, groundwater (Saccò et al., 2022b, van der Heyde et al., 2023). eDNA detects extracellular DNA or cell debris that organisms leave behind in environmental samples, such as water or sediment (Bohmann et al., 2014, Saccò et al., 2022b). Species presence can, therefore, be detected without the need for an entire specimen. For example, eDNA analysis detected an

[1] Microorganisms consist of bacteria and archaea in this context.

underground species of blind shrimp in bores where specimens had not been previously retrieved (Oberprieler et al., 2021).

eDNA-based methods target specific host DNA using primers[2] (short sequences of DNA that bind to the target DNA). Polymerase chain reaction (PCR) amplifies the DNA to generate thousands to millions of sequences (next-generation sequencing). eDNA metabarcoding[3] enables the characterisation of eDNA from multiple species within a single groundwater sample (Takahashi et al., 2023). Analysis of the sequence data is performed to assign taxonomies to the sequence reads so that individual host organisms can be identified. From this data, species prevalence can be determined, and overall biodiversity can be assessed (Figure 1).

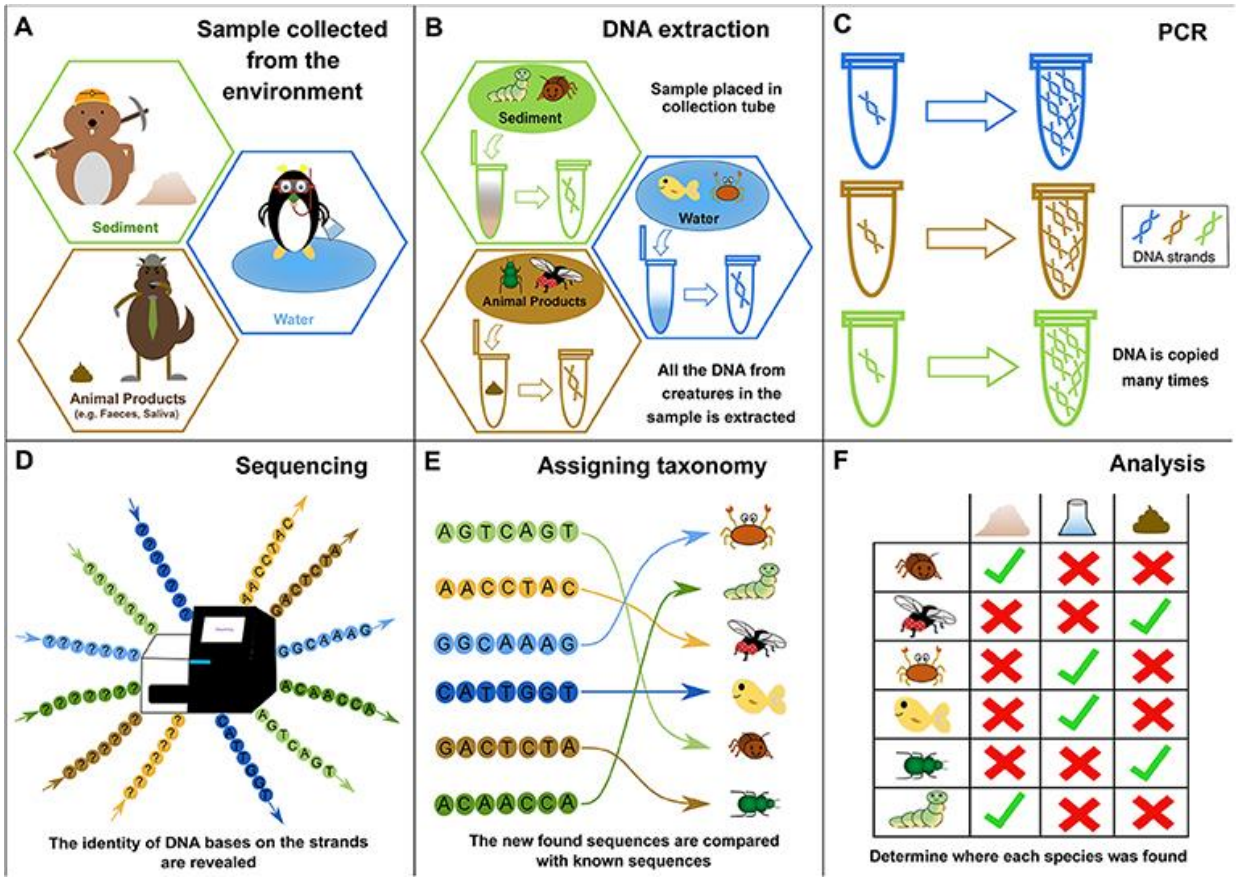

Figure 1. How can environmental DNA be used to identify species?

A) Samples are collected from the environment (e.g., water, soil, animal products). B) eDNA is extracted from the samples. C) Target DNA sequences are multiplied using PCR. D) The sequences are read on a sequencing machine, showing the order of bases on the DNA strands. E) Sequences are then matched to known sequences in worldwide databases. F) A list is produced of species found in that environment (Schallenberg et al., 2020).

[2] Primers are short sequences of DNA that are found in specific organisms (barcoding), groups of organisms or a range of organisms (metabarcoding). The primers bind to specific regions in the target organisms DNA and are then copied (amplified) many times so the organism can be detected. We can think of primers as barcodes that you see on food packaging to identify a product.

[3] Metabarcoding is the use of a primer sequence (or multiple primers) that enables multiple taxa (types of organisms) to be identified in one sample.

Globally, eDNA studies of subterranean ecosystems are still relatively rare, with most investigations being undertaken in caves located in karst aquifers (Table 1).

Table 1. Examples of overseas research using eDNA methodology in subterranean ecosystems

Author and year	Location	Target	Aquifer	Outcome
West et al. (2020)	Australia	Eukaryotes	Karst	Characterised eukaryotic[4] subterranean diversity from sediment and water collected from caves and springs.
Boyd et al. (2020)	Alabama, USA	Rare groundwater crustacean	Karst	Detected crustacean DNA from water samples collected from caves and springs.
Gorički et al. (2017)	Europe	Cryptic cave-dwelling amphibian	Karst	Concluded that eDNA methodology provided a rapid detection of a rare subterranean species inhabiting karst groundwater.
Niemiller et al. (2018)	Washington, USA	Amphipod	Shallow groundwater-fed seepage spring	Demonstrated the ability of eDNA to detect rare and endangered groundwater amphipods.
Vörös et al. (2017)	Croatia, Southeast Europe	Rare and elusive cave-dwelling amphibian	Karst	Confirmed the presence of a cave-dwelling amphibian in ten caves and detected the species for the first time in five others.
White et al. (2020)	Australia	Rare, blind cave eel	Karst	Results demonstrated that the newly designed assays effectively detect this rare and vulnerable subterranean species.
Korbel et al. (2017)	Australia	Community-level detection of groundwater fauna in a large range of habitats and for all life stages	Alluvial	This study used DNA community profiling (metabarcoding) of 16S rDNA and 18S rDNA, combined with traditional stygofauna sampling methods, to characterise groundwater biota from four catchments within eastern Australia.

