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eDNA in subterranean ecosystems: Applications, technical aspects, and future prospects

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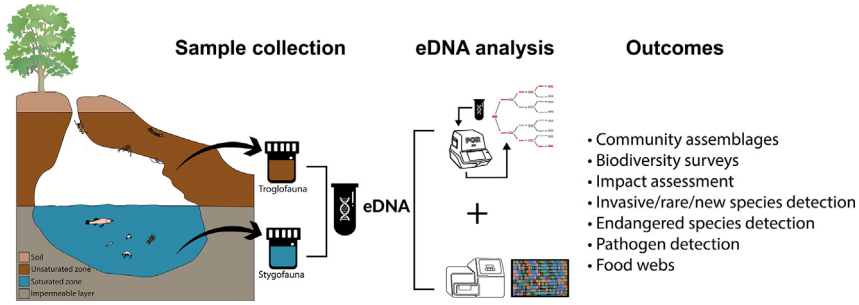
HIGHLIGHTS

- eDNA is a powerful but underused biomonitoring tool in subterranean environments.
- Metabarcoding data can unveil community assemblages and invasive/rare/new species.
- Recent advances can overcome limitations and caveats for eDNA analysis underground.
- Combination of eDNA with conventional tests will allow improved ecological surveys.

GRAPHICAL ABSTRACT



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ABSTRACT

Monitoring of biota is pivotal for the assessment and conservation of ecosystems. Environments worldwide are being continuously and increasingly exposed to multiple adverse impacts, and the accuracy and reliability of the biomonitoring tools that can be employed shape not only the present, but more importantly, the future of entire habitats. The analysis of environmental DNA (eDNA) metabarcoding data provides a quick, affordable, and reliable molecular approach for biodiversity assessments. However, while extensively employed in aquatic and terrestrial surface environments, eDNA-based studies targeting subterranean ecosystems are still uncommon due to the lack of accessibility and the cryptic nature of these environments and their species. Recent advances in genetic and genomic analyses have established a promising framework for shedding new light on subterranean biodiversity and ecology. To address current knowledge and the future use of eDNA methods in groundwaters and caves, this review explores conceptual and technical aspects of the application and its potential in subterranean systems. We briefly introduce subterranean biota and describe the most used traditional sampling techniques. Next, eDNA characteristics, application, and limitations in the subsurface environment are outlined. Last, we provide suggestions on how to overcome caveats and delineate some of the research avenues that will likely shape this field in the near future. We advocate that eDNA analyses, when carefully conducted and ideally combined with conventional sampling techniques, will substantially increase understanding and enable crucial expansion of subterranean community characterisation. Given the importance of groundwater

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and cave ecosystems for nature and humans, eDNA can bring to the surface essential insights, such as study of ecosystem assemblages and rare species detection, which are critical for the preservation of life below, as well as above, the ground.

Contents

1. The hidden world of subterranean environments

Subterranean environments not only provide vital resources to humanity, such as key water reservoirs for industry, agriculture, and human consumption (Danielopol et al., 2003), but also host complex ecosystems, the diversity and function of which are just being revealed (Mammola et al., 2020; Saccò et al., 2019a). Compared to their surficial freshwater counterparts, subterranean habitats, due to their poor accessibility and the cryptic nature of their inhabitants (Hancock et al., 2005), provide challenging and fascinating natural laboratories to investigate ecological dynamics (Griebler et al., 2014) and evolutionary trends (Mammola, 2019). Moreover, the lack of light triggers a characteristic absence of primary producers in caves and groundwaters (Danielopol et al., 2000), environments that have been long mistakenly considered a simplified version of surficial habitats (Tomlinson et al., 2007 and references therein). In fact, recent studies focusing on subterranean biotic interactions and evolutionary patterns are unravelling complex adaptive processes, including highly divergent rates of molecular evolution (e.g., Deharveng et al., 2008; Langille et al., 2021), extensive short-range endemism (Guzik et al., 2019; Harvey et al., 2011), resistance to starvation (e.g., Ercoli et al., 2019; Francois et al., 2020; Iliffe and Kornicker, 2009), and species-specific energy flows and food web interactions (Herrmann et al., 2020; Saccò et al., 2019b, 2021a; Weitowitz et al., 2019).

