RESOURCE ARTICLE

Taking eDNA underground: Factors affecting eDNA detection of subterranean fauna in groundwater

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Abstract

Stygofauna are aquatic fauna that have evolved to live underground. The impacts of anthropogenic climate change, extraction and pollution on groundwater pose major threats to groundwater health, prompting the need for efficient and reliable means to detect and monitor stygofaunal communities. Conventional survey techniques for these species rely on morphological identification and can be biased, labour-intensive and often indeterminate to lower taxonomic levels. By contrast, environmental DNA (eDNA)-based methods have the potential to dramatically improve on existing stygofaunal survey methods in a large range of habitats and for all life stages, reducing the need for the destructive manual collection of often critically endangered species or for specialized taxonomic expertise. We compared eDNA and haul-net samples collected in 2020 and 2021 from 19 groundwater bores and a cave on Barrow Island, northwest Western Australia, and assessed how sampling factors influenced the quality of eDNA detection of stygofauna. The two detection methods were complementary; eDNA metabarcoding was able to detect soft-bodied taxa and fish often missed by nets, but only detected seven of the nine stygofaunal crustacean orders identified from haul-net specimens. Our results also indicated that eDNA metabarcoding could detect 54%–100% of stygofauna from shallow-water samples and 82%–90% from sediment samples. However, there was significant variation in stygofaunal diversity between sample years and sampling types. The findings of this study demonstrate that haul-net sampling has a tendency to underestimate stygofaunal diversity and that eDNA metabarcoding of groundwater can substantially improve the efficiency of stygofaunal surveys.

KEYWORDS
Crustaceans, environmental DNA, invertebrates, Stygofauna, subterranean Fauna

1 | INTRODUCTION

Groundwater comprises over 90% of the available freshwater on Earth and is a key resource for surface life (i.e., natural ecosystems, human consumption, agriculture, industry and urbanization; Danielopol et al., 2003). Groundwater also supports diverse, complex ecosystems of subterranean fauna, largely comprising short-range endemic invertebrate and vertebrate species that represent exceptional levels of species richness and often ancient evolutionary lineages reflective of the past climatic and biogeographical history (Des Châtelliers et al., 2009; Fenwick et al., 2021; Juan et al., 2010; Ribera et al., 2010). Stygofauna (aquatic subterranean fauna) perform critical water purification services; they consume and stimulate microbial processes and biomasses which are essential to the ecological integrity of groundwater systems (Smith et al., 2016), and can facilitate hydraulic connectivity. Concerns over the impact of human-mediated disturbances (i.e., aquifer drawdown, contamination and climate change; Mammola et al., 2019) on groundwater-dependent communities has led to an increased need to characterize, describe and monitor them (Saccò, Guzik, et al., 2022; Stumpp & Hose, 2013).

Except for a few examples—Switzerland (SWPO, 1998) and Australia (EPA (WA), 2003; NSW-SGDEP, 2002)—predicting and monitoring the impacts of proposed developments on subterranean fauna are still rarely required within a regulatory setting (Danielopol & Griebler, 2007; Niemiller, Taylor, & Bichuette, 2018). In part, this is because several major impediments still exist that limit the ability to monitor and assess the diversity and distribution of stygofauna species (Cardoso et al., 2011; Mammola et al., 2019), including (i) specialist taxonomic expertise required to morphologically identify the small and highly adaptive forms of stygofauna (Bork et al., 2008; Dumas & Fontanini, 2001); (ii) DNA barcoding is often used to verify morphological species identification due to the high numbers of unknown and cryptic species (Bradford et al., 2010); (iii) subterranean environments are difficult to access and, in most cases, sampling is often limited to low-yield methods that require specialist equipment (Saccò, Guzik, et al., 2022); (iv) visual observation and assessment is extremely challenging at subterranean sampling access points such as caves, springs and boreholes (see Section 2 for details; Sorensen et al., 2013). There is therefore a need for new methods that will increase the accessibility, efficiency and accuracy of subterranean monitoring (Gibson et al., 2019; Mammola et al., 2022; Saccò, Guzik, et al., 2022).

Environmental DNA is genetic material that has been shed into the environment by organisms, and can be collected by extracting DNA from various substrates including water and sediment (Taberlet et al., 2012). Research in marine systems, combining next generation sequencing (NGS) and eDNA, has demonstrated that eDNA metabarcoding is an important new molecular tool for biodiversity assessment (Miya et al., 2015; Stat et al., 2017). In other ecosystems, eDNA-based monitoring can significantly decrease the time, expense and resources required compared to traditional survey methods in other ecosystems (Fediajevaite et al., 2021), and has the potential to improve on existing stygofauna sampling and monitoring approaches. Improvements have already been observed; recent studies have successfully used single-species assays to detect rare and endangered subterranean species (Boyd et al., 2020; Goricki et al., 2017; Niemiller, Porter, et al., 2018; Vörös et al., 2017) and investigate genetic differences (haplotypes) between populations (White et al., 2020). Additionally, Saccò, Blyth, Humphreys, Karasiewicz, et al. (2020), Saccò, Guzik, et al., 2022) have highlighted the potential value of eDNA for community-level detection of subterranean fauna and their diets (Korbel et al., 2017; Saccò, Campbell, et al., 2022; West et al., 2020) in a large range of habitats and for all life stages, thereby avoiding the need for: (i) specialized taxonomic expertise (Bohmann et al., 2014; Thomsen & Willerslev, 2015); and (ii) destructive sampling for identification. However, before an eDNA metabarcoding approach can be used as a standard method for monitoring and assessment of groundwater communities, it is essential that the appropriate testing and development of the methods are undertaken to assess the efficacy, accuracy and speed of species detection. Here we outline some ecosystem-specific factors that may impact the development of eDNA protocols for subterranean habitats.

To date, the utility of eDNA-based surveys in subterranean habitats has been undertaken for the detection of priority species (Baker et al., 2020; White et al., 2020), stygobitic communities from caves (West et al., 2020), and from boreholes and springs (Korbel et al., 2017; Oberprieler et al., 2021). Common challenges identified from eDNA studies for subterranean ecosystems include a critical lack of reference sequence availability and biases in metabarcoding assays (Korbel et al., 2017; Oberprieler et al., 2021). Further, the most appropriate sampling protocols for underground systems have yet to be formally tested and standardized. For instance, sample type (water, sediment, etc.), volume and depth are known to influence the diversity detected through eDNA (Jeunen et al., 2020; Koziol et al., 2018; West et al., 2020). Conversely, it is unknown if bore characteristics (e.g., diameter) or order of sampling (i.e., performing haul-net sampling first) will affect the species diversity that can be detected from eDNA.

Environmental factors are known to influence the detection and persistence of eDNA (e.g., high temperatures promote DNA degradation through enzymatic activity and denaturation, while anoxic environments limit degradation and stabilize eDNA; Barnes et al., 2014). However, for stygofauna, ecological factors, such as life history strategies, niche occupation and possible seasonal/temporal variability that might affect eDNA shedding rates and longevity, are poorly known. We know that there is a general pattern of decreasing species richness with depth, attributed to lower levels of oxygen and nutrients in deeper water (Brunke & Gonser, 1999; Datry et al., 2005). Further, previous studies have indicated that groundwater recharge can influence the abundance and life-history stages of some stygofauna (Datry et al., 2005; Hyde et al., 2018; Saccò, Blyth, Humphreys, Karasiewicz, et al., 2020) probably through increases in organic carbon leaching into the groundwater (Datry et al., 2005; Herrmann et al., 2020; Saccò, Blyth, Humphreys, Middleton, et al., 2020). However, recharge rates can

be quite low (Crosbie et al., 2010), with the groundwater in many parts of Australia being tens of thousands, if not millions, of years old (Meredith, 2009). There is much developmental work required to establish protocols for subterranean environments.

Barrow Island, located ~55 km off the Pilbara coast of Western Australia, is the largest island in the tropical waters of the northwest continental shelf of Western Australia (Moro & Lagdon, 2013). At 202 km^2 the island has been listed as a Class A nature reserve since 1910 for its extreme short-range endemic mammal, herpetological and subterranean fauna, including stygofauna and troglofauna (terrestrial subterranean fauna). The island also supports resource infrastructure including an operating oilfield (since 1960) and natural gas plant (Chevron, 2006). The subterranean biodiversity value of Barrow Island is internationally recognized (Burbidge, 2004) with an unprecedented 63 stygobitic species representing 12 orders (Humphreys et al., 2013). Endemic taxonomic groups recovered from Barrow Island groundwater include crustaceans, worms, (Eberhard et al., 2005; Humphreys et al., 2013) and the rare and endangered teleosts, the blind cave gudgeon and blind cave eel (Larson et al., 2013; Moore et al., 2018). Intensive sampling of groundwater has occurred since 2001, through Environmental Impact Assessment and monitoring in accordance with Western Australia's EPA guidelines (Eberhard et al., 2005; EPA, 2003; Humphreys et al., 2013). Given the high conservation status, the potential impacts of resource development and the legislated requirement to monitor for environmental impacts on Barrow Island, there is a real need to detect stygofauna as accurately as possible.

Here, we used the groundwater ecosystem at Barrow Island as an exemplar for understanding the factors that may affect eDNA detection of a stygofaunal community from groundwater. In addition to its distinct ecosystem, Barrow Island is also suited for scientific study due to the extensive number of sampling bores, many of which have been purpose-drilled to access the groundwater and stygofauna community for monitoring purposes. These bores have been cased and capped to retain the integrity of the bore and its groundwater profile. Using key parameters that are known to impact eDNA detection in various environments (i.e., substrate; Jeunen et al., 2020; Koziol et al., 2018) we investigated factors important in developing a robust eDNA sampling approach for effective assessment and monitoring of stygofaunal communities. Our aims were as follows:

1. To compare eDNA metabarcoding with a universal eukaryote assay (18S) to conventional stygofaunal survey methods (i.e., whole specimens were collected and identified; see Methods below). This comparison can be used to determine the strengths and limitations of both methods and the degree to which they complement each other. This comparison includes stygofauna and troglofauna which can also be detected using both methods.
2. To investigate whether *substrate type* (shallow water, deep water, sediment) and *sampling protocol* (order of sampling) affect the stygofaunal diversity and community composition detected through eDNA metabarcoding. The degree of overlap between substrate types will determine whether it is necessary to sample multiple substrates to detect the most diversity.

3. To examine whether temporal differences affect stygofauna communities detected through haul-nets and eDNA. Dynamism in the system and/or stochasticity would indicate the need for multiple sampling events to monitor the system.

2 | METHODS

2.1 | Study site

Barrow and Boodie islands are located northwest of mainland Western Australia on the edge of the Australian continental shelf. The climate of the islands is arid and subtropical with hot, humid summers with daily mean maxima reaching 34°C and daily mean minima of 20°C. The winter daily mean maxima are lower at 26°C and daily mean minima of 17°C (Moro & Lagdon, 2013). Rainfall is seasonal, most prevalent in November–April, and dependent on the passage of summer monsoons. The average annual rainfall has historically been 320 mm; however, the first year of sampling for this study (2020) was during a particularly dry period when the average annual rain for the previous 3 years was only 105 mm (www.bom.gov.au). The following year of sampling (2021) was during a wetter year with 377 mm of rain, the majority occurring between March and May.

Groundwater on Barrow Island comprises an exceptional example of an anchialine system (Humphreys, 2001); that is, stratified waters where a freshwater lens that originates from seasonal rainfall overlies seawater with a transitional zone in between. The transitional zone is of comparable thickness to the freshwater layer having been expanded due to tidal forces. As a smaller island just south of Barrow, Boodie Island has a slimmer freshwater lens and is almost entirely marine. Access to the groundwater and in situ stygofaunal sampling is conducted via existing bores that have been installed for specific operational or monitoring purposes. The bores are typically constructed of 50- or 100-mm-diameter, PVC casing that is slotted at discrete intervals depending on their purpose. Slots vary from 0.5 to 5 mm in width. Bores with larger slot sizes can have mesh across them to prevent debris from accumulating inside the bore. Depth of bores varies depending on the location on the island (bores at higher elevation need to be deeper to reach the water column) and the initial purpose of the bore. Bores designed to monitor stygofauna or groundwater have slotted intervals in shallower parts of the aquifer (i.e., water several metres deep below the water table) compared to those constructed for the cathodic protection system (tens of metres below the water table). Many bores are slotted below the water table.