[4] Eukaryotic – organisms where the cell contains a nucelus and other membrane-bound organelles e.g. Protists, fungi, all animals, plants. Eukaryotes can be single-celled or multicellular.

While studies using eDNA in surface freshwater have been conducted in New Zealand, the use of eDNA in groundwater research is very limited. Table 2 summarises the extent of eDNA studies in groundwater research across New Zealand (to 2023).

Table 2. Examples of New Zealand research using eDNA methodology in subterranean ecosystems

Author and year	Location	Target	Aquifer	Success
University of Auckland-led MBIE Smart Idea research project that included ESR, 2017 to 2020	Auckland, Waikato, Wellington, and Canterbury	Denitrifying microorganisms	Alluvial	Examined the genomic novelty and functional capacity of typical groundwater ecosystems and the impact of nutrient gradients on the groundwater communities.
Fenwick et al. (2021)	North and South Island	Stygofauna	Alluvial	High biodiversity and short-range endemism were found across the North and South Islands.
ESR, 2013 to present	Otago, Canterbury, Takaka, Nelson Waikato, Hamilton, and Auckland	Bacteria, eukaryotes and fungi	Alluvial, marble, fractured basalt and coarse sand	eDNA has been used to monitor the spatial and temporal diversity of microorganisms across New Zealand.

The following sections provide an overview of the advantages and limitations of eDNA-based methods in groundwater ecosystems.

2.1 ADVANTAGES OF ENVIRONMENTAL DNA METHODOLOGY:

eDNA methods are advantageous in biodiversity studies of groundwater ecosystems as multiple species (micro to macrofauna) can be detected simultaneously using a single groundwater sample. Detecting multiple species is advantageous for studying complex aquifer systems where numerous species may co-exist.

The use of wells allows replicate sampling to be undertaken, enabling in-depth studies to capture more accurate data for community analysis and species diversity (Dickie et al., 2018).

Collecting groundwater samples is typically non-invasive and minimally disruptive to the environment (Couton et al., 2023b, Nakagawa et al., 2018, Shaw et al., 2016).

eDNA analysis has benefits for identifying groundwater macrofauna and has been found to detect soft-bodied stygofauna species more efficiently than haul-net sampling (van der Heyde et al., 2023). It is much easier and less time-consuming to compare macrofauna species sequences through online databases rather than define and describe morphological characteristics where highly specialised taxonomic expertise is required (Deiner et al., 2017, Shaw et al., 2016).

In some cases, eDNA can provide higher taxonomic resolution, especially when species cannot be distinguished based on morphological characteristics, such as microorganisms and juvenile life stages of some macrofauna species.

eDNA methodology can detect very small protozoans as well as rare and inconspicuous species in groundwater ecosystems (Shogren et al., 2018) and can clarify the genetic difference between morphologically similar specimens (Zakšek et al., 2007).

2.2 LIMITATIONS OF ENVIRONMENTAL DNA METHODOLOGY:

While eDNA can offer valuable insights, it is essential to recognise its constraints. Two important errors in eDNA methodology are false positives and false negatives.

2.2.1 False positive errors

False positives occur when the species is “detected” but is not present in the ecosystem. They can result from (1) contamination, (2) incomplete reference databases, or (3) non-specific amplification.

(1) Field sampling is often undertaken in non-sterile and challenging conditions. DNA can easily be transferred to a sample from aerosols (microscopic droplets of fluid), skin, gloves, clothing, shoes, and environmental influences. Groundwater samples typically have very small amounts of DNA, and any contamination will significantly affect the sequencing results.

(2) The lack of reference sequence availability in public databases is a challenge for studies using eDNA (Couton et al., 2023a, Korbel et al., 2017, Korbel et al., 2022, Oberprieler et al., 2021, West et al., 2020). Public databases do not contain sequences for all groundwater species, as many have yet to be identified (Couton et al., 2023a). False positive identification of a species can occur when an unknown sequence cannot be matched exactly to a sequence in the database and is instead assigned to the closest matching sequence, which can sometimes be a completely different organism. To mitigate the occurrence of a false positive error, specific criteria are used so that

only sequences with a defined similarity (e.g., 97% alignment at the species level for bacteria) are considered.

(3) eDNA samples contain the DNA of many organisms, and the slightest amplification of any non-target organism's DNA has the potential for non-specific false positives.

2.2.2 False negative errors

False negatives occur when taxa are present in the ecosystem but not detected and can result from (1) low DNA yield, (2) non-specific primer design and lack of suitable primers, (3) inhibition and (4) degraded DNA.

(1) eDNA is typically present at low concentrations in groundwater samples and the eDNA of interest may not be captured during sample collection.

(2) The accuracy of eDNA metabarcoding relies heavily on the choice of primers for PCR amplification. If incorrect or poorly designed primers are used, the target DNA will not be amplified, resulting in a false negative error. Additionally, there is a lack of suitable primers for groundwater organisms (Saccò et al., 2022b), especially macrofauna, which are generally not well-amplified by primers traditionally used in metabarcoding (Brantschen et al., 2022; Elbrecht and Leese, 2017; Leray et al., 2013).

(3) Humic and non-humic substances can inhibit PCR amplification, leading to a failure of DNA amplification (Opel et al., 2010, Williams et al., 2017).

(4) Degraded eDNA is often highly fragmented, which affects the efficiency of PCR amplification. DNA can rapidly degrade to sub-detectable levels within days to weeks due to environmental factors such as temperature, pH, microbial activity (including predation), microbial enzymes (nucleases), and oxygenation reactions (Shogren et al., 2018).

2.2.3 Bias

Sampling methods may bias the types of organisms collected. For example, when pumping water from a well, the sample may be biased against the larger, sessile, or more active macrofauna species that may evade extraction by the pump.

The depth at which samples are taken can also cause bias as species richness decreases with depth due to the lower levels of oxygen and nutrients in deeper groundwater.

Deposits of organic sediments can result in a disproportionate representation of single-source DNA material from dominant taxa if sampling is concentrated around these sediments.

Different bacterial groups (gram-negative, gram-positive) may resist the chemical agents applied during DNA extraction (von Wintzingerode et al., 1997). In some cases, the chemical agents cannot completely break the bacterial cell walls, preventing the DNA from being released into the solution. In contrast, the chemical agents might lyse the cells well but damage the DNA. These factors could lead to underestimating or overestimating different species present in the groundwater community (Mthethwa et al., 2022, Shapiro et al., 2019, Valeix et al., 2020).