Animal life underground can be split into two major groups according to the life history strategies of their inhabitants: stygofauna and troglofauna. Stygofaunal species are groundwater-dwelling aquatic fauna that occupy the interstitial spaces, voids, and fissures in alluvial, karstic, or fractured rock aquifers, while troglofauna refer to biota which occur in the same habitats but above the water table (see Fig. 1 for examples) (Culver and Pipan, 2019). The major taxon groups of stygofauna include: Pisces, Decapoda, Coleoptera (Dytiscidae), Amphipoda, Isopoda, Bathynellacea, Ostracoda, Copepoda, Gastropoda as well as water mites and annelids; and for troglofauna: Hexapoda, Arachnida, Myriapoda, Crustacea at a minimum (e.g., Australia: Guzik et al., 2011, Saccò et al., 2019a; Europe: Deharveng et al., 2009; USA: Niemiller et al., 2019; South-East Asia: Brancelj et al., 2013). Both groups are essential for the ecological functioning of subterranean ecosystems, allowing the transition from microbially-mediated nutrients and organic sources to the upper levels in food chains characterised by a scarcity of top-predators (Saccò et al., 2021b and references therein; Simon et al., 2003; Venarsky and Huntsman, 2018; Venarsky et al., 2022). Biotic communities underground have been investigated through multiple sampling techniques, depending on the accessibility of the environments (i.e., caves, groundwater aquifers, alluvium, etc.), and the taxonomic groups being targeted for study (i.e., stygofauna vs troglofauna), among many other factors (Fig. 1).

Environmental DNA (eDNA) refers to DNA found in the environment as a result of biotic activities which can be collected via sampling the environment (e.g., water, soil, and/or air) in which organisms reside (Barnes and Turner, 2015; Rees et al., 2014a). This DNA is shed into the environment and comes from skin, faeces, saliva or reproductive fluids or from decomposing tissues, and typically exists in a highly fragmented state at ultra-low concentrations. eDNA, in combination with Next Generation Sequencing (NGS), is quickly becoming a go-to for environmental biomonitoring (Ficetola et al., 2008; Leese et al., 2016, 2018) as a cheaper (Wang et al., 2018), less invasive (Beja-Pereira et al., 2009), and potentially more accurate (Lejzerowicz et al., 2015) method for assessing and exploring biodiversity. Species identification via eDNA has been used for an array of biological studies including invasive species monitoring (Takahara et al., 2013), monitoring rare and endangered species (Biggs et al., 2015; Dougherty et al., 2016; Thomsen et al., 2012; White et al., 2020), and biodiversity estimates in different environments (Bista et al., 2017; Bohmann et al., 2014; Cristescu, 2014). In aquatic habitats, the analysis of eDNA has already proved to be highly accurate for identifying species assemblages, including novel studies of marine (Lobo et al., 2017; Stat et al., 2017), hypersaline