2.2 | Sample collection and processing

Stygofaunal specimens and eDNA samples were collected from 17 bores in December 2020 and November 2021 (Figure 1). An additional two bores were sampled in 2021 (November) from Boodie Island. Groundwater at the base of a cave on the west side of Barrow Island was also sampled in November 2021. The cave and Boodie

Island sites are particularly difficult to access and were unavailable for sampling during the 2020 sampling period. Bores chosen for sampling were spatially stratified across the island and high in stygofaunal diversity according to previous monitoring efforts (Humphreys et al., 2013). Two "control" bores (BGMW08, WFS2MW04) with no previous detection of stygofauna were also selected.

2.3 | Stygofaunal collection and processing

Whole specimens used to assess a baseline for morphological species diversity were collected using haul-nets (similar to plankton-nets—see Saccò, Campbell, et al., 2022; Saccò, Guzik, et al., 2022), which were 45 or 95 mm in diameter, depending on the size of the PVC casing. Five (Allford et al., 2008) to six (EPA guidance) haul-net samples were taken at each bore, half with 150-μm mesh and half with 50-μm

mesh, alternating mesh size between hauls. We favoured haul-net sampling for stygofauna prior to collection of eDNA samples to ensure that live specimens were not dispersed by the eDNA sampling equipment. Haul-net samples were sorted and identified using a dissecting microscope and specimens of each morphotype were stored in glass vials in 100% ethanol and kept frozen at −20°C. Stygofauna experts (N.S. and M.T.G.) provided the morphological identifications.

2.4 | eDNA sample collection

Water samples were collected for eDNA analysis after hauling. Four 1-L water samples were collected from the upper 2 m of the water column using a 1-L plastic disposable bailer (shallow water samples). One 1-L water sample was collected from the bottom of the bore using a 1-L steel bailer (deeper water sample). Sediment samples
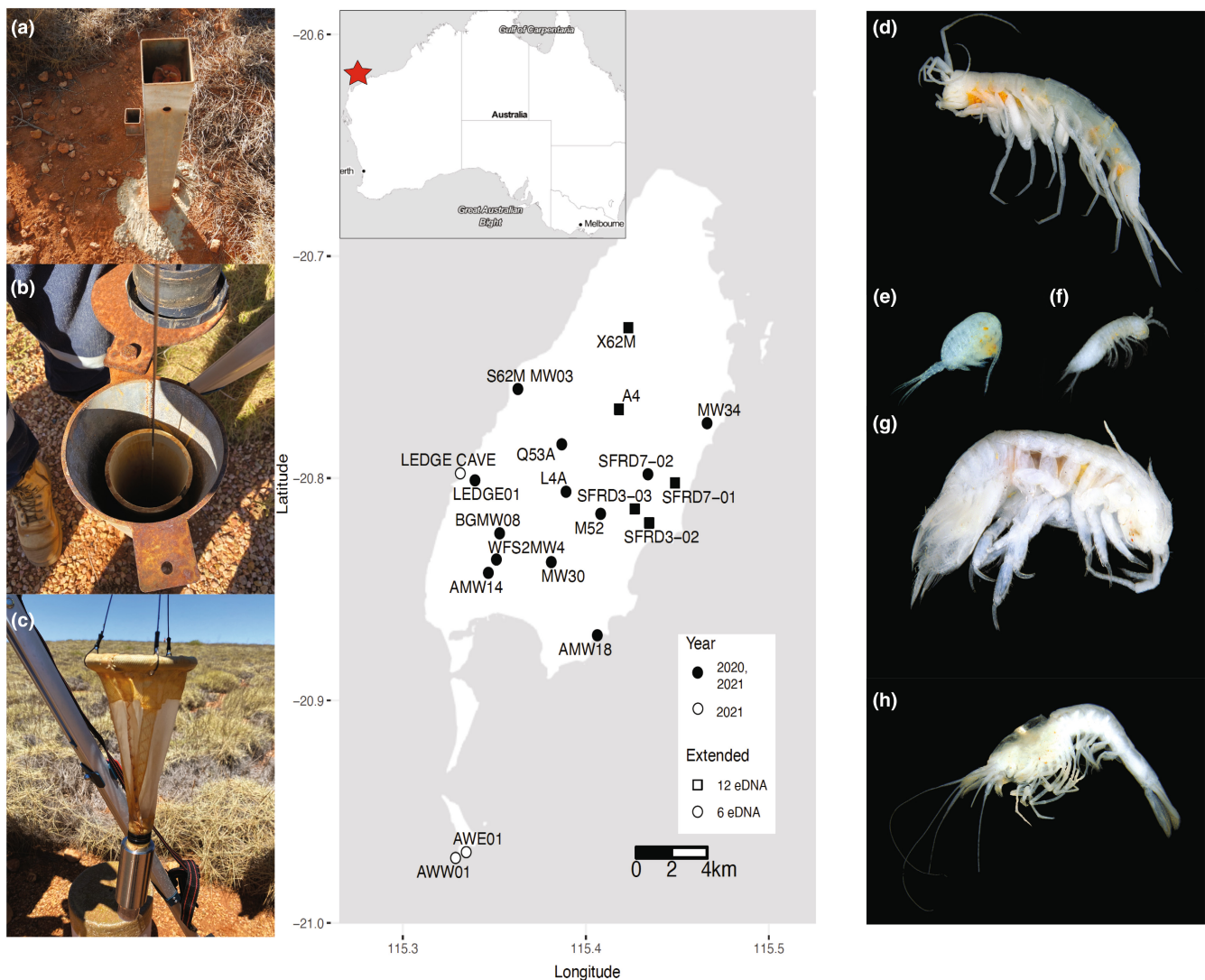

FIGURE 1 Map of all boreholes sampled on Barrow Island showing the year of sampling and bores selected for the extended scope to investigate if order of eDNA sample collection makes a difference to stygofauna detected. Photos on left show (a) 50-mm anode bores, (b) 100-mm stygofauna monitoring bores and (c) a stygofauna haul-net. On the right are images of some stygofauna collected during this study including (d) Amphipoda *Nedsia*, (e) Calanoida *Stygoridgewayia trispinosa*, (f) Parabathynella, (a) Isopoda and (h) Decapoda *Stygiocaris stylifera*.

(one per bore) were collected from the deeper eDNA samples where there was an abundance of sediment left over that could not be filtered. With six samples per bore (i.e., 4× 1-L eDNA, 1× 1-L deeper, 1× sediment), there were 102 samples collected in 2020, and 120 samples in 2021. For five bores the scope was extended to include collecting the eDNA samples before *and* after the haul-net samples to determine if the order of sampling was significant. This resulted in 12 eDNA samples per bore for these five bores with a total of 30 samples collected before nets and 30 after. All equipment was sterilized between bores in 10% bleach for 10 min and rinsed in reverse osmosis (RO) water, and disposable gloves were used at each borehole to prevent contamination. Fresh bleach solution and RO water was used for every borehole for equipment that was not decontaminated in the laboratory ahead of time, and samples of the RO water used for rinsing equipment (i.e., field controls) were collected to assess sources of contamination. Additional quality control samples included: two blank filter membranes carried in the field and a sample of all RO water sources for a total of 34 field controls.

eDNA and haul-net samples were kept on ice until they could be transferred to a 4°C refrigerator at the end of the collecting day. eDNA water samples were filtered within 24 h across 0.45-μm Supor polyethersulfone membranes using a Pall Sentino Microbiology pump (Pall Corporation). For samples with high turbidity, up to two membranes were used to increase the volume of water filtered until the filters clogged. The samples were immediately frozen and stored at −20°C prior to, and on-ice during, their transportation to the Trace & Environmental DNA (TrEnD) Laboratory at Curtin University in Perth, Western Australia.

Water physicochemical properties were measured for each borehole using a YSI ProDSS either 2 days before or the day after sampling, to avoid cross-contamination between boreholes. Measurements of turbidity (FNU), conductivity (μS cm^{-1}), salinity (psu), temperature (°C), dissolved oxygen (%sat, mg L^{-1}), pH, and depth (m) were recorded for each borehole. Water chemistry at various depths was only measured for bores with a water column that was greater than several metres.

2.5 | DNA barcoding of specimens and custom reference library

A leg was removed from 15 morphologically identified specimens (Table S1) collected via haul-nets and DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's instructions with the following modification: genomic DNA was eluted from the silica column with 50 μL of AE buffer. The DNA was then amplified using PCR with universal eukaryote18S primers (Table S2; Pochon et al., 2013). These primers have previously been used for metabarcoding assays (~400 bp) to detect stygofauna (West et al., 2020) and broad enough to detect a wide range of organisms, which might otherwise require multiple assays (Pochon et al., 2013). Each PCR for barcoding was carried out in duplicate in a total volume of 25 μL containing: 1× AmpliTaq Gold PCR buffer (Life Technologies), 2.5 mM MgCl2, 0.25 mM dNTPs, 0.4 μM each of forward and reverse primers (Integrated DNA Technologies), 0.4 mg mL^{-1} bovine serum albumin (Fisher Biotec), 0.6 μL of 5× SYBR Green (Life Technologies), 1 U AmpliTaq Gold DNA Polymerase (Life Technologies), 2 μL of genomic DNA template, and made up to volume with Ultrapure Distilled Water (Life Technologies). The cycling conditions were initial denaturation at 95°C for 5 min, followed by 40–45 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 45 s, a melt curve stage of 95°C for 15 s, 60°C for 1 min and 95°C for 15 s, and a final extension at 72°C for 10 min. The expected PCR product size (~400 bp) was visualized on a 2% agarose gel and sent to the Australian Genome Research Facility (Perth) for Sanger sequencing in both directions. The forward and reverse sequences were then aligned, the primers were removed and the consensus sequences were used to create a custom library for reference analyses (i.e., taxonomic identification) of the 18S eDNA sequences obtained from the water and sediment samples. All barcoding sequences generated were submitted to GenBank (Table S1). The custom DNA sequence library also included sequences of stygofauna from the Pilbara region sourced from BOLD and GenBank (www.ncbi.nlm.nih.gov/genbank), as well as previous consulting work done in the area for a total of 207 18S sequences.

2.6 | eDNA laboratory processing

DNA was extracted from filter membranes within 1 month of collection and filtering using a DNeasy Blood and Tissue Kit (Qiagen) with the following modifications: 540 μL of ATL lysis buffer and 60 μL of Proteinase K were used during the cell lysis phase. A total of seven DNA extraction controls, containing the solutions and plastics supplied in the extraction kit, were used and processed alongside all eDNA and Sanger sequencing samples in order to detect any laboratory or cross-contamination of samples. Sediment samples were homogenized for 1 min at 30 Hz in a TissueLyser (Qiagen) and DNA was extracted from 0.25 g of homogenized sediment using a DNeasy PowerLyser PowerSoil kit (Qiagen). All eDNA extractions (post-lysis stage) were performed using an automated DNA extraction platform (QIAcube; Qiagen) with a customized eDNA protocol that elutes the DNA off the silica membrane in 100 μL of AE buffer.

DNA extracts were screened for quality and quantity of DNA using qPCR to determine the presence of inhibitors and the quantity of target template molecules present in each DNA extract (Murray et al., 2015). Three dilutions (neat, 1:5 and 1:10) were used for a subset of 30 samples, while the remaining were assessed using only the neat and 1:10 dilution. Each PCR for the 18S universal assay was carried out with the same master mix as described above. The cycling conditions were initial denaturation at 95°C for 5 min, followed by 45 cycles of 95°C for 30 s, annealing at 52°C for 30 s, 72°C for 45 s, a melt curve stage of 95°C for 15 s, 60°C for 1 min and 95°C for 15 s, and a final extension at 72°C for 10 min. All PCR plates included a negative control and a positive control of chicken DNA.