2.2.4 Other limitations

eDNA does not provide phenotypic information (e.g., sex, life stage) nor differentiate between DNA from live and dead organisms, as both shed DNA into the environment, contributing to the eDNA pool. Additionally, while eDNA methodology aids in identifying the different species found in a groundwater community, it cannot determine how many individual members of that species are present in the ecosystem (i.e., their abundance). Even if more eDNA from a particular species is found, that does not necessarily mean more organisms are present. As a

result, eDNA cannot be relied upon to accurately assess the number of organisms in a community. However, estimations of relative abundance by eDNA can be helpful when evaluating multiple and temporal samples, providing researchers with an understanding of a species' contribution to a groundwater ecosystem (Couton et al., 2023b).

The limited understanding of local hydrogeology at most sampling locations complicates the interpretation of eDNA detections. eDNA can be transported through an aquifer system via the passive movement of intra-, extracellular, or particle-bound DNA in the environment (e.g., by flow). As such, eDNA can be sampled at a different place than where it was produced, affecting inferences about fine-scale spatiotemporal trends in species and communities (Deiner et al., 2017, Eichmiller et al., 2016, Goldberg et al., 2016, Hering et al., 2018, Pawlowski et al., 2020, Taberlet et al., 2012).

Groundwater organisms vary within an aquifer system, with a high endemism. Therefore, eDNA detected at one sampling well may not be indicative of the entire aquifer, requiring a larger number of sampling points to obtain representative results (Gibert and Deharveng, 2002, Hahn and Matzke, 2005, Mösslacher, 1998, Thulin and Hahn, 2008).

The description of new species, which generally depends on the identification of actual specimens using traditional morphological methods, is limited due to the numerous undescribed species in the subterranean environment (Couton et al., 2023b, Goldberg et al., 2020). Undescribed species are present in databases due to the lack of taxonomic expertise required to identify them morphologically.

Estimating DNA longevity and persistence in environments is difficult. DNA can absorb onto particles, such as sands, clays, and minerals, which protect eDNA from nuclease degradation. This allows the DNA to persist for longer in the environment, making it harder to determine reliably if the detected DNA belongs to recent or historical populations (Foucher et al., 2020).

Results can be confusing to interpret, for example, when soil organisms such as earthworms or above-ground creatures such as spiders are detected in a groundwater sample. Therefore, eDNA results must be used with expert knowledge of the taxa and their biology. In some cases, it may be concluded that the genetic traces of surface-dwelling organisms have likely been washed down into the groundwater by rain (Couton et al., 2023a).

It can be difficult to extract enough DNA from groundwater organisms with delicate exoskeletons that degrade relatively quickly and are often present in small numbers (Perina et al., 2018).

Some organisms shed low amounts of DNA, resulting in low DNA concentrations and low detection rates. (Deiner and Altermatt, 2014, Korbel et al., 2024). Crustaceans shed less DNA in their environment than soft-bodied organisms, which may cause biased results against these taxa (Andruszkiewicz Allan et al., 2021).

3 DISCUSSION

eDNA is highly sensitive and allows for cost-efficient, rapid, and non-invasive assessment of species biodiversity. It is increasingly used in short- and long-term biomonitoring programs, biodiversity assessments, and conservation (Lodge, 2022). Within groundwater ecosystems, eDNA can detect traces of DNA left behind by large, active macrofauna, even if they occur at low densities. Taking groundwater samples for eDNA metabarcoding is faster, less work-intensive to process, and is thus easier to scale up spatially (i.e., sample at the regional or national level) and temporally (e.g., yearly monitoring) than the traditional biodiversity assessment methods. Moreover, it gives information on the whole community, not only a restricted set of species of interest. However, eDNA appears to be not as efficient as traditional sampling in detecting rare macrofauna (Keck et al., 2022) and fails to detect some key organisms, such as amphipods or syncarids that are collected with net samples (Korbel et al., 2017). Several reasons have been suggested to explain these discrepancies, either related to the nature of eDNA (e.g., shedding rates, eDNA degradation, low concentration of eDNA) or to technical difficulties (e.g., primer bias, sampling bias) (Andruszkiewicz Allan et al., 2021, Brantschen et al., 2022). Many studies suggest that eDNA and traditional morphological identification methods should be integrated when identifying macrofauna specimens, as this would allow both methods' benefits without being hindered by the drawbacks of using only one. More effort is also required to improve specific aspects of DNA methodology, including reducing contamination, improving database references and PCR design, and gaining a more thorough understanding of DNA's behaviour in groundwater ecosystems.

3.1 REDUCTION IN CONTAMINATION

There is a high likelihood of contamination influencing the DNA result, especially during non-sterile field sampling, a common cause of false positive results. Negative controls should be included simultaneously with the field sampling to assess for possible contamination leading to false positives. An appropriate field negative control for microorganisms is a clean, ultra-pure water sample brought into the field and filtered at the same time and location as the collected field samples (Sepulveda et al., 2020). However, it is more difficult to control for net and pump samples as, inevitably, the environment cannot be sterilised, and there are pathways from which surface organism DNA can enter groundwater.

3.2 DATABASES

False positives due to incomplete reference databases are also a major challenge (Abbott et al., 2021), with the gaps in reference databases especially pronounced in under-studied ecosystems such as groundwater (Weigand et al., 2019). Expanding the reference databases for groundwater species would increase precise taxonomic assignment and knowledge, reducing the likelihood of assigning a sequence to an incorrect species (Emilson et al., 2017, Maggia et al., 2017, Yang et al., 2017). Globally, work is underway to help improve the gaps in online databases. For example, an online database dedicated to subterranean taxa, called Stygofauna Mundi, is in development (Martinez et al., 2018), which could stimulate the discovery of new species and help future eDNA metabarcoding works. In 2024, researchers from the University of Waikato, ESR, Massey University, Wilderlab, the University of Auckland, and NIWA undertook a project to optimise the extraction of DNA and generate barcodes of recent and historical New Zealand stygofauna that could be deposited into online databases (van der Reis et al., 2024).

3.3 PRIMER DESIGN[5]

A molecular primer is a short sequence of nucleotides (usually DNA or RNA) that serves as a starting point for DNA synthesis. Synthetic oligonucleotide primers are designed to amplify a region of DNA on a sequence of interest during processes like polymerase chain reaction (PCR) or DNA replication. Primers are designed to be complementary to specific sequences on a target DNA strand, allowing enzymes like DNA polymerase to bind and begin copying the DNA at the correct location. Primers for eDNA metabarcoding need to be short enough to amplify degraded samples, identical within but variable between species, and flanked by highly conserved regions to amplify a variety of species without sacrificing the specificity of the target group (Epp et al., 2012). Primer choice has the potential to bias results by preferentially amplifying some target sequences more than others, as well as amplifying non-target groups (Cristescu, 2014). One potential solution is using multiple primer sets, particularly evolutionarily independent primer sets coinciding with standardised barcodes for the target taxonomic groups (Drummond et al., 2015). Although it can reduce primer bias and increase taxonomic coverage, this method suffers from being more costly and time-consuming (Alberdi et al., 2018, Creer et al., 2016, Cristescu, 2014). Another important factor in PCR and primer design is in replicates; multiple PCR replicates increase species detection and decrease the likelihood of false negatives, but the number of replicates used often differs between studies and depends on detection probabilities, research objectives, sequencing depth, primer choice, cost constraints, and the sequencing platform (Alberdi et al., 2018, Ficetola et al., 2015).