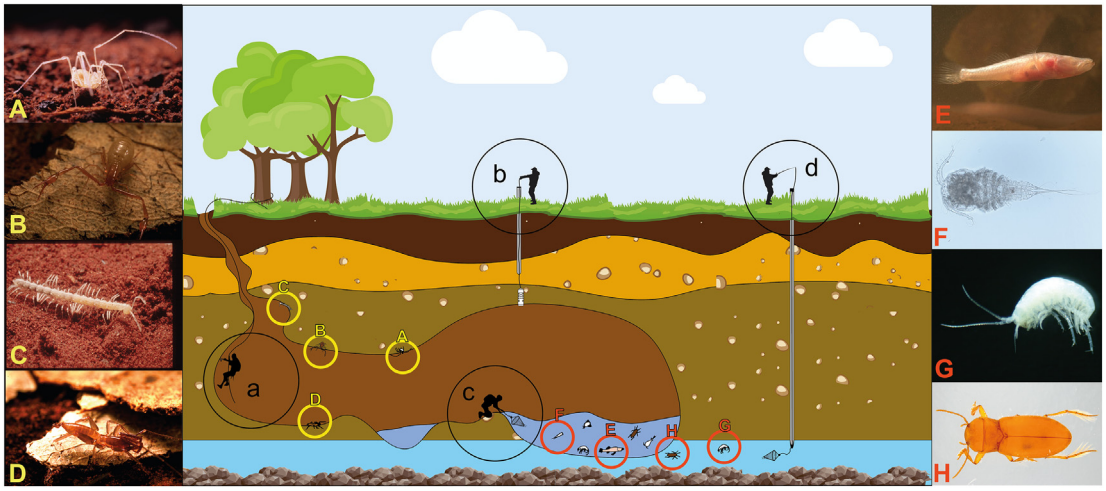

Fig. 1. Main sampling techniques for troglofauna (a - direct collection, and b - litter trapping) and stygofauna (c - direct collection and d - net hauling) together with some of the taxon groups present. Troglofauna in yellow from A to D (A - *Glennhuntia glennhunti* Shear, 2001 (Opiliones: Phalangodidae) (©Patrick Baker, Western Australian Museum); B - *Indohya humphreysi* Harvey, 1993 (Pseudoscorpiones: Hyidae) (©Patrick Baker, Western Australian Museum); C - *Stygiochiropus communis* Humphreys & Shear, 1993 (Polydesmida: Paradaxosomatidae) (©Bill Humphreys, Western Australian Museum); D - *Draculoides brooksi* Harvey, 2001 (Schizomida: Hubbardiidae) (©Patrick Baker, Western Australian Museum). Stygofauna in red from E to H (E - *Milyeringa veritas* Whitley, 1945 (Gobiiformes: Milyeringidae) (©Douglas Elford, Western Australian Museum); F – unknown (Copepoda) (©Mattia Saccò, Curtin University); G - *Scutachiltonia axfordi* King, 2012 (Amphipoda: Chiltoniidae) (©Mattia Saccò, Curtin University); H - *Paroster macrosturtensis* Watts & Humphreys, 2006 (Coleoptera: Dytiscidae) (©Mattia Saccò, Curtin University).

(Saccò et al., 2021c) and freshwater (Rees et al., 2014b; Thomsen et al., 2012; Valentini et al., 2009) communities. In contrast, eDNA for subterranean fauna has great potential to extend biodiversity assessment and exploration (Niemiller et al., 2018a; Saccò et al., 2019a) but, to date, very few studies have been published (e.g., Gorički et al., 2017; White et al., 2020). As the cost of high-throughput sequencing (HTS) becomes less of a limiting factor, we anticipate the field will move forward in line with that of studies on surface habitats.

The principal aim of this review is to describe the characteristics and applications of eDNA as a biomonitoring tool for subterranean biota and to outline future research opportunities. First, we briefly review the main conventional sampling techniques employed in the field of subterranean ecology. Second, we focus on the analytical and technical aspects of eDNA, including its potential and limitations for subterranean environments, and third, we propose research avenues that will advance molecular-based studies of subterranean ecology. Indeed, the vastness and magnitude of subterranean environments requires effective and standardised sampling strategies to accurately monitor the taxonomically and functionally diverse communities comprising subterranean ecosystems (Mammola et al., 2019a). Beyond its analytical value, eDNA has the potential to focus a spotlight on some of the most essential, but undervalued and threatened, ecosystems in the world, and may dismantle conceptual boundaries (e.g., subterranean vs surface), creating a more holistic approach to studying freshwater and terrestrial ecology.

2. Monitoring biota underground: Brief review of conventional methods

Subterranean fauna was first reported nearly 500 years ago from the Alugu Cave in Yunnan Province in China (Borowsky, 2018). However, studies on these fauna for ecological assessments and biomonitoring have only gathered pace over the last 60 years. These studies have employed many sampling techniques for troglofauna and stygofauna, which differ in efficacy, affordability, accuracy and representation (Hahn, 2002) (Fig. 2). Of note, Camacho (1992) reviewed the different sampling methods for subterranean fauna and, more recently Wynne et al. (2018), in comparing different methods, focused on the difficulties when sampling for troglofauna. Whilst different countries and jurisdictions deal with surveying and monitoring of subterranean fauna in different ways, the manual for the assessment of regional groundwater biodiversity, as developed for the PASCALIS (Protocols for the Assessment and Conservation of Aquatic Life In the Subsurface) project (Malard et al., 2002), is among the most comprehensive. Here, we briefly outline the four most extensively used sampling approaches for collection of troglofauna (scraping, litter trap, pitfall traps and direct collection) and four equivalent methods for stygofauna (haul net, pumping, unbaited phreatic traps, direct collection). The advantages and disadvantages of each of these eight techniques are summarised in Fig. 2.