Each sample, including all controls, was assigned a unique combination of multiplex identifier (MID) tags for the 18S universal assay. These MID tags were incorporated into fusion tagged primers, and none of the primer–MID tag combinations had been used previously in the laboratory to prevent cross-contamination. Fusion PCRs were done in duplicate to minimize PCR stochasticity, and the mixes were prepared in a dedicated ultraclean room before DNA was added. The PCRs were done with the same conditions as the standard qPCRs described above. Samples were then pooled into approximately equimolar concentrations to produce a PCR amplicon library that was size-selected to remove any primer-dimer that may have accumulated during fusion PCR. Size selection was performed (250–600 bp) using a PippinPrep 2% ethidium bromide cassette (Sage Science). Libraries were cleaned using a QIAquick PCR Purification Kit (Qiagen) and quantified using Qubit Fluorometric Quantitation (Thermo Fisher Scientific). Sequencing was performed on the Illumina MiSeq platform using the 500-cycle paired-end V2 as per the manufacturer's instructions.

2.7 | Bioinformatics

DNA sequences were processed using eDNAFlow, an automated bioinformatics workflow designed for analysis of eDNA metabarcoding data (Mousavi-Derazmahalleh et al., 2021). This pipeline uses ADAPTORREMOVAL (Schubert et al., 2016) and FASTQC (Andrews, 2010) to quality filter and demultiplex sequences using "obitools" (Boyer et al., 2016). The minimum Phred quality score was set at 20, with a minimum alignment of 12 for the paired-end reads, minimum length of 100 bp and maximum two primer mismatches allowed, but no mismatches allowed in the MID-tag sequences. The demultiplexed sequences were then denoised using USEARCH unoise3 (Edgar, 2016) to create zero-radius operational taxonomic units (ZOTUs) with a minimum abundance of 8, the recommended value for the denoising algorithm (Edgar, 2016). The ZOTU sequences were then queried against a custom barcode reference library for stygofauna and GenBank (NCBI) using BLASTN with the following parameters (–outfmt "5"-perc_identity 95-qcov_hsp_perc 95-query-max_target_seqs 10). Taxonomic assignment was performed using a simple lowest common ancestor algorithm on MEGAN (Huson et al., 2007) with a minimum score of 450.

Niches (i.e., stygofaunal, troglofaunal, nonsubterranean taxon groups) were assigned to all metazoan taxa identified from ZOTUs. This allowed us to parse stygofauna and troglofaunal taxa from non-subterranean taxa. ZOTUs belonging to subterranean taxa were then extracted an aligned along with sequences from the custom reference database using MUSCLE (Edgar et al., 2004) in GENEIOUS 2021.0.3 (https://www.geneious.com) with 10 iterations of the default settings. The resulting tree was used to infer putative species with a Poisson tree processes (PTP) model (Zhang et al., 2013). Some subterranean ZOTU taxon names were modified to reflect the species inferences from the model such that all ZOTUs assigned to a species had the same taxa name, and no two subterranean "species" had the same name. For example, three ZOTUs identified as "Atyidae" were renamed to *Stygiocaris stylifera* because they belonged to the same putative species. Details on initial and modified taxon names can be found in the taxon table in the dryad digital repository.

2.8 | Statistical analysis

Statistical analyses were performed in R 4.0.5 (R Core Team, 2018). We used the R package "phyloseq" (McMurdie & Holmes, 2013) to filter the data set. This included removing samples with fewer than 2000 reads, where rarefaction curves appeared to asymptote (Figure S1) and pruning ZOTUs that were present in extraction controls and rinsate blanks (>5 reads). This removed 18 ZOTUs that were detected in extraction controls and 166 ZOTUs from the rinsate controls, although only three of those were stygofauna. Two of the potential stygofaunal ZOTUs removed were nematode ZOTUs identified as Monhysterida found in RO water used to rinse equipment. ZOTUs with fewer than five reads were removed, and abundances were converted to presence or absence. We selected only the ZOTUs from presumed subterranean taxa (both stygofaunal and troglofaunal), and combined the detections from all sample types for the comparison between subterranean taxa detected using morphological identification of haul-net samples, and taxa detected using eDNA. Differences in the taxa detected using each method were visualized using a bubble plot to compare taxa at the order level for both stygofauna and troglofauna.

The effect of substrate types (deeper, shallow, sediment) on stygofauna community composition was tested on a data set containing the one sediment sample, one deeper water sample and one randomly selected shallow water sample per borehole. The effect of sample type on stygofaunal ZOTU richness was tested using a Friedman rank sum test for repeated measures. Pairwise comparisons were performed using paired Wilcoxon sign rank tests with a Holm adjustment for multiple comparisons (Holm, 1979). For the data from 2021, the analysis was performed on the data for all sites, and without the three extra sites (Boodie Island and Ledge cave), to ensure the addition of those sites was not influencing the patterns observed. To examine which stygofaunal taxa could be detected using each sample type, the ZOTUs were collapsed by taxon using the *tax_glom* function from "phyloseq" (McMurdie & Holmes, 2013). The taxa detected by each substrate were then illustrated using a Venn diagram function (*ps_venn*) from the "MicEco" package (Russel, 2021).

For the five bores where we collected eDNA samples both before and after using the haul-nets, we tested whether the order of sampling had an effect on stygofauna ZOTU richness using an analysis of variance (ANOVA) with "bore" as a random factor. Differences between community composition before and after haul-net sampling were likewise tested using a permutational multivariate analysis of variance (PERMANOVA, distance = "jaccard", permutations = 999) with bore as a random factor. Finally, a multipattern analysis was run

using the R package "indicspecies" (Cáceres & Legendre, 2009) to determine which taxa were more prevalent in the sampling before or after haul-net samples.

An asymptotic regression curve was fitted to the accumulation curves for each sampling replicate using the "AR.2" function in the R package "drc" (Ritz et al., 2015). The replicates for each borehole were then merged and a Mantel test from the R package "ade4" (Dray & Dufour, 2007) was used to test the correlations between similarity in community composition and the distances between the bores. The clustering of bores was analysed using the SIMPROF (++Similarity Profile Analysis) function in the "CLUSTIG" package (Whitaker & Christman, 2014).

3 | RESULTS

We generated 13,744,116 18S sequences from 300 samples including controls with a mean of $45,813 \pm 1934$ reads per sample and a total of 4013 eukaryote ZOTUs. After removing the samples with low sequencing depth and filtering taxa (see Section 2.8) there were 231 samples and 3800 ZOTUs remaining for analyses. A large proportion of the total ZOTUs could not be assigned a taxonomic rank (57.9%). Other groups detected included SAR (Stramenopiles+Alveolata+Rhizaria, e.g., Cercozoa, Ciliophora, Bigyra, 14%), fungi (Ascomycota, Basidiomycota, etc., 10.4%), Amoebozoa (2.4%) and many other unicellular eukaryotes. There were 348 Metazoan ZOTUs. Of these, 120 ZOTUs were identified as stygofauna, and 64 ZOTUs were identified as troglofauna based on the existing nomenclature. The niche (troglofaunal, stygofaunal, not subterranean) could not be determined for 44 Metazoan ZOTUs because the taxonomic identification was of a higher rank. This included ZOTUs identified to Aranae/Arachnida, Hemipteran superfamilies and arthropod superfamilies such as Peracarida.

3.1 | Comparison of morphological vs. eDNA identification of subterranean fauna

Twenty-four subterranean taxa were morphologically identified from the net-haul samples taken at Barrow Island during 2020 and 2021. Of these, 17 were stygofaunal (Table 1) and seven were troglofaunal. Using the 18S universal metabarcoding assay we identified 36 different subterranean taxa: 26 stygofaunal and 10 troglofaunal. Here "taxa" refers to the lowest taxonomic identification available for the specimen, and all the ZOTUs assigned to the same "putative

species" using the species delimitation model. Overall, more subterranean taxa were identified using an eDNA metabarcoding approach than with morphology alone (36 vs. 24; Table 1).

The stygofauna orders identified using a morphological approach were three copepod groups (Calanoida, Cyclopoida, Harpacticoida), Amphipoda, Decapoda, Isopoda, Ostracoda, Bathynellacea, Thermosbaenacea, Platyhelminthes and Perciformes from the haul-net samples. Of the 11 stygofauna orders identified morphologically, nine (82%) were also detected with the eDNA metabarcoding 18S assay (Figure 2) using GenBank and a custom reference library of Barrow Island stygofauna sequences. However, these orders were not detected with the same frequency for both methods. Some crustacean orders, such as Calanoida, Cyclopoida, Bathynellacea and Decapoda, were detected by both methods in approximately the same number of bores (Figure 2). Other orders, such as Amphipoda and Ostracoda, were detected from more bores using morphological identification than eDNA metabarcoding while Isopoda and Thermosbaenacea were not detected using an eDNA approach (Figure 2). The cavefish blind gudgeon (*Milyeringa justitia*) was detected from four bores using eDNA, and only one bore using haul nets. eDNA metabarcoding detected five stygofaunal orders from the water and sediment samples that were not identified using morphology from the haul-net samples. The eDNA metabarcoding findings included the soft-bodied orders that can have stygofaunal representatives, such as catenulid flatworms (phylum Platyhelminthes) detected from 17 bores and annelid worms (11 bores). In all the stygofauna haul-net samples, only one platyhelminth specimen was captured, and this could not be identified beyond the phylum level. Some troglofauna (e.g., Collembola, Schizomida, Pseudoscorpiones) were also detected occasionally with both eDNA and haul-net samples (Figure 2), while other troglofauna could only be detected using eDNA (e.g., Polyxenida). Several surface-dwelling taxa were detected (e.g., Blattodea—cockroaches, Squamata—reptiles, Hymenoptera—ants) from eDNA samples, although there were also some surface-dwelling specimens collected with the haul-net samples. During the specimen sorting, stygofauna and troglofauna were targeted, and surface taxa were often ignored. Several terrestrial taxa were detected (e.g., Hymenoptera, Coleoptera) in some bores, but these were incidental and the data cannot provide accurate comparison between methods for surface-dwelling taxa.

Some discordance between methods (eDNA vs. morphology) was observed at a few bore locations. For example, bore WFS2MW4, a "control" bore where no stygofauna have been detected on previous surveys (unpublished data from Chevron), was confirmed as a control bore using the haul-net sampling approach during both sampling

TABLE 1 Number of subterranean taxa detected from Barrow Island boreholes using morphological and eDNA metabarcoding-based identification in both sample years.