3.4 LOW-YIELD EDNA

Increasing the number of collected groundwater samples (replicates) could enhance the likelihood of capturing eDNA. Large volumes of water may need to be filtered (compared to surface freshwater) (Couton et al., 2023a) since the genetic material in groundwater is even more diluted.

A recent mesocosm study comparing recovery of (e)DNA from a range of specimens found that crustacean species DNA was detected sporadically and unpredictably compared to non-crustacean stygofauna. They suggest that crustacean species shed very little DNA compared to other taxa (Korbel et al 2024).

The number of replicates taken at a sampling point has been found to impact the detection of taxa (Rees et al., 2014, Shaw et al., 2016, Takahashi et al., 2023). In a groundwater ecosystem, replicates are particularly crucial due to the inherent randomness of sampling heterogeneous aquifer habitats and the possibility of uneven distribution of eDNA molecules (Hunter et al., 2015).

In New Zealand, eDNA metabarcoding validation trials have shown that six groundwater sample replicates at a single site for macrofauna provide the most robust data (Melchior and Baker, 2023).

3.5 EDNA PERSISTENCE

A better understanding of DNA longevity and persistence in groundwater ecosystems will help improve our knowledge and understanding of the origin and fate of eDNA in groundwater, the likelihood of its detection over time, and the reliability of eDNA as an indicator.

[5] Primers are short sequences of DNA or RNA that are specific to an organism/group of organisms or a wide range of taxa. Primers initial building of DNA/RNA within an organism and are used to bind specifically to DNA/RNA found and can then be amplified (copied) many times.
Primer design is undertaken in Laboratories to be used in molecular methods such as sequencing of eDNA. To design a primer, DNA is scanned to look for specific and unique regions that the primer will bind to. The primer is then manufactured and used in sequencing.

3.6 FOREIGN DNA

Other challenges of eDNA methodology include the difficulty of interpreting whether DNA truly originates from the sample or has migrated into the groundwater ecosystem from another location. For example, eDNA from surface-dwelling organisms can be washed from surface locations into deeper subterranean ecosystems. As such, interpreting eDNA results should consider all available biological information on habitat ecology, eDNA behaviour, and the transport of DNA through the aquifer system.

3.7 EDNA APPLICATIONS IN NEW ZEALAND

eDNA methodology has many uses, including in the conservation of New Zealand's endemic and distinctive flora and fauna. For example, eDNA is used to assess declines in biodiversity due to habitat loss and fragmentation, invasive species, disease, hunting, and climate change. Most New Zealand eDNA studies have been conducted on lake and river water and sediments to detect tuna, crayfish, and mussels to preserve their cultural value and ecological importance (Thomson-Laing et al., 2021). eDNA has also been used in biosecurity efforts to detect pests and other unwanted species (De Brauwer et al., 2023) and to examine distribution or biogeographical patterns of organisms of interest (von Ammon et al., 2023). Commercial companies such as Wilderlab and Cawthron now offer eDNA analysis to detect thousands of species of fish, macroinvertebrates, birds, mammals, reptiles, amphibians, plants, fungi, protists, bacteria, and other organisms.

While research using molecular techniques to assess the biodiversity of groundwater across New Zealand is increasing, only a small percentage of this has been submitted for scientific publication. The largest New Zealand study using molecular techniques (*not* eDNA) from Fenwick et al. (2021), who researched the diversity of New Zealand groundwater crustaceans. They collected 186 amphipods and 42 isopods from wells, of which DNA from 154 of these specimens was successfully sequenced and identified as amphipods or isopods (68% overall success rate).

The use of eDNA in groundwater samples has recently been compared with taxonomic identification in New Zealand through funding at ESR (Bolton and Weaver, 2023). Initial analysis points to a difference in the diversity and abundance of species when using traditional taxonomic identification compared to eDNA sequencing which are similar findings to studies overseas. This research is in preparation for publication at present, but the data suggests that taxonomic identification has a bias to larger, crustacean taxa whereas eDNA has a bias to smaller single cellular eukaryotic taxa such as Protists and soft-bodied taxa such as worms.

4 CONCLUSION

eDNA is a new and rapidly developing field that is opening new avenues for biodiversity assessments in groundwater and emerging as a powerful tool for ecological research and environmental monitoring. eDNA is a non-invasive and cost-effective method of identifying species in their natural habitat. The ability of eDNA to detect cryptic and rare groundwater species and its use as a bioindicator of ecosystem health are some of the advantages of eDNA. eDNA is not, however, without limitations and in order to effectively interpret eDNA data, it is important to understand the key factors outlined in Section 3, such as contamination sources, database completeness, primer design and use, eDNA quality and quantity, and eDNA persistence and transport.

While continued advancements and decreasing costs of eDNA make it a useful method for assessing groundwater biodiversity, it is still an emerging technology. More research is therefore needed before it can be confidently implemented as a routine sampling method for assessing groundwater biodiversity and be included into New Zealand groundwater policy and planning documentation.

5 REFERENCES

ABBOTT, C., COULSON, M., GAGNÉ, N., LACOURSIÈRE-ROUSSEL, A., PARENT, G. J., BAJNO, R., DIETRICH, C. & MAY-MCNALLY, S. 2021. *Guidance on the use of targeted environmental DNA (eDNA) analysis for the management of aquatic invasive species and species at risk*, Canadian Science Advisory Secretariat (CSAS) Ottawa, ON, Canada.

ALBERDI, A., AIZPURUA, O., GILBERT, M. T. P. & BOHMANN, K. 2018. Scrutinising key steps for reliable metabarcoding of environmental samples. *Methods in Ecology and Evolution,* 9**,** 134-147.

ANDRUSZKIEWICZ ALLAN, E., ZHANG, W. G., C LAVERY, A. & F GOVINDARAJAN, A. 2021. Environmental DNA shedding and decay rates from diverse animal forms and thermal regimes. *Environmental DNA,* 3**,** 492-514.

BARNES, M. A. & TURNER, C. R. 2016. The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics,* 17**,** 1-17.

BOHMANN, K., EVANS, A., GILBERT, M. T. P., CARVALHO, G. R., CREER, S., KNAPP, M., DOUGLAS, W. Y. & DE BRUYN, M. 2014. Environmental DNA for wildlife biology and biodiversity monitoring. *Trends in Ecology and Evolution,* 29**,** 358-367.

BOLTON, A., & WEAVER, L., (2023), Preliminary Assessment of Macrofauna, Meiofauna and Bacterial Communities within Otago Groundwater Aquifers, A report prepared for Otago Regional Council, ESR Report Number CSC23013.

BOYD, S. H., NIEMILLER, K. D. K., DOOLEY, K. E., NIX, J. & NIEMILLER, M. L. 2020. Using environmental DNA methods to survey for rare groundwater fauna: Detection of an endangered endemic cave crayfish in northern Alabama. *PLoS One,* 15**,** e0242741.

BRANTSCHEN, J., PELLISSIER, L., WALSER, J. C. & ALTERMATT, F. 2022. Evaluation of primer pairs for eDNA-based assessment of Ephemeroptera, Plecoptera, and Trichoptera across a biogeographically diverse region. *Environmental DNA,* 4**,** 1356-1368.

CHEN, L., ZHANG, J., DAI, H., HU, B. X., TONG, J., GUI, D., ZHANG, X. & XIA, C. 2020. Comparison of the groundwater microbial community in a salt-freshwater mixing zone during the dry and wet seasons. *Journal of Environmental Management,* 271**,** 110969.

COUTON, M., HÜRLEMANN, S., STUDER, A., ALTHER, R. & ALTERMATT, F. 2023a. Groundwater environmental DNA metabarcoding reveals hidden diversity and reflects land-use and geology. *Molecular Ecology,* 32**,** 3497-3512.

COUTON, M., STUDER, A., HÜRLEMANN, S., LOCHER, N., KNÜSEL, M., ALTHER, R. & ALTERMATT, F. 2023b. Integrating citizen science and environmental DNA metabarcoding to study biodiversity of groundwater amphipods in Switzerland. *Scientific Reports,* 13**,** 18097.

CREER, S., DEINER, K., FREY, S., PORAZINSKA, D., TABERLET, P., THOMAS, W. K., POTTER, C. & BIK, H. M. 2016. The ecologist's field guide to sequence-based identification of biodiversity. *Methods in Ecology and Evolution,* 7**,** 1008-1018.

CRISTESCU, M. E. 2014. From barcoding single individuals to metabarcoding biological communities: towards an integrative approach to the study of global biodiversity. *Trends in Ecology and Evolution,* 29**,** 566-571.

DE BRAUWER, M., CLARKE, L. J., CHARITON, A., COOPER, M. K., DE BRUYN, M., FURLAN, E., MACDONALD, A. J., ROURKE, M. L., SHERMAN, C. D. & SUTER, L. 2023. Best practice guidelines for environmental DNA biomonitoring in Australia and New Zealand. *Environmental DNA,* 5**,** 417-423.

DEINER, K. & ALTERMATT, F. 2014. Transport distance of invertebrate environmental DNA in a natural river. *PloS one,* 9**,** e88786.

DEINER, K., BIK, H. M., MÄCHLER, E., SEYMOUR, M., LACOURSIÈRE-ROUSSEL, A., ALTERMATT, F., CREER, S., BISTA, I., LODGE, D. M. & DE VERE, N. 2017. Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. *Molecular Ecology,* 26**,** 5872-5895.

DICKIE, I. A., BOYER, S., BUCKLEY, H. L., DUNCAN, R. P., GARDNER, P. P., HOGG, I. D., HOLDAWAY, R. J., LEAR, G., MAKIOLA, A. & MORALES, S. E. 2018. Towards robust and repeatable sampling methods in eDNA-based studies. *Molecular Ecology Resources,* 18**,** 940-952.

GROOTE-WOORTMANN, W., KORBEL, K. & HOSE, G. C. 2024. STYGOTOX: A Quality-Assessed Database of (Eco) Toxicological Data on Stygofauna and Other Aquatic Subterranean Organisms. *Environmental Toxicology and Chemistry*.

DRUMMOND, A. J., NEWCOMB, R. D., BUCKLEY, T. R., XIE, D., DOPHEIDE, A., POTTER, B. C., HELED, J., ROSS, H. A., TOOMAN, L. & GROSSER, S. 2015. Evaluating a multigene environmental DNA approach for biodiversity assessment. *GigaScience,* 4**,** s13742-015-0086-1.

EICHMILLER, J. J., MILLER, L. M. & SORENSEN, P. W. 2016. Optimising techniques to capture and extract environmental DNA for detection and quantification of fish. *Molecular Ecology Resources,* 16**,** 56-68.

ELBRECHT, V. & LEESE, F. 2017. Validation and development of COI metabarcoding primers for freshwater macroinvertebrate bioassessment. *Frontiers in Environmental Science,* 5**,** 11.

EMILSON, C. E., THOMPSON, D. G., VENIER, L. A., PORTER, T. M., SWYSTUN, T., CHARTRAND, D., CAPELL, S. & HAJIBABAEI, M. 2017. DNA metabarcoding and morphological macroinvertebrate metrics reveal the same changes in boreal watersheds across an environmental gradient. *Scientific Reports,* 7**,** 12777.

EPP, L. S., BOESSENKOOL, S., BELLEMAIN, E. P., HAILE, J., ESPOSITO, A., RIAZ, T., ERSEUS, C., GUSAROV, V. I., EDWARDS, M. E. & JOHNSEN, A. 2012. New environmental metabarcodes for analysing soil DNA: potential for studying past and present ecosystems. *Molecular Ecology,* 21**,** 1821-1833.

FENWICK, G. D., GREENWOOD, M., WILLIAMS, E., MILNE, J. R. & WATENE-RAWIRI, E. M. 2018. *Groundwater Ecosystems: Functions, values, impacts and management*, Horizons Regional Council.

FENWICK, G. D., GREENWOOD, M. J., HOGG, I. D. & MEYER, S. J. 2021. High diversity and local endemism in Aotearoa New Zealand's groundwater crustacean fauna. *Ecology and Evolution,* 11**,** 15664-15682.

FICETOLA, G. F., PANSU, J., BONIN, A., COISSAC, E., GIGUET-COVEX, C., DE BARBA, M., GIELLY, L., LOPES, C. M., BOYER, F. & POMPANON, F. 2015. Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. *Molecular Ecology Resources,* 15**,** 543-556.

FOUCHER, A., EVRARD, O., FICETOLA, G. F., GIELLY, L., POULAIN, J., GIGUET-COVEX, C., LACEBY, J. P., SALVADOR-BLANES, S., CERDAN, O. & POULENARD, J. 2020. Persistence of environmental DNA in cultivated soils:

implication of this memory effect for reconstructing the dynamics of land use and cover changes. *Scientific Reports,* 10**,** 10502.

GIBERT, J. & DEHARVENG, L. 2002. Subterranean Ecosystems: A Truncated Functional Biodiversity: This article emphasises the truncated nature of subterranean biodiversity at both the bottom (no primary producers) and the top (very few strict predators) of food webs and discusses the implications of this truncation both from functional and evolutionary perspectives. *BioScience,* 52**,** 473-481.