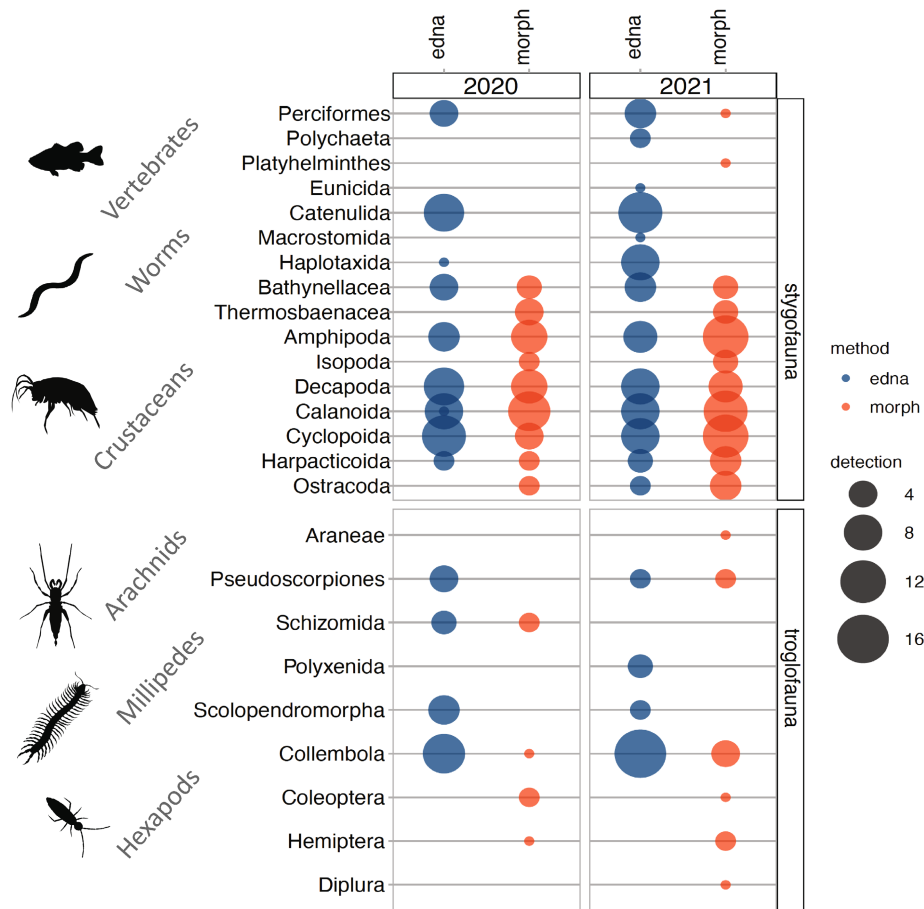| Year	eDNA			Morph		
	2020	2021	Total	2020	2021	Total
Stygofauna	11	19	21	13	12	16
Potential stygofauna	2	5	5	0	1	1
Troglofauna	3	2	3	3	3	4
Potential troglofauna	5	6	7	1	3	3

FIGURE 2 Comparison of Metazoan orders detected through morphological identification of haul-net specimens (morph), or eDNA from water and sediment samples with the 18S universal assay (edna). Size of bubble indicates the number of bores where the order was detected. Detections are separated into stygofauna and troglofauna and only taxa that could be classified as probable subterranean fauna are included.

periods (2020–2021). However, eDNA metabarcoding of water and sediment detected Cylcopoida (Copepoda) at this bore. Similarly, BGMW08 was previously identified as a "control" bore, although in 2020 a copepod taxon (Harpaticoida) was identified from haul-net samples and in 2021 no specimens were collected. However, the eDNA sampling at BGMW08 detected several nonarthropod invertebrates or "worm" taxa (Catenulida and Haplotaxida), but not copepods. Another bore that revealed inconsistent results was MW34, which historically hosted a relatively rich (seven taxa) stygofauna community (unpublished data from Chevron), but in 2020 no stygofauna were identified using the haul net. With eDNA, however, we detected five stygofaunal taxa, including some previously detected, such as Decapoda. In 2021, after a year of heavy rainfall, Amphipoda, Cyclopoida and Ostracoda specimens were again collected at this site using the haul-net samples, but were not detected using eDNA metabarcoding.

although not significantly so (Figure 3). In 2021, our results showed a strong decrease in stygofauna ZOTU richness in shallow water samples ($p = .016$), a weak increase in the sediment ($p = .12$) and no change in the deeper water samples ($p = .77$; Figure S2). As a result, in 2021, the stygofauna ZOTU richness in sediment samples was significantly higher than the other two substrates ($p < .05$; Figure 3). In terms of compositional differences between the sampled substrates, 100% of stygofauna taxa identified could be detected from the shallow water samples in 2020 and 82% from sediment samples (Figure 3). In 2021, those proportions decreased, and shallow samples detected half of the stygofauna (55%) while sediment samples detected 90%. In comparison, there was only one taxon (Polychaeta A) detected from only deeper water samples in 2021. The majority of stygofauna (82%) could be detected by all three substrates in 2020, but this proportion dropped to 32% in 2021.

## 3.2 | Effect of substrate on eDNA metabarcoding-based detection of stygofauna

In 2020, we detected higher stygofaunal ZOTU richness in the shallow water and sediment samples than the deeper water samples,

3.3 | Effect of sampling order on eDNA metabarcoding-based detection of stygofauna

Water samples collected before haul nets were visibly observed to contain whole specimens of stygofauna on two occasions;

FIGURE 3 Stygofauna richness and composition differences between the eDNA sample types: deeper water samples, regular shallow water samples and sediment samples. (a) Stygofauna ZOTU richness is shown above and below (b) is a Venn diagram depicting the number of stygofaunal taxa detected in each sample type.
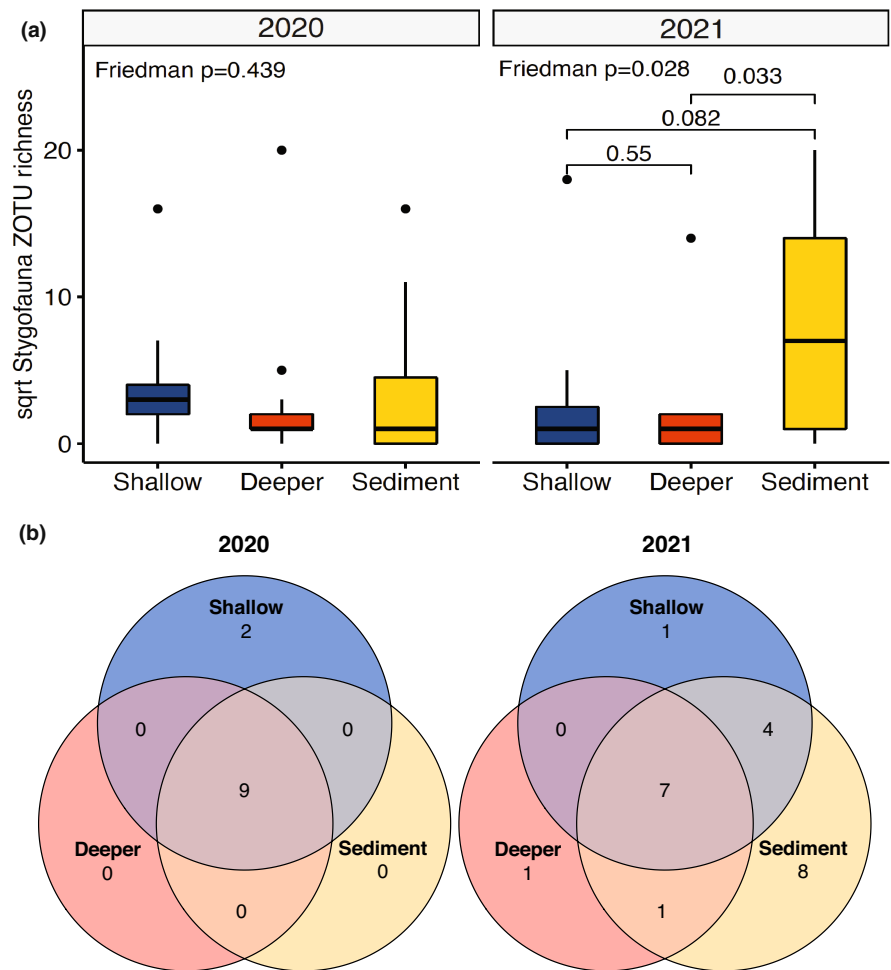
these individuals were removed from the sample with sterilized forceps before filtering. For some of the samples collected before haul-nets there were one or two stygofauna ZOTUs with very high read abundances (Figure S3), indicating some specimens may have been caught on the filter paper. However, from presence/absence data the eDNA samples collected prior to haul-net sampling were no richer in stygofauna ZOTUs than the samples collected after haul-nets ($p = .115$) although "bore" was a significant factor ($p = .001$; Figure S4). Similarly, the order of sampling was not significant in explaining variation in stygofauna community composition ($p = .131$), but communities varied significantly between bores ($p = .001$; Figure S5). Multipattern analysis indicated ZOTUs such as those identified as *Stygoridgewayia trispinosa* could be associated with eDNA samples taken before or after haul-nets depending on the bore (Table S3). The number of specimens per bore collected through haul-net samples was similar between the 2 years of sampling, ranging from one to three at bore SFRD3-03 to 362-45 at bore X62M (see Table S4).

3.4 | Physicochemical properties

Dissolved oxygen and salinity showed an inverse relationship with increasing water depth, with oxygenated freshwater found
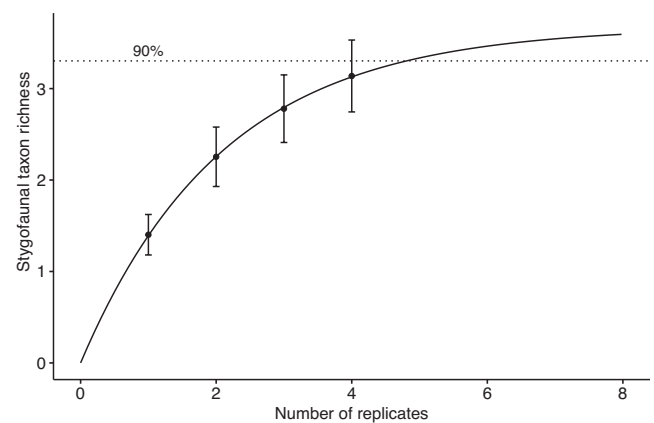

FIGURE 4 Accumulation of Stygofauna taxa with each additional 1-L water replicate. Points indicate mean richness, with standard error bars. The solid line indicates the fitted asymptotic regression model with an upper limit $d = 3.67$, and steepness of increase $e = 2.101$.

at the surface and oxygen-depleted salt water in deeper water (Figure S5). Temperature remained relatively constant at ~30°C, while pH remained neutral (~7) for most bores though some became slightly more acidic (pH=6.5) with depth (M52, L4A; Figure 1).

3.5 | Community composition

The asymptotic regression models showed that 90% of the stygofaunal taxa in each borehole could be detected with five 1-L water replicates (Figure 4). SIMPROF analysis indicated that the communities detected in the different years tended to cluster together based on the bore (Figure 5). However, there were some exceptions such as MW34 and S62M MW03, where the communities from the different years were found in separate clusters (Figure 5). Boreholes that were geographically close did not necessarily have more similar stygofauna community composition than those that were geographically distant in either 2020 ($r = -.008$, $p = .514$) or 2021 ($r = -.116$, $p = .681$). There were more stygofaunal taxa detected in 2021 than in 2020, using eDNA metabarcoding (24 vs. 13, respectively), but no difference was observed with the specimens that were morphologically identified (13 vs. 13; Table 1). The most common stygofaunal taxa included Cyclopoida (14 bores), Catenulida (13 bores), Calanoida (12 bores) and Decapoda (10 bores).