GOLDBERG, C. S., TURNER, C. R., DEINER, K., KLYMUS, K. E., THOMSEN, P. F., MURPHY, M. A., SPEAR, S. F., MCKEE, A., OYLER-MCCANCE, S. J. & CORNMAN, R. S. 2016. Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution,* 7**,** 1299-1307.

GOLDBERG, L., LAGOMASINO, D., THOMAS, N. & FATOYINBO, T. 2020. Global declines in human-driven mangrove loss. *Global Change Biology,* 26**,** 5844-5855.

GORIČKI, Š., STANKOVIĆ, D., SNOJ, A., KUNTNER, M., JEFFERY, W. R., TRONTELJ, P., PAVIĆEVIĆ, M., GRIZELJ, Z., NĂPĂRUŞ-ALJANČIČ, M. & ALJANČIČ, G. 2017. Environmental DNA in subterranean biology: range extension and taxonomic implications for Proteus. *Scientific Reports,* 7**,** 45054.

GRIEBLER, C. & AVRAMOV, M. 2015. Groundwater ecosystem services: a review. *Freshwater Science,* 34**,** 355-367.

GROOTE-WOORTMANN, W., KORBEL, K. & HOSE, G. C. 2024. STYGOTOX: A Quality-Assessed Database of (Eco) Toxicological Data on Stygofauna and Other Aquatic Subterranean Organisms. *Environmental Toxicology and Chemistry*.

HAHN, H. J. 2006. The GW-Fauna-Index: A first approach to a quantitative ecological assessment of groundwater habitats. *Limnologica,* 36**,** 119-137.

HAHN, H. J. & MATZKE, D. 2005. A comparison of stygofauna communities inside and outside groundwater bores. *Limnologica,* 35**,** 31-44.

HANCOCK, P. J. 2002. Human impacts on the stream–groundwater exchange zone. *Environmental Management,* 29**,** 763-781.

HANCOCK, P. J., BOULTON, A. J. & HUMPHREYS, W. F. 2005. Aquifers and hyporheic zones: Towards an ecological understanding of groundwater. *Hydrogeology Journal,* 13**,** 98-111.

HERING, D., BORJA, A., JONES, J. I., PONT, D., BOETS, P., BOUCHEZ, A., BRUCE, K., DRAKARE, S., HÄNFLING, B. & KAHLERT, M. 2018. Implementation options for DNA-based identification into ecological status assessment under the European Water Framework Directive. *Water Research,* 138**,** 192-205.

HOSE, G. C. & STUMPP, C. 2019. Architects of the underworld: bioturbation by groundwater invertebrates influences aquifer hydraulic properties. *Aquatic Sciences,* 81**,** 1-9.

HUNTER, M. E., OYLER-MCCANCE, S. J., DORAZIO, R. M., FIKE, J. A., SMITH, B. J., HUNTER, C. T., REED, R. N. & HART, K. M. 2015. Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive Burmese pythons. *PloS one,* 10**,** e0121655.

KECK, F., BLACKMAN, R. C., BOSSART, R., BRANTSCHEN, J., COUTON, M., HÜRLEMANN, S., KIRSCHNER, D., LOCHER, N., ZHANG, H. & ALTERMATT, F. 2022. Meta-analysis shows both congruence and complementarity of DNA and eDNA metabarcoding to traditional methods for biological community assessment. *Molecular Ecology,* 31**,** 1820-1835.

KINSEY, J., COONEY, T. J. & SIMON, K. S. 2007. A comparison of the leaf shredding ability and influence on microbial films of surface and cave forms of Gammarus minus Say. *Hydrobiologia,* 589**,** 199-205.

KOCH, F., MENBERG, K., SCHWEIKERT, S., SPENGLER, C., HAHN, H. J. & BLUM, P. 2021. Groundwater fauna in an urban area–natural or affected? *Hydrology and Earth System Sciences,* 25**,** 3053-3070.

KORBEL, K., CHARITON, A., STEPHENSON, S., GREENFIELD, P. & HOSE, G. C. 2017. Wells provide a distorted view of life in the aquifer: implications for sampling, monitoring and assessment of groundwater ecosystems. *Scientific Reports,* 7**,** 40702.

KORBEL, K., HOSE, G., KARWAUTZ, C., GREENFIELD, P., WANG, H., CHARITON, A. & GRIEBLER, C. 2024. Detection, movement and persistence of invertebrate eDNA in groundwater. *Scientific Reports,* 14**,** 17151.

KORBEL, K., RUTLIDGE, H., HOSE, G., EBERHARD, S. & ANDERSEN, M. 2022. Dynamics of microbiotic patterns reveal surface water groundwater interactions in intermittent and perennial streams. *Science of the Total Environment,* 811**,** 152380.

LERAY, M., YANG, J. Y., MEYER, C. P., MILLS, S. C., AGUDELO, N., RANWEZ, V., BOEHM, J. T. & MACHIDA, R. J. 2013. A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. *Frontiers in Zoology,* 10**,** 1-14.

LODGE, D. M. 2022. Policy action needed to unlock eDNA potential. *Frontiers in Ecology and the Environment,* 20**,** 448-449.

MAGGIA, M., VIGOUROUX, Y., RENNO, J.-F., DUPONCHELLE, F., DESMARAIS, E., NÚÑEZ, J., GARCÍA-DÁVILA, C., CARVAJAL-VALLEJOS, F., PARADIS, E. & MARTIN, J.-F. 2017. DNA metabarcoding of Amazonian ichthyoplankton swarms. *PLoS One,* 12**,** e0170009.

MARMONIER, P., MAAZOUZI, C., BARAN, N., BLANCHET, S., RITTER, A., SAPLAIROLES, M., DOLE-OLIVIER, M.-J., GALASSI, D. M., EME, D. & DOLÉDEC, S. 2018. Ecology-based evaluation of groundwater ecosystems under intensive agriculture: A combination of community analysis and sentinel exposure. *Science of the Total Environment,* 613**,** 1353-1366.

MARTINEZ, A., ANICIC, N., CALVARUSO, S., SANCHEZ, N., PUPPIENI, L., SFORZI, T., ZAUPA, S., ALVAREZ, F., BRANKOVITS, D. & GĄSIOROWSKI, L. A new insight into the Stygofauna Mundi: assembling a global dataset for aquatic fauna in subterranean environments. ARPHA conference abstracts, 2018. Pensoft Publishers, e29514.

MATEOS-CÁRDENAS, A., SCOTT, D. T., SEITMAGANBETOVA, G., VAN PELT FRANK, N. & AK, J. M. 2019. Polyethylene microplastics adhere to *Lemna minor* (L.), yet have no effects on plant growth or feeding by *Gammarus duebeni* (Lillj.). *Science of the Total Environment,* 689**,** 413-421.