4 | DISCUSSION

Here we present the first metabarcoding study which examines the factors affecting community-wide eDNA metabarcoding-based biodiversity assessments in groundwater. As such, it is intended as the first step in developing a robust protocol for effective eDNA-based stygofaunal detection and monitoring. Environmental impact assessment and monitoring for subterranean fauna is currently conducted using traditional approaches such as haul-net sampling, but, as with most sampling protocols, these methods have inherent biases (Saccò, Campbell, et al., 2022; Saccò, Guzik, et al., 2022). We show that broad eukaryote 18S metabarcoding assays were more successful than haul-net sampling in detecting the breadth of major stygofaunal taxon groups known from groundwater on Barrow Island, but that key taxa were notably absent from metabarcoding results compared to those collected using haul nets and identified morphologically. eDNA was also able to detect stygofauna at locations that haul nets did not. In testing the protocols for sampling groundwater fauna for eDNA metabarcoding, we show the shallow
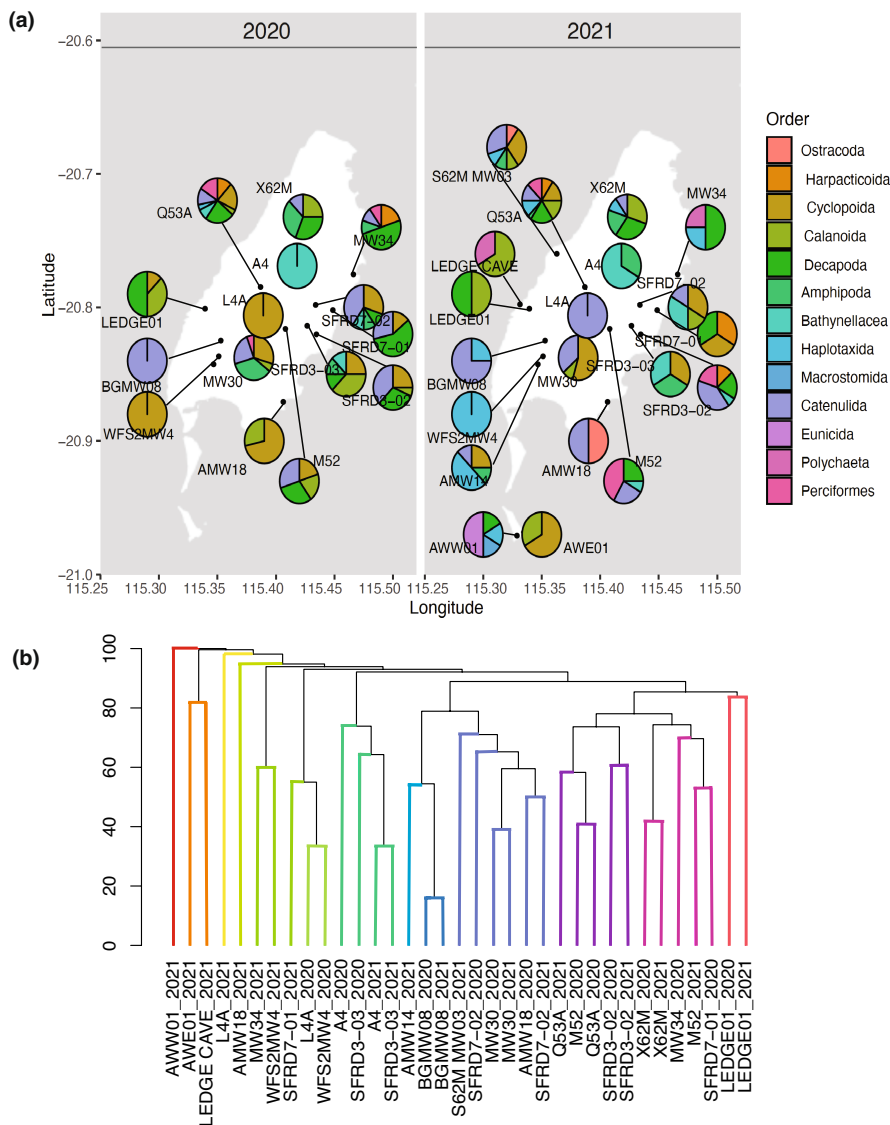

FIGURE 5 Stygofauna community composition from bore samples. Scatterpie plots show the stygofaunal orders detected at each borehole using eDNA metabarcoding. Dendogram depicts SIMPROF cluster analysis of bore stygofauna community composition. Different colours indicate significant clusters ($p < .01$).

water and sediment samples were the most effective substrate for detecting diversity in the majority of taxa. However, temporal variation in detection of taxonomic diversity in all samples was observed between 2020 and 2021.

4.1 | Comparison of eDNA metabarcoding vs. morphological identification detection of stygofauna

eDNA metabarcoding consistently detected more taxa than were identified morphologically from haul-net samples (Figure 2). Some of the taxa detected using eDNA metabarcoding but missed by haul-nets included soft-bodied taxa (catenulid flatworms, Annelida) that may be more difficult to catch in haul-nets or identify because of their small size, but leave sufficient DNA for eDNA detection (Figure 2). These soft-bodied taxa have been identified as first- and second-level predators common to groundwater habitats. In particular, they feed on protozoan and metazoan prey and bacterial biomass which contribute to groundwater purification and nutrient cycling, important for ecosystem functioning (Cunillera-Montcusí et al., 2022; Herrmann et al., 2020). Platyhelminthes (Catenulida) and copepods (e.g., Cyclopoida) are considered high-level predators in some groundwater systems (Herrmann et al., 2020), although recent studies suggest they may also be feeding on roots (Saccò, Campbell, et al., 2022) and bacteria (Saccò et al., 2021). These organisms are themselves preyed on by stygobiont teleosts, if present (Humphreys & Feinbergz, 1995), and are important for the food web structure and trophic dynamics of groundwater systems. Previous haul-net specimens from Barrow Island have been dominated by crustaceans, with Annelida and Chordata being far less numerous in collections (Humphreys et al., 2013). The differences in abundance between these groups may be an artefact of the sampling method, rather than a reflection of their true abundances in this groundwater ecosystem (Herrmann et al., 2020; Humphreys & Feinbergz, 1995). While morphological identification detected exclusively the larger organisms, eDNA metabarcoding could detect smaller, lower-level consumers, such as Cercozoa and Ciliophora, as well as fungi and bacteria (Herrmann et al., 2020; Oberprieler et al., 2021). The absence of taxon groups from trophic levels other than those that are free-swimming, in haul-nets, indicates that traditional methods of sampling are frequently incapable of detecting organisms below tertiary consumers, providing a narrow window into the diversity of stygofauna communities inhabiting groundwater systems.

With haul-nets, we were able to collect specimens from all crustacean orders known to occur in groundwater ecosystems at Barrow Island (Humphreys et al., 2013). Most of these taxa were also detected using 18S eDNA metabarcoding. Some crustaceans were reliably detected with both methods (Calanoida, Cyclopoida, Bathynellacea and Decapoda) while Ostacoda and Amphipoda were detected more often from haul-net samples than by eDNA metabarcoding. We know that the 18S eukaryote assay can amplify these taxa (based on in silico studies; our unpublished data), but primer bias against crustaceans appears to have resulted in false negatives as

the DNA from these taxa are "drowned out" by the many other taxa this broad assay can detect (Korbel et al., 2017; Pochon et al., 2013). Crustaceans tend to shed less DNA in their environment than soft-bodied organisms (Andruszkiewicz Allan et al., 2021), which may further bias results against these taxa.

Thermosbaenacea and Isopoda are important components of the subterranean ecosystem at Barrow Island that probably represent Tethyan relicts (Humphreys, 1993; Page et al., 2018) However, these taxa were not detected using eDNA metabarcoding even though they are known to occur on the island and were collected using haul-nets. Further investigation showed there were no mismatches in the primer binding sites for these taxa, but the amplicon length for thermosbanaeceans and isopods was found to be ~500 bp, compared to the other taxon groups (~400 bp; our unpublished data). Our retrospective investigation into this apparent failure of DNA amplification led to the conclusion that the 18S amplicons are likely to have been amplified but failed to be stitched together after sequencing due to the additional ~100-bp size difference. At this length, the sequencing chemistry used (Illumina Miseq 500v2; 250-bp paired-end) would not have sequenced the DNA fragments long enough to ensure the two paired-ends could be joined (i.e., stitched) to make a complete 18S sequence for further downstream analyses. Instead, with the primers, MID tags and amplicon length, there was a 20- to 30-bp gap (i.e., nonsequenced bases) between the forward and reverse reads that resulted from the paired-end run. This resulted in thermosbaenaceans and isopods being excluded from the analysis despite the assay amplifying these taxa during PCR. The development of stygofauna metabarcoding assays will be an important part of operationalizing this tool in the future for biodiversity monitoring.

Similar to Oberprieler et al. (2021), we found both morphological and eDNA-based detection was limited in taxonomic resolution (Table 2). The lack of specificity in the morphological identifications probably occurred because of the cryptic differences between species, and poorly resolved taxonomy (Guzik et al., 2010; Humphreys et al., 2013). Like Korbel et al. (2017), the inconsistent detection of key stygofaunal taxa using eDNA metabarcoding indicates that an 18S assay alone is not yet good enough as a "stand-alone" method for stygofaunal surveys, even with the use of a custom reference database. While the development of more specific assays may increase the specificity and detection probability of eDNA metabarcoding-based surveys, currently, using a combination of both morphological characteristics and eDNA-based survey methods yields a greater diversity than haul-net sampling and morphological identification alone (as is currently standard protocol). Additionally, eDNA-based methods are notoriously unreliable for abundance estimates (Elbrecht & Leese, 2015), while morphological identification provides some population data on relative abundances of taxa from the specimens collected (Eberhard et al., 2009; Halse et al., 2014; Humphreys et al., 2013). Morphological and eDNA-based assessments of stygofaunal diversity are thus complementary, and combining these survey approaches provides a more complete assessment of the communities present in the aquifer under investigation.

TABLE 2 Stygofauna taxa detected using eDNA metabarcoding and morphological identification of haul-net specimens.

Order	eDNA taxon	Morph taxon
Ostracoda	Ostracoda BOLD:AEE1979	Ostracoda
Harpacticoida	*Cancrincola plumipes*	Harpacticoida
	Phyllognathopus	
Cyclopoida	*Diacyclops*	Cyclopidae
	Halicyclops rochai BOLD:AEE3240	Cyclopoida
Calanoida	*Stygoridgewayia trispinosa*	*Stygoridgewayia trispinosa*
	Calanoida	
Decapoda	*Stygiocaris stylifera*	*Stygiocaris stylifera*
	Pleocyemata	
Isopoda		*Haptolana pholeta*
		Isopoda
		Oniscidae
Amphipoda	*Nedsia A*	Amphipoda/*Nedsia/* Eriopsidae
		Nedsia? macrosculptilis
		Bogidomma
Thermosbaenacea		*Halosbaena tulki*
Bathynellacea	Parabathynellidae sp. Pilbara 17 EM-2019	Notobathynella
	Parabathynellidae sp. Pilbara 24 EM-2019	Parabathynellidae
Haplotaxida	Phallodrilinae A	
	Phallodrilinae B	
	Tubificina	
Macrostomida	Macrostomum	
Catenulida/Platyhelminthes	Paracatenula	Platyhelminthes
	Stenostomidae	
	Stenostomum A	
	Stenostomum B	
Polychaeta	Polychaeta A	
	Polychaeta B	
Eunicida	Ophryotrocha	
Perciformes	*Milyeringa justitia*	*Milyeringa justitia*

4.2 | Troglofauna

Like West et al. (2020) we detected troglofauna (e.g., Schizomida, Pseudoscorpiones) from eDNA metabarcoding of groundwater, and troglofauna were also incidentally collected in haul-nets as they were raised up from the borehole access point from the groundwater. These organisms typically live in geology surrounding the groundwater habitat and can fall in and can be eaten by stygofaunal predators; in this way troglofaunal detections from groundwater are analogous to using water samples from estuaries (Polanco et al., 2021) and ponds (Harper et al., 2019) to detect terrestrial diversity. While bores can act as large pitfall traps, depending on the location and size of the slots in the pipe, such results are not consistent between bores and frequently unknown. While there

are methods to assess troglofaunal communities (e.g., litter traps, Javidkar et al., 2018), the application of eDNA-based methods in this area is untested. Scrapes of soil from inside drilled holes may provide an adequate eDNA substrate; however, this is difficult on Barrow Island because the PVC casing of the bores prevents the collection of scrapes.

In addition to troglofauna, other taxa opportunistically detected using eDNA and occasionally haul-nets were surface-dwelling organisms (e.g., reptiles, ants, cockroaches, collembolans), animals that either fell in the boreholes or DNA being leeched through the soil to the groundwater. Other subterranean eDNA studies have also detected terrestrial taxa from groundwater, whether they sampled boreholes (Oberprieler et al., 2021) or caves (West et al., 2020). We avoided contact between sampling equipment and the ground to

minimize contamination, but any access to groundwater also risks exposing the groundwater to animals/DNA from the surface. This is why eDNA detection of an organism in groundwater is not necessarily proof of occupancy. However, this potential problem can be at least partly ameliorated by expert knowledge of the taxa concerned and their biology.

4.3 | Effect of substrate and sampling order on the diversity of stygofauna detected using eDNA

Methodological protocols for sampling eDNA metabarcoding are known to be critical considerations if these methods are to be used in future environmental impact assessment and monitoring (Díaz-Ferguson & Moyer, 2014; Dickie et al., 2018). In applying this knowledge to groundwater, we investigated the effectiveness of sampling various substrate types and the order of sampling to examine the impact of detecting community-wide diversity and composition using eDNA metabarcoding. For substrate type, we found that the treatments tested identified different components of the community (Figure 3). In shallow water, eDNA metabarcoding detected 100% of the total stygofauna detected in 2020, and 55% in 2021. In contrast, the deeper water samples detected only one additional stygofaunal taxon, and was also the substrate with the lowest total richness (Figure 3). This result agrees with previous traditional monitoring surveys using sampling techniques that were able to sample at various depths (Bork et al., 2008; Hahn, 2005). The drop in diversity in deeper water has generally been attributed to the decrease in oxygen and food supply with increasing depth (Figure S5; Brunke & Gonser, 1999; Datry et al., 2005).