MELCHIOR, M. & BAKER, C. 2023. eDNA Guidelines and field protocols for lotic systems. NIWA.

MERMILLOD-BLONDIN, F., HOSE, G. C., SIMON, K. S., KORBEL, K., AVRAMOV, M. & VANDER VORSTE, R. 2023. Role of invertebrates in groundwater ecosystem processes and services. *Groundwater Ecology and Evolution.* Elsevier.

MÖSSLACHER, F. 1998. Subsurface dwelling crustaceans as indicators of hydrological conditions, oxygen concentrations, and sediment structure in an alluvial aquifer. *International Review of Hydrobiology,* 83**,** 349-364.

MTHETHWA, N., AMOAH, I., REDDY, P., BUX, F. & KUMARI, S. 2022. Development and evaluation of a molecular-based protocol for detection and quantification of Cryptosporidium spp. in wastewater. *Experimental Parasitology,* 234**,** 108216.

NAKAGAWA, H., YAMAMOTO, S., SATO, Y., SADO, T., MINAMOTO, T. & MIYA, M. 2018. Comparing local-and regional-scale estimations of the diversity of stream fish using eDNA metabarcoding and conventional observation methods. *Freshwater Biology,* 63**,** 569-580.

NIEMILLER, M. L., PORTER, M. L., KEANY, J., GILBERT, H., FONG, D. W., CULVER, D. C., HOBSON, C. S., KENDALL, K. D., DAVIS, M. A. & TAYLOR, S. J. 2018. Evaluation of eDNA for groundwater invertebrate detection and monitoring: a case study with endangered Stygobromus (Amphipoda: Crangonyctidae). *Conservation Genetics Resources,* 10**,** 247-257.

NPSFM 2020. National Policy Statement for Freshwater Management 2020, amended February 2023.

OBERPRIELER, S., REES, G., NIELSEN, D., SHACKLETON, M., WATSON, G., CHANDLER, L. & DAVIS, J. 2021. Connectivity, not short-range endemism, characterises the groundwater biota of a northern Australian karst system. *Science of the Total Environment,* 796**,** 148955.

OPEL, K. L., CHUNG, D. & MCCORD, B. R. 2010. A study of PCR inhibition mechanisms using real time PCR. *Journal of Forensic Sciences,* 55**,** 25-33.

PAWLOWSKI, J., APOTHÉLOZ-PERRET-GENTIL, L. & ALTERMATT, F. 2020. Environmental DNA: What's behind the term? Clarifying the terminology and recommendations for its future use in biomonitoring. *Molecular Ecology,* 29**,** 4258-4264.

PAWLOWSKI, J., KELLY-QUINN, M., ALTERMATT, F., APOTHÉLOZ-PERRET-GENTIL, L., BEJA, P., BOGGERO, A., BORJA, A., BOUCHEZ, A., CORDIER, T. & DOMAIZON, I. 2018. The future of biotic indices in the ecogenomic era: Integrating (e) DNA metabarcoding in biological assessment of aquatic ecosystems. *Science of the Total Environment,* 637**,** 1295-1310.

PERINA, G., CAMACHO, A., HUEY, J., HORWITZ, P. & KOENDERS, A. 2018. Understanding subterranean variability: the first genus of Bathynellidae (Bathynellacea, Crustacea) from Western Australia described through a morphological and multigene approach. *Invertebrate Systematics,* 32**,** 423-447.

REDŽOVIĆ, Z., ERK, M., GOTTSTEIN, S., PERIĆ, M. S., DAUTOVIĆ, J., FIKET, Ž., BRKIĆ, A. L. & CINDRIĆ, M. 2023. Metal bioaccumulation in stygophilous amphipod Synurella ambulans in the hyporheic zone: The influence of environmental factors. *Science of the Total Environment,* 866**,** 161350.

REES, H. C., MADDISON, B. C., MIDDLEDITCH, D. J., PATMORE, J. R. & GOUGH, K. C. 2014. The detection of aquatic animal species using environmental DNA–a review of eDNA as a survey tool in ecology. *Journal of Applied Ecology,* 51**,** 1450-1459.

SACCÒ, M., BLYTH, A. J., VENARSKY, M. & HUMPHREYS, W. F. 2022a. Trophic interactions in subterranean environments. *Encyclopedia of Inland Waters, Second Edition.* Elsevier.

SACCÒ, M., GUZIK, M. T., VAN DER HEYDE, M., NEVILL, P., COOPER, S. J. B., AUSTIN, A. D., COATES, P. J., ALLENTOFT, M. E. & WHITE, N. E. 2022b. eDNA in subterranean ecosystems: Applications, technical aspects, and future prospects. *Science of The Total Environment,* 820**,** 153223.

SANSOM, B. J. & SASSOUBRE, L. M. 2017. Environmental DNA (eDNA) shedding and decay rates to model freshwater mussel eDNA transport in a river. *Environmental Science and Technology,* 51**,** 14244-14253.

SCHALLENBERG, L., WOOD, S. A., POCHON, X. & PEARMAN, J. K. 2020. What Can DNA in the Environment Tell Us About an Ecosystem? *Frontiers for Young Minds*.

SEPULVEDA, A. J., HUTCHINS, P. R., FORSTCHEN, M., MCKEEFRY, M. N. & SWIGRIS, A. M. 2020. The elephant in the lab (and field): Contamination in aquatic environmental DNA studies. *Frontiers in Ecology and Evolution,* 8**,** 609973.

SHA, C., WU, J., SHEN, C., WU, J., YAN, Z. & WANG, M. 2023. The ecology of bacterial communities in groundwater of industrial areas: Diversity, composition, network, and assembly. *Environmental Pollution,* 322**,** 121207.

SHAPIRO, K., BAHIA-OLIVEIRA, L., DIXON, B., DUMÈTRE, A., DE WIT, L. A., VANWORMER, E. & VILLENA, I. 2019. Environmental transmission of Toxoplasma gondii: Oocysts in water, soil and food. *Food and Waterborne Parasitology,* 15**,** e00049.

SHAW, J. L., CLARKE, L. J., WEDDERBURN, S. D., BARNES, T. C., WEYRICH, L. S. & COOPER, A. 2016. Comparison of environmental DNA metabarcoding and conventional fish survey methods in a river system. *Biological Conservation,* 197**,** 131-138.

SHOGREN, A. J., TANK, J. L., EGAN, S. P., AUGUST, O., ROSI, E. J., HANRAHAN, B. R., RENSHAW, M. A., GANTZ, C. A. & BOLSTER, D. 2018. Water flow and biofilm cover influence environmental DNA detection in recirculating streams. *Environmental Science & Technology,* 52**,** 8530-8537.

SIMON, K. & BENFIELD, E. 2001. Leaf and wood breakdown in cave streams. *Journal of the North American Benthological Society,* 20**,** 550-563.

STEUBE, C., RICHTER, S. & GRIEBLER, C. 2009. First attempts towards an integrative concept for the ecological assessment of groundwater ecosystems. *Hydrogeology Journal,* 17**,** 23.

STUMPP, C. & HOSE, G. C. 2017. Groundwater amphipods alter aquifer sediment structure. *Hydrological Processes,* 31.

TABERLET, P., COISSAC, E., HAJIBABAEI, M. & RIESEBERG, L. H. 2012. Environmental DNA. *Molecular Ecology,* 21.

TAKAHASHI, M., SACCÒ, M., KESTEL, J. H., NESTER, G., CAMPBELL, M. A., VAN DER HEYDE, M., HEYDENRYCH, M. J., JUSZKIEWICZ, D. J., NEVILL, P. & DAWKINS, K. L. 2023. Aquatic environmental DNA: A review of the macro-organismal biomonitoring revolution. *Science of the Total Environment,* 873**,** 162322.

THOMSON-LAING, G., PARAI, R., KELLY, L. T., POCHON, X., NEWNHAM, R., VANDERGOES, M. J., HOWARTH, J. D. & WOOD, S. A. 2021. Development of droplet digital Polymerase Chain Reaction assays for the detection of long-finned (Anguilla dieffenbachii) and short-finned (Anguilla australis) eels in environmental samples. PeerJ, 9, e12157.

THULIN, B. & HAHN, H. J. 2008. Ecology and living conditions of groundwater fauna.

VALEIX, N., COSTA, D., BASMACIYAN, L., VALOT, S., VINCENT, A., RAZAKANDRAINIBE, R., ROBERT-GANGNEUX, F., NOURRISSON, C., PEREIRA, B. & FRÉALLE, E. 2020. Multicenter comparative study of six Cryptosporidium parvum DNA extraction protocols including mechanical pretreatment from stool samples. *Microorganisms,* 8**,** 1450.

VAN DER HEYDE, M., WHITE, N. E., NEVILL, P., AUSTIN, A. D., STEVENS, N., JONES, M. & GUZIK, M. T. 2023. Taking e DNA underground: Factors affecting e DNA detection of subterranean fauna in groundwater. *Molecular Ecology Resources,* 23**,** 1257-1274.

VAN DER REIS, A., BOLTON, A., SMITH, B., GERNEKE, D., JACKSON, H., RIDDEN, J., WEBBER, J., KASPER, J., HANDLEY, K., HUDSON, M., CLOSE, M., LIGGINS, L., WEAVER, L., GOWO, P., STERLING, R. & WILKINSON, S. 2024. Molecular library of groundwater fauna. Report prepared for: Ministry for the Environment, Project 25872.

VON AMMON, U., CASANOVAS, P., POCHON, X., ZIRNGIBL, M., LEONARD, K., SMITH, A., CHETHAM, J., MILNER, D. & ZAIKO, A. 2023. Harnessing environmental DNA to reveal biogeographical patterns of non-indigenous species for improved co-governance of the marine environment in Aotearoa New Zealand. *Scientific Reports,* 13**,** 17061.

VON WINTZINGERODE, F., GÖBEL, U. B. & STACKEBRANDT, E. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiology Reviews,* 21**,** 213-229.

VÖRÖS, J., MÁRTON, O., SCHMIDT, B. R., GÁL, J. T. & JELIĆ, D. 2017. Surveying Europe's only cave-dwelling chordate species (Proteus anguinus) using environmental DNA. *PloS one,* 12**,** e0170945.

WEAVER, L., BOLTON, A. & ABRAHAM, P. 2021. Updated sampling considerations and protocols for assessing groundwater ecosystems. *Tasman District Council under Envirolink contract C03X2002-2.*

WEIGAND, H., BEERMANN, A. J., ČIAMPOR, F., COSTA, F. O., CSABAI, Z., DUARTE, S., GEIGER, M. F., GRABOWSKI, M., RIMET, F. & RULIK, B. 2019. DNA barcode reference libraries for the monitoring of aquatic biota in Europe: Gap-analysis and recommendations for future work. *Science of the Total Environment,* 678**,** 499-524.

WEST, K. M., RICHARDS, Z. T., HARVEY, E. S., SUSAC, R., GREALY, A. & BUNCE, M. 2020. Under the karst: detecting hidden subterranean assemblages using eDNA metabarcoding in the caves of Christmas Island, Australia. *Scientific Reports,* 10**,** 21479.

WHITE, N. E., GUZIK, M. T., AUSTIN, A. D., MOORE, G. I., HUMPHREYS, W. F., ALEXANDER, J. & BUNCE, M. 2020. Detection of the rare Australian endemic blind cave eel (Ophisternon candidum) with environmental DNA: implications for threatened species management in subterranean environments. *Hydrobiologia,* 847**,** 3201-3211.

WILLIAMS, K. E., HUYVAERT, K. P. & PIAGGIO, A. J. 2017. Clearing muddied waters: Capture of environmental DNA from turbid waters. *PLoS One,* 12**,** e0179282.

XIU, W., LLOYD, J., GUO, H., DAI, W., NIXON, S., BASSIL, N. M., REN, C., ZHANG, C., KE, T. & POLYA, D. 2020. Linking microbial community composition to hydrogeochemistry in the western Hetao Basin: Potential importance of ammonium as an electron donor during arsenic mobilisation. *Environment International,* 136**,** 105489.

YANG, J., ZHANG, X., XIE, Y., SONG, C., ZHANG, Y., YU, H. & BURTON, G. A. 2017. Zooplankton community profiling in a eutrophic freshwater ecosystem-lake tai basin by DNA metabarcoding. *Scientific Reports,* 7**,** 1773.

ZAKŠEK, V., SKET, B. & TRONTELJ, P. 2007. Phylogeny of the cave shrimp Troglocaris: evidence of a young connection between Balkans and Caucasus. *Molecular Phylogenetics and Evolution,* 42**,** 223-235.

INSTITUTE OF ENVIRONMENTAL SCIENCE AND RESEARCH LIMITED

Kenepuru Science Centre
34 Kenepuru Drive, Kenepuru, Porirua 5022
PO Box 50348, Porirua 5240
New Zealand
T: +64 4 914 0700 **F:** +64 4 914 0770

Mt Albert Science Centre
120 Mt Albert Road, Sandringham, Auckland 1025
Private Bag 92021, Auckland 1142
New Zealand
T: +64 9 815 3670 **F:** +64 9 849 6046

NCBID – Wallaceville
66 Ward Street, Wallaceville, Upper Hutt 5018
PO Box 40158, Upper Hutt 5140
New Zealand
T: +64 4 529 0600 **F:** +64 4 529 0601

Christchurch Science Centre
27 Creyke Road, Ilam, Christchurch 8041
PO Box 29181, Christchurch 8540
New Zealand
T: +64 3 351 6019 **F:** +64 3 351 0010

www.esr.cri.nz