Inverse to oxygen, salinity on Barrow Island increases with depth, and variation in salinity is known to influence stygofaunal community composition in coastal aquifers (Shapouri et al., 2016) and island cave systems (West et al., 2020). Salinity is one of the main drivers of adaptation, speciation and community assemblage in aquatic systems (Cunillera-Montcusí et al., 2022). Salinization can also alter bottom-up affects with the mobilization of base cations, changes in pH and the mobilization of nutrients (Cunillera-Montcusí et al., 2022). Several stygofaunal taxa (e.g., Harpacticoida, Decapoda, Ostracoda) have been previously associated with freshwater or brackish environments (West et al., 2020) while others (Thermosbaenacea) are primarily marine and appear to tolerate variable levels of salinity (Jaume, 2008). The taxa we detected primarily in deeper water samples (more saline, less oxygen) tended to be worms (Polychaeta) that can survive a range of salinities (Glasby, 1999). The stygofauna richness in deeper water samples was consistently low, as was the proportion of unique taxa in this substrate. In conclusion, if we were to exclude the deeper water samples, we would still be able to detect 95%–100% of the total taxonomic diversity detected using eDNA metabarcoding.

The diversity detected by sediment samples varied from 2020 (82%) to 2021 (90%). DNA tends to last longer in sediment than free floating in water (Corinaldesi et al., 2008; Turner et al., 2015). Mineral particles form electrostatic bonds that can protect the DNA

from damage (Hou et al., 2014) resulting in concentrations of DNA in sediments being up to 1800 times higher than in water (Turner et al., 2015). Consequently, the sediment samples were expected to contain the greatest diversity of stygofauna; however, our results were variable. In 2021, sediment samples were the richest, and detected the most stygofauna taxa, while in 2020 all the taxa detected from sediment samples could also be detected using shallow water samples (Figure 3). Rainfall events are responsible for both carbon and nutrient infiltration into groundwater systems, which is exploited by microbes and are direct and indirect diet sources of stygofauna (Saccò et al., 2021). Buxton et al. (2018) also found increased detection probability in pond water rather than sediment, and that there were seasonal fluctuations in both substrates. Considering the magnitude of change observed in the sediment samples, it is probable that we detected organisms that were alive in the last year, rather than very old DNA. This is reassuring because it means we were detecting primarily recent diversity rather than legacy eDNA, but an investigation of eDNA degradation in groundwater systems under different conditions is necessary to indicate how long eDNA can persist, especially in sediment. Combining sediment and water samples resulted in up to 43% more stygofauna detected, and the reduced overlap in the diversity detected by different substrates in 2021 (Figure 3b) indicates multiple substrates are necessary for effective detection of all aspects of the groundwater community.

Order of sampling (nets or eDNA first) did not make a significant difference to assessment of stygofauna richness or community composition, although there appeared to be variation between bores (Figure S4). For example, at one bore (SFRD3-03), *S. trispinosa* was associated with eDNA samples collected prior to nets, but for another (X62M) they were associated with samples collected after nets. The samples collected before disturbing the water column are typically less turbid and therefore easier to filter with fewer inhibitors than those collected after. Therefore, for improved field sampling methodologies (eDNA and haul-nets), it is possible to conduct the collection of groundwater first for eDNA work and then proceed with the haul-net collection of fauna for manual identification in the laboratory. However, we have not explicitly tested whether sampling for eDNA might impact the haul-net results. Such an undertaking would be extremely difficult because additional haul-net samples are more likely to be affected by the preceding net hauls than the preceding eDNA samples. In the present study, specimens were occasionally observed in the water samples, and larger individuals could be easily removed at the water filtering stage. Further, even if organisms were caught on the filter membrane, they did not overwhelm the results of stygofaunal diversity (Figure S4). In future, water samples could be prefiltered to ensure any specimens caught in eDNA samples are removed and identified, if required.

4.4 | Bore properties

There was not much overlap in stygofauna communities between sampling locations (bores), and bores closer together did not reveal

any greater similarity in community composition (Figure 5). Since hydrogeological studies have indicated that the superficial aquifer on Barrow Island is well connected, the distinct stygofauna communities within a bore are likely to reflect localized geological heterogeneities and microclimates. Suggesting minimal DNA dispersion, stygofaunal detections from groundwater eDNA probably reflect the stygofauna present in the bore, rather than the surrounding groundwater. Our result of geographical dissimilarity is likely to represent an artefact of sampling of bore habitats. While we know of almost no other way to sample groundwater aquifers in the absence of caves or surface incursions, it is well established that bores are an artificial representation of an aquifer community (Hahn & Matzke, 2005; Korbel et al., 2017). Bores act like large traps that attract fauna, and the abundance of stygofauna in unbaited phreatic traps is typically five times higher than the surrounding aquifer matrix (Bork et al., 2008). Bores (and traps) can have enriched oxygen and organic matter, as they comprise large volumes of open water which attract stygofauna (Hahn & Matzke, 2005). However, while the abundance of the taxa may be skewed, the diversity of taxa tends to remain the same in the bore/trap as in the aquifer (Bork et al., 2008; Hahn & Matzke, 2005). The exception to this may represent the ability of different methods to detect naturally rare taxa (Saccò, Guzik, et al., 2022). In fact, the increased abundance of taxa in bores may increase the detectability of stygofauna using eDNA methods, allowing sampling of an enriched community. Therefore, purging bores before sampling is not recommended for stygofaunal surveys specifically. Furthermore, if pumps are to be used, then the first flow-through will always contain the highest number of specimens (Oberprieler et al., 2021; Sorensen et al., 2013), and, consequently, the most DNA. However, purging a bore may be appropriate when the aim is to characterize the microbial communities of the aquifer (Korbel et al., 2017). Sampling the aquifer directly may require a greater volume of water and/or more replication, possibly using pumps with in-line filtration to sample a larger area.

This study took place at a single location, on an island with freshwater overlying saltwater. The results provide a valuable case study; however, they may not be representative of mainland groundwater systems. For example, Mouser et al. (2021) found that the velocity of groundwater increased the detectability of cavefish and cave crayfish using eDNA-based surveys, while the groundwater on Barrow is relatively still. Flowing water may also reduce the difference between inside and outside the bore, and further render purging the bore unnecessary. Similarly, while 5×1-L eDNA samples may be sufficient to obtain ~90% of the stygofaunal diversity (Figure 4), our model may be specific to this groundwater system, location and metabarcoding assay. Further studies in diverse ecosystems are needed to determine the across-system applicability of these results.

4.5 | Temporal variation

There was considerable variability in communities and substrates between the two sample years, as was expected since sampling occurred during a 3-year drought (2020), and the following year when rainfall increased (2021). Groundwater recharge plays an important role in nutrient availability and oxygenation (Datry et al., 2005; Saccò, Blyth, Humphreys, Middleton, et al., 2020), with recharge being related to rainfall (Hendrickx & Walker, 2017). Under high rainfall, even with low recharge rates, there are increases in carbon inputs that increase the food resources for stygofauna (Saccò et al., 2021; Saccò, Blyth, Humphreys, Middleton, et al., 2020). Not only were there significant shifts in stygofaunal ZOTU richness found in the different substrates (Figure 2), several bores also showed inconsistent results between years. For example, MW34, historically rich in stygofauna, produced no specimens using haul-nets in 2020, while eDNA of previously caught taxa could still be detected. During the drought period between 2017 and 2020 the stygofauna in MW34 may have still been present but at lower abundance or different life stages such that they were not found in haul-net samples, but were still detected using eDNA metabarcoding. This hypothesis is supported by the fact that stygofauna were again collected from MW34 in 2021. The findings of Herrmann et al. (2020) show that bacteria depend on the availability of organic or inorganic electron donors, while protists and Metazoa often have a broad prey spectrum and the hydrochemistry-driven distribution patterns were stronger for bacteria than for the eukaryotic communities. There may also be an element of stochasticity involved, considering not all bores showed the same trends. BGMW04, for example, produced specimens of Harpacticoida in 2020 but not in 2021. As we only had two sampling periods a year apart, we cannot comment on the rate at which stygofauna communities may change and how they may vary across smaller timescales. Seasonality can affect not only stygofauna communities (Saccò, Blyth, Humphreys, Karasiewicz, et al., 2020), but also eDNA degradation and detection probabilities (Troth et al., 2021). Repeated sampling, preferably in different seasons, is necessary to assess stygofaunal diversity using both morphological identification and eDNA metabarcoding.

5 | CONCLUSION

This study demonstrated that eDNA metabarcoding has the potential to improve the assessment of stygofaunal communities by widening the breadth of biodiversity that can be detected. It can act as a noninvasive tool that is complementary to morphological assessments and able to detect additional taxa (i.e., food web complexity and trophic levels) that are often missed (e.g., Platyhelminthes, Annelida). The study has also demonstrated the need for multiple substrates and sampling events to account for natural temporal variability in an arid and subtropical continental island off the coast of Western Australia. To standardize subterranean eDNA metabarcoding for environmental monitoring we must address the bias in taxa detected by improving and validating metabarcoding assays for stygofaunal groups so that all major taxa can be detected. Sequencing specimens for primer design will naturally also improve reference databases inclusive of provenance and intraspecies variability, which

is particularly important when working with ancient lineages of short-range subterranean endemic species with the future goal of making reliable molecular, species-level identifications possible.

While further optimization is necessary, we have shown the feasibility of this method for assessing stygofaunal communities in these diverse and largely unstudied subterranean ecosystems. Groundwater is an important global resource that hosts ancient lineages, with many short-range endemics. If we do not have the tools to accurately assess these communities, there is no way of knowing how they are affected by human activity.

CONFLICT OF INTEREST STATEMENT
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available on the dryad digital repository at https://doi.org/10.5061/dryad.dv41ns22g.

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REFERENCES
Allford, A., Cooper, S. J. B., Humphreys, W. F., & Austin, A. D. (2008). Diversity and distribution of groundwater fauna in a calcrete aquifer: Does sampling method influence the story? *Invertebrate Systematics*, 22(2), 127. https://doi.org/10.1071/IS07058

Andrews, S. (2010). *FastQC*: A quality control tool for high throughput sequence data [Online]. Retrieved from http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

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(2), 492–514. https://doi.org/10.1002/edn3.141

Baker, S. J., Niemiller, M. L., Stites, A. J., Ash, K. T., Davis, M. A., Dreslik, M. J., & Phillips, C. A. (2020). Evaluation of environmental DNA to detect Sistrurus catenatus and *Ophidiomyces ophiodiicola* in crayfish burrows. *Conservation Genetics Resources*, 12, 13–15.

Barnes, M. A., Turner, C. R., Jerde, C. L., Renshaw, M. A., Chadderton, W. L., & Lodge, D. M. (2014). Environmental conditions influence eDNA persistence in aquatic systems. *Environmental Science and Technology*, 48(3), 1819–1827. https://doi.org/10.1021/es404734p

Bohmann, K., Evans, A., Gilbert, M. T. P., Carvalho, G. R., Creer, S., Knapp, M., Yu, D. W., & de Bruyn, M. (2014). Environmental DNA for wildlife biology and biodiversity monitoring. *Trends in Ecology and Evolution*, 29(6), 358–367. https://doi.org/10.1016/j.tree.2014.04.003

Bork, J., Bork, S., Berkhoff, S. E., & Hahn, H. J. (2008). Testing unbaited stygofauna traps for sampling performance. *Limnologica*, 38(2), 105–115. https://doi.org/10.1016/j.limno.2007.10.001

Boyd, S. H., Niemiller, 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(12), 1–13. https://doi.org/10.1371/journal.pone.0242741

Boyer, F., Mercier, C., Bonin, A., Le Bras, Y., Taberlet, P., & Coissac, E. (2016). Obitools: A unix-inspired software package for DNA metabarcoding. *Molecular Ecology Resources*, 16(1), 176–182. https://doi.org/10.1111/1755-0998.12428

Bradford, T., Adams, M., Humphreys, W. F., Austin, A. D., & Cooper, S. J. B. (2010). DNA barcoding of stygofauna uncovers cryptic amphipod diversity in a calcrete aquifer in Western Australia's arid zone. *Molecular Ecology Resources*, 10(1), 41–50. https://doi.org/10.1111/j.1755-0998.2009.02706.x

Brunke, M., & Gonser, T. (1999). Hyporheic invertebrates: The clinal nature of interstitial communities structured by hydrological exchange and environmental gradients. *Journal of the North American*, 18(3), 344–362.

Burbidge, A. (2004). *Threatened animals of Western Australia*. Department of Conservation and Land Management. Chevron.

Buxton, A. S., Groombridge, J. J., & Griffiths, R. A. (2018). Seasonal variation in environmental DNA detection in sediment and water samples. *PLoS One*, 13(1), 1–14. https://doi.org/10.1371/journal.pone.0191737

Cardoso, P., Erwin, T. L., Borges, P. A. V., & New, T. R. (2011). The seven impediments in invertebrate conservation and how to overcome them. *Biological Conservation*, 144(11), 2647–2655. https://doi.org/10.1016/j.biocon.2011.07.024

Cáceres, M. D., & Legendre, P. (2009). Associations between species and groups of sites: Indices and statistical inference. *Ecology*, 90, 3566–3574. https://doi.org/10.1890/08-1823.1

Chevron. (2006). *Final environmental impact statement/response to submissions on the environmental review and management Programme for the proposed gorgon development*. Chevron.

Corinaldesi, C., Beolchini, F., & Dell'Anno, A. (2008). Damage and degradation rates of extracellular DNA in marine sediments: Implications for the preservation of gene sequences. *Molecular Ecology*, 17(17), 3939–3951. https://doi.org/10.1111/j.1365-294X.2008.03880.x

Crosbie, R., Jolly, I., Leaney, F., Petheram, C., & Wohling, D. (2010). *Review of Australian groundwater recharge studies*. CSIRO: Water for a Healthy Country National Research Flagship (April), 8 pp. Retrieved from www.csiro.au/org/HealthyCountry.html%0Ahttp://www.clw.csiro.au/publications/waterforahealthycountry/2010/wfhc-review-Australian-recharge.pdf

Cunillera-Montcusí, D., Beklioğlu, M., Cañedo-Argüelles, M., Jeppesen, E., Ptacnik, R., Amorim, C. A., Arnott, S. E., Berger, S. A., Brucet, S., Dugan, H. A., Gerhard, M., Horváth, Z., Langenheder, S., Nejstgaard, J. C., Reinikainen, M., Striebel, M., Urrutia-Cordero, P., Vad, C. F., Zadereev, E., & Matias, M. (2022). Freshwater salinisation: A research agenda for a saltier world. *Trends in Ecology and Evolution*, 37(5), 440–453. https://doi.org/10.1016/j.tree.2021.12.005

Danielopol, D. A. N. L., & Griebler, C. (2007). Incorporation of groundwater ecology in environmental policy. In *Groundwater science and policy: An international overview* (pp.671689). Royal Society of Chemistry.

Danielopol, D. L., Griebler, C., Gunatilaka, A., & Notenboom, J. (2003). Present state and future prospects for groundwater ecosystems. *Environmental Conservation*, 30(2), 104–130. https://doi.org/10.1017/S0376892903000109

Datry, T., Malard, F., & Gibert, J. (2005). Response of invertebrate assemblages to increased groundwater recharge rates in a phreatic aquifer. *Journal of the North American Benthological Society*, 24(3), 461–477.

Des Châtelliers, M. C., Juget, J., Lafont, M., & Martin, P. (2009). Subterranean aquatic Oligochaeta. *Freshwater Biology*, 54(4), 678–690. https://doi.org/10.1111/j.1365-2427.2009.02173.x

Díaz-Ferguson, E. E., & Moyer, G. R. (2014). History, applications, methodological issues and perspectives for the use environmental DNA (eDNA) in marine and freshwater environments. *Revista de Biología Tropical*, 62(4), 1273–1284. https://doi.org/10.15517/rbt.v62i4.13231

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., Powell, J. R., & Weaver, L. (2018). Towards robust and repeatable sampling methods in eDNA-based studies. *Molecular Ecology Resources*, 18(5), 940–952. https://doi.org/10.1111/1755-0998.12907

Dray, S., & Dufour, A.-B. (2007). The ade4 package: Implementing the duality diagram for ecologists. *Journal of Statistical Software*, 22(4), 1–20. https://doi.org/10.18637/jss.v022.i04

Dumas, P., & Fontanini, G. (2001). Sampling fauna in aquifers: A comparison of net-sampling and pumping. *Archiv Fur Hydrobiologie*, 150(4), 661–676. https://doi.org/10.1127/archiv-hydrobiol/150/2001/661

Eberhard, S. M., Halse, S. A., & Humphreys, W. F. (2005). Stygofauna in the Pilbara region, north-West Western Australia: A review. *Journal of the Royal Society of Western Australia*, 88(4), 167–176.

Eberhard, S. M., Halse, S. A., Williams, M. R., Scanlon, M. D., Cocking, J., & Barron, H. J. (2009). Exploring the relationship between sampling efficiency and short-range endemism for groundwater fauna in the Pilbara region, Western Australia. *Freshwater Biology*, 54(4), 885–901. https://doi.org/10.1111/j.1365-2427.2007.01863.x

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, 32(5), 1792–1797.

Edgar, R. C. (2016). UNOISE2: Improved error-correction for Illumina 16S and ITS amplicon sequencing. *BioRxiv*, 081257. https://doi.org/10.1101/081257

Elbrecht, V., & Leese, F. (2015). Can DNA-based ecosystem assessments quantify species abundance? Testing primer bias and biomass-sequence relationships with an innovative metabarcoding protocol. *PLoS ONE*, 10, e0130324.

EPA (WA). (2003). Guidance for the assessment of environmental factors: Consideration of subterranean Fauna in groundwater and caves during environmental impact assessment in Western Australia Environmental Protection Authority, Perth, Australia.

Fediajevaite, J., Priestley, V., Arnold, R., & Savolainen, V. (2021). Meta-analysis shows that environmental DNA outperforms traditional surveys, but warrants better reporting standards. *Ecology and Evolution*, 11(9), 4803–4815. https://doi.org/10.1002/ece3.7382

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(22), 15664–15682. https://doi.org/10.1002/ece3.8220

Gibson, L., Humphreys, W. F., Harvey, M., Hyder, B., & Winzer, A. (2019). Shedding light on the hidden world of subterranean fauna: A transdisciplinary research approach. *Science of the Total Environment*, 684, 381–389. https://doi.org/10.1016/j.scitotenv.2019.05.316

Glasby, C. (1999). The Namanereidinae (polychaeta: Nereididae). Part 1, taxonomy and phylogeny. *Records of the Australian Museum, supplement* (Vol. 25, 1-129). The Australian Museum. http://www.annelida.net/docs/glasby99all.pdf

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(February), 91–93. https://doi.org/10.1038/srep45054

Guzik, M. T., Austin, A. D., Cooper, S. J. B., Harvey, M. S., Humphreys, W. F., Bradford, T., Eberhard, S. M., King, R. A., Leys, R., Muirhead, K. A., & Tomlinson, M. (2010). Is the Australian subterranean fauna uniquely diverse? *Invertebrate Systematics*, 24(5), 407–418. https://doi.org/10.1071/IS10038

Hahn, H. J. (2005). Unbaited phreatic traps: A new method of sampling stygofauna. *Limnologica*, 35(4), 248–261. https://doi.org/10.1016/j.limno.2005.04.004

Hahn, H. J., & Matzke, D. (2005). A comparison of stygofauna communities inside and outside groundwater bores. *Limnologica*, 35(1–2), 31–44. https://doi.org/10.1016/j.limno.2004.09.002

Halse, S. A., Scanlon, M. D., Cocking, J. S., Barron, H. J., Richardson, J. B., & Eberhard, S. M. (2014). Pilbara stygofauna: Deep groundwater of an arid landscape contains globally significant radiation of biodiversity. *Records of the Western Australian Museum, Supplement*, 78(2), 443. https://doi.org/10.18195/issn.0313-122x.78(2).2014.443-483

Harper, L. R., Lawson Handley, L., Carpenter, A. I., Ghazali, M., Di Muri, C., Macgregor, C. J., Logan, T. W., Law, A., Breithaupt, T., Read, D. S., McDevitt, A. D., & Hänfling, B. (2019). Environmental DNA (eDNA) metabarcoding of pond water as a tool to survey conservation and management priority mammals. *Biological Conservation*, 238(August), 108225. https://doi.org/10.1016/j.biocon.2019.108225

Hendrickx, J. M., & Walker, G. R. (2017). *Recharge from precipitation. Recharge of phreatic aquifers in (semi-) arid areas*. Routledge. IAH International Contributions to Hydrogeology.

Herrmann, M., Geesink, P., Yan, L., Lehmann, R., Totsche, K. U., & Küsel, K. (2020). Complex food webs coincide with high genetic potential for chemolithoautotrophy in fractured bedrock groundwater. *Water Research*, 170, 115306. https://doi.org/10.1016/j.watres.2019.115306

Holm, S. (1979). A simple sequentially rejective multiple test procedure. *Scandinavian Journal of Statistics*, 6(2), 65–70.

Hou, Y., Wu, P., & Zhu, N. (2014). The protective effect of clay minerals against damage to adsorbed DNA induced by cadmium and mercury. *Chemosphere*, 95, 206–212. https://doi.org/10.1016/j.chemosphere.2013.08.069

Humphreys, G., Alexander, J., Harvey, M. S., & Humphreys, W. F. (2013). The subterranean fauna of Barrow Island, North-Western Australia: 10 years on. *Records of the Western Australian Museum, Supplement*, 83(1), 145. https://doi.org/10.18195/issn.0313-122x.83.2013.145-158

Humphreys, W. F. (1993). Stygofauna in semi-arid tropical Western Australia: A tethyan connection? *Memoires de Biospeologie*, 20, 111–116.

Humphreys, W. F., & Feinbergz, M. N. (1995). Food of the blind cave fishes of northwestern Australia. *Records of the Western Australian Museum*, 33, 29–33.

Humphreys, W. H. (2001). Groundwater calcrete aquifers in the Australian arid zone: The context to an unfolding plethora of stygal biodiversity. *Records of the Western Australian Museum, Supplement*, 64(1), 63. https://doi.org/10.18195/issn.0313-122x.64.2001.063-083

Huson, D. H., Auch, A. F., Qi, J., & Schuster, S. C. (2007). MEGAN analysis of metagenomic data. *Genome Research*, 17(3), 377–386. https://doi.org/10.1101/gr.5969107

Hyde, J., Cooper, S. J. B., Humphreys, W. F., Austin, A. D., & Munguia, P. (2018). Diversity patterns of subterranean invertebrate fauna in calcretes of the Yilgarn region, Western Australia. *Marine and Freshwater Research*, 69(1), 114–121. https://doi.org/10.1071/MF17005

Jaume, D. (2008). Global diversity of spelaeogriphaceans & thermosbaenaceans (crustacea; Spelaeogriphacea & Thermosbaenacea) in freshwater. *Hydrobiologia*, 595(1), 219–224. https://doi.org/10.1007/s10750-007-9017-1

Javidkar, M., Cooper, S. J. B., Humphreys, W. F., King, R. A., Judd, S., & Austin, A. D. (2018). Biogeographic history of subterranean isopods from groundwater calcrete islands in Western Australia. *Zoologica Scripta*, 47(2), 206–220. https://doi.org/10.1111/zsc.12265

Jeunen, G. J., Lamare, M. D., Knapp, M., Spencer, H. G., Taylor, H. R., Stat, M., Bunce, M., & Gemmell, N. J. (2020). Water stratification in the marine biome restricts vertical environmental DNA (eDNA) signal dispersal. *Environmental DNA*, 2(1), 99–111. https://doi.org/10.1002/edn3.49

Juan, C., Guzik, M. T., Jaume, D., & Cooper, S. J. B. (2010). Evolution in caves: Darwin's "wrecks of ancient life" in the molecular era. *Molecular Ecology*, 19(18), 3865–3880. https://doi.org/10.1111/j.1365-294X.2010.04759.x

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(January), 1–13. https://doi.org/10.1038/srep40702

Koziol, A., Stat, M., Simpson, T., Jarman, S., Harvey, E. S., Marnane, M., McDonald, J., & Bunce, M. (2018). Environmental DNA metabarcoding studies are critically affected by substrate selection. *Molecular Ecology Resources*, 19(September), 1–11. https://doi.org/10.1111/1755-0998.12971

Larson, H. K., Foster, R., Humphreys, W. F., & Stevens, M. I. (2013). A new species of the blind cave gudgeon Milyeringa (Pisces: Gobioidei, Eleotridae) from Barrow Island, Western Australia, with a redescription of M. veritas Whitley. *Zootaxa*, 3616(2), 135–150. https://doi.org/10.11646/zootaxa.3616.2.3

Mammola, S., Cardoso, P., Culver, D. C., Deharveng, L., Ferreira, R. L., Fišer, C., Galassi, D. M. P., Griebler, C., Halse, S., Humphreys, W. F., Isaia, M., Malard, F., Martinez, A., Moldovan, O. T., Niemiller, M. L., Pavlek, M., Reboleira, A. S. P. S., Souza-Silva, M., Teeling, E. C., … Zagmajster, M. (2019). Scientists' warning on the conservation of subterranean ecosystems. *Bioscience*, 69(8), 641–650. https://doi.org/10.1093/biosci/biz064

Mammola, S., Meierhofer, M. B., Borges, P. A. V., Colado, R., Culver, D. C., Deharveng, L., Delić, T., di Lorenzo, T., Dražina, T., Ferreira, R. L., Fiasca, B., Fišer, C., Galassi, D. M. P., Garzoli, L., Gerovasileiou, V., Griebler, C., Halse, S., Howarth, F. G., Isaia, M., … Cardoso, P. (2022). Towards evidence-based conservation of subterranean ecosystems. *Biol Rev Camb Philos Soc*, 97, 1476–1510. https://doi.org/10.1111/brv.12851

McMurdie, P. J., & Holmes, S. (2013). Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*, 8(4), e61217. https://doi.org/10.1371/journal.pone.0061217

Meredith, K. (2009). Radiocarbon age dating groundwaters of the West Canning Basin, Western Australia. (March), 47.

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

Moore, G. I., Humphreys, W. F., & Foster, R. (2018). New populations of the rare subterranean blind cave eel Ophisternon candidum (Synbranchidae) reveal recent historical connections throughout North-Western Australia. *Marine and Freshwater Research*, 69(10), 1517–1524. https://doi.org/10.1071/MF18006

Moro, D., & Lagdon, R. (2013). History and environment of Barrow Island. *Records of the Western Australian Museum, Supplement*, 83(1), 1. https://doi.org/10.18195/issn.0313-122x.83.2013.001-008

Mousavi-Derazmahalleh, M., Stott, A., Lines, R., Peverley, G., Nester, G., Simpson, T., Zawierta, M., de la Pierre, M., Bunce, M., & Christophersen, C. T. (2021). eDNAFlow, an automated, reproducible and scalable workflow for analysis of environmental DNA sequences exploiting Nextflow and singularity. *Molecular Ecology Resources*, 21(5), 1697–1704. https://doi.org/10.1111/1755-0998.13356

Mouser, J. B., Brewer, S. K., Niemiller, M. L., Mollenhauer, R., & Van Den Bussche, R. A. (2021). Refining sampling protocols for cavefishes and cave crayfishes to account for environmental variation. *Subterranean Biology*, 39, 79–105. https://doi.org/10.3897/subtbiol.39.64279

Murray, D. C., Coghlan, M. L., & Bunce, M. (2015). From benchtop to desktop: Important considerations when designing amplicon sequencing workflows. *PLoS One*, 10(4), e0124671. https://doi.org/10.1371/journal.pone.0124671

Niemiller, M. L., Taylor, S. J., & Bichuette, M. E. (2018). Conservation of cave fauna, with an emphasis on Europe and the Americas. In *Cave ecology* (pp. 451–478). Springer.

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(2), 247–257. https://doi.org/10.1007/s12686-017-0785-2

NSW-SGDEP. (2002). The NSW state groundwater dependent ecosystem policy New South Wales government—Department of Land and Water Conservation, New South Wales, Australia.

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. https://doi.org/10.1016/j.scitotenv.2021.148955

Page, T. J., Hughes, J. M., Real, K. M., Stevens, M. I., King, R. A., & Humphreys, W. F. (2018). Allegory of a cave crustacean: Systematic and biogeographic reality of Halosbaena (Peracarida: Thermosbaenacea) sought with molecular data at multiple scales. *Marine Biodiversity*, 48(2), 1185–1202. https://doi.org/10.1007/s12526-016-0565-3

Pochon, X., Bott, N. J., Smith, K. F., & Wood, S. A. (2013). Evaluating detection limits of next-generation sequencing for the surveillance and monitoring of international marine pests. *PLoS One*, 8(9), e73935. https://doi.org/10.1371/journal.pone.0073935

Polanco, F. A., Mutis Martinezguerra, M., Marques, V., Villa-Navarro, F., Borrero Pérez, G. H., Cheutin, M. C., Dejean, T., Hocdé, R., Juhel, J.-B., Maire, E., Manel, S., Spescha, M., Valentini, A., Mouillot, D., Albouy, C., & Pellissier, L. (2021). Detecting aquatic and terrestrial biodiversity in a tropical estuary using environmental DNA. *Biotropica*, 53(6), 1606–1619. https://doi.org/10.1111/btp.13009

R Core Team. (2018). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. https://www.R-project.org

Ribera, I., Fresneda, J., Bucur, R., Izquierdo, A., Vogler, A. P., Salgado, J. M., & Cieslak, A. (2010). Ancient origin of a Western Mediterranean radiation of subterranean beetles. *BMC Evolutionary Biology*, 10(1), 29. https://doi.org/10.1186/1471-2148-10-29

Ritz, C., Baty, F., Streibig, J. C., & Gerhard, D. (2015). Dose-response analysis using R. *PLOS One*, 10(12), e0146021. https://doi.org/10.1371/journal.pone.0146021

Russel, J. (2021). MicEco: Various functions for microbial community data. R package version 0.9.15.

Saccò, M., Blyth, A. J., Humphreys, W. F., Cooper, S. J. B., White, N. E., Campbell, M., Mousavi-Derazmahalleh, M., Hua, Q., Mazumder, D.,

Smith, C., Griebler, C., & Grice, K. (2021). Rainfall as a trigger of ecological cascade effects in an Australian groundwater ecosystem. *Scientific Reports*, *11*(1), 1–15. https://doi.org/10.1038/s41598-021-83286-x

Saccò, M., Blyth, A. J., Humphreys, W. F., Karasiewicz, S., Meredith, K. T., Laini, A., Cooper, S. J. B., Bateman, P. W., & Grice, K. (2020). Stygofaunal community trends along varied rainfall conditions: Deciphering ecological niche dynamics of a shallow calcrete in Western Australia. *Ecohydrology*, *13*(1), 1–19. https://doi.org/10.1002/eco.2150

Saccò, M., Blyth, A. J., Humphreys, W. F., Middleton, J. A., White, N. E., Campbell, M., Mousavi-Derazmahalleh, M., Laini, A., Hua, Q., Meredith, K., Cooper, S. J. B., Griebler, C., Allard, S., Grierson, P., & Grice, K. (2020). Tracking down carbon inputs underground from an arid zone Australian calcrete. *PLoS One*, *15*(8), 1–22. https://doi.org/10.1371/journal.pone.0237730

Saccò, M., Campbell, M. A., Nevill, P., Humphreys, W. F., Blyth, A. J., Grierson, P. F., & White, N. E. (2022). Getting to the root of organic inputs in groundwaters: Stygofaunal plant consumption in a Calcrete aquifer. *Frontiers in Ecology and Evolution*, *10*(March), 1–9. https://doi.org/10.3389/fevo.2022.854591

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. (2022). eDNA in subterranean ecosystems: Applications, technical aspects, and future prospects. *Science of the Total Environment*, *820*, 153223. https://doi.org/10.1016/j.scitotenv.2022.153223

Schubert, M., Lindgreen, S., & Orlando, L. (2016). AdapterRemoval v2: Rapid adapter trimming, identification, and read merging. *BMC Research Notes*, *9*(1), 1–7. https://doi.org/10.1186/s13104-016-1900-2

Shapouri, M., Cancela da Fonseca, L., Iepure, S., Stigter, T., Ribeiro, L., & Silva, A. (2016). The variation of stygofauna along a gradient of salinization in a coastal aquifer. *Hydrology Research*, *47*(1), 89–103. https://doi.org/10.2166/nh.2015.153

Smith, R. J., Paterson, J. S., Launer, E., Tobe, S. S., Morello, E., Leijs, R., Marri, S., & Mitchell, J. G. (2016). Stygofauna enhance prokaryotic transport in groundwater ecosystems. *Scientific Reports*, *6*(August), 1–7. https://doi.org/10.1038/srep32738

Sorensen, J. P. R., Maurice, L., Edwards, F. K., Lapworth, D. J., Read, D. S., Allen, D., Butcher, A. S., Newbold, L. K., Townsend, B. R., & Williams, P. J. (2013). Using boreholes as windows into groundwater ecosystems. *PLoS One*, *8*(7), e70264. https://doi.org/10.1371/journal.pone.0070264

Stat, M., Huggett, M. J., Bernasconi, R., DiBattista, J., Berry, T. E., Newman, S. J., Harvey, E. S., & Bunce, M. (2017). Ecosystem biomonitoring with eDNA: Metabarcoding across the tree of life in a tropical marine environment. *Scientific Reports*, *7*(1), 12240. https://doi.org/10.1038/s41598-017-12501-5

Stumpp, C., & Hose, G. C. (2013). The impact of water table drawdown and drying on subterranean aquatic fauna in in-vitro experiments. *PLoS One*, *8*(11), e78502. https://doi.org/10.1371/journal.pone.0078502

SWPO. (1998). Swiss Water Protection Ordinance (Gewässerschutzverordnung) Swiss Federal Council (Schweizer Bundesrat), Bern, Switzerland.

Taberlet, P., Coissac, E., Hajibabaei, M., & Rieseberg, L. H. L. H. (2012). Environmental DNA. *Molecular Ecology*, *21*(8, SI), 1789–1793. https://doi.org/10.1111/j.1365-294X.2012.05542.x

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

Troth, C. R., Sweet, M. J., Nightingale, J., & Burian, A. (2021). Seasonality, DNA degradation and spatial heterogeneity as drivers of eDNA detection dynamics. *Science of the Total Environment*, *768*, 144466. https://doi.org/10.1016/j.scitotenv.2020.144466

Turner, C. R., Uy, K. L., & Everhart, R. C. (2015). Fish environmental DNA is more concentrated in aquatic sediments than surface water. *Biological Conservation*, *183*, 93–102. https://doi.org/10.1016/j.biocon.2014.11.017

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*(1), 12–14. https://doi.org/10.1371/journal.pone.0170945

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*(1), 1–15. https://doi.org/10.1038/s41598-020-78525-6

Whitaker, D., & Christman, M. (2014). clustsig: Significant cluster analysis. R package version 1.1. https://CRAN.R-Project.Org/Package=clustsig. Retrieved from http://www.douglaswhitaker.com

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*(15), 3201–3211. https://doi.org/10.1007/s10750-020-04304-z

Zhang, J., Kapli, P., Pavlidis, P., & Stamatakis, A. (2013). A general species delimitation method with applications to phylogenetic placements. *Bioinformatics*, *29*(22), 2869–2876. https://doi.org/10.1093/bioinformatics/btt499

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.