Collection of troglofauna through scraping (Fig. 2a.1) is a method developed by modifying a haul net that is usually used to sample stygofauna in wells/bores. “A cone-shaped net is dropped down a drill hole and dragged back up against the wall of the hole. Troglofauna crawling along the walls are disturbed/displaced by the net and fall into the net” (Halse and Pearson, 2014). Leaf litter traps (Fig. 2a.2) are another technique for the capture of litter-dwelling organisms which consists of a short length of PVC pipe containing damp, sterilised, leaf litter with holes drilled into the upper quarter to allow the entry of troglofauna. The fauna is usually retained in situ for long periods of time (~8 weeks). An alternative frequently used approach is baited pitfall traps (Fig. 2a.3), where a PVC tube is buried in the ground so the lip is flushed with the surrounding sediment allowing organisms to fall into the trap but not to escape. These may be also baited with sweet potato, peanut butter, chicken liver or fish entrails to attract certain species (Howarth et al., 2007). In some cases, pitfall traps cannot be buried deep enough in cave sediments to be flush with the floor and ‘ramps’ built from local materials have been used to allow arthropods to access the traps and fall in (Wynne et al., 2018). Finally, direct collection (Fig. 2a.4) in caves can include visual searching and hand collection with a paintbrush or aspirator (Paquin and Provost, 2010 and references therein).

Stygofaunal specimens are often collected using a haul net which comprises a cone-shaped, weighted fine mesh net with a collection tube attached (Fig. 2b.1). The net is dropped into a well/bore/open aquifer and then retrieved slowly through the water column, capturing animals in the collection tube on the way up (Allford et al., 2008). Another sampling option is the use of several different types of pumps, such as membrane pumps, piston pumps used with the Bou-Rouch, and motor pumps (see Pospisil (1992) for further detail). The extracted water is then filtered through a fine mesh net (Fig. 2b.2) (Allford et al., 2008). A method mostly employed in Europe is unbaited phreatic traps (Fig. 2b.3), consisting of an inert plastic chamber with holes in the upper parts (for fauna entry) and

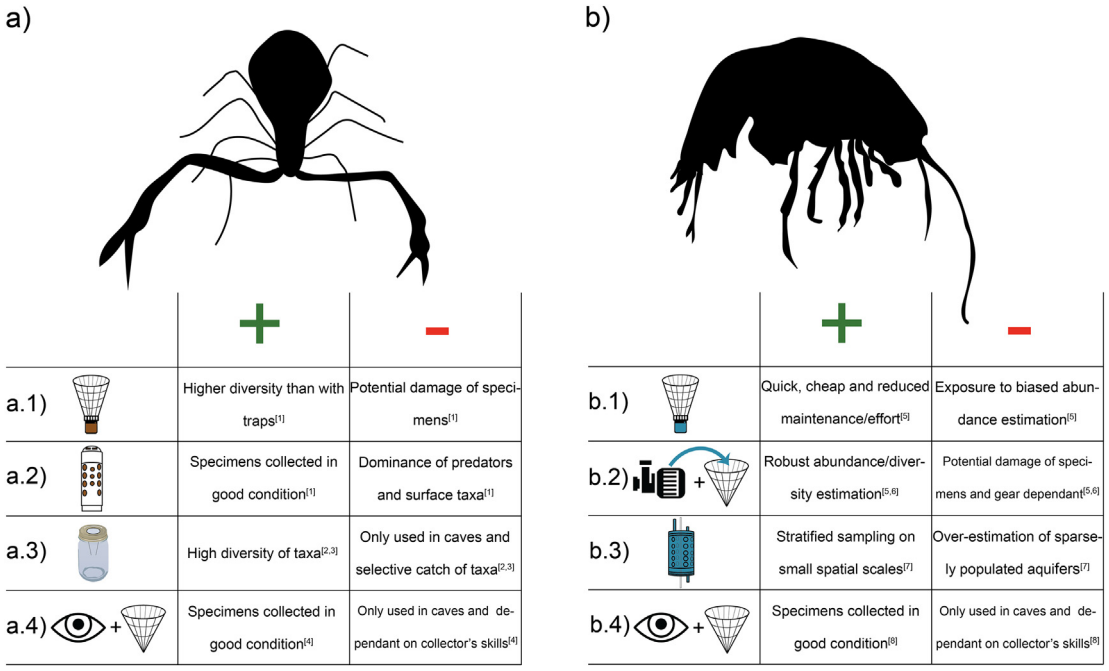

Fig. 2. Conventional sampling techniques for collection of troglofauna (a) and stygofauna (b) specimens in subterranean environments. +: main advantages; −: main disadvantages. a.1: scrape; a.2: litter trap; a.3: pitfall traps; a.4: direct collection; b.1: haul netting; b.2: pumping and filtering; b.3 unbaited phreatic traps; b.4: direct collection. References used in the tables: [1] Halse and Pearson, 2014; [2] Howarth et al., 2007; [3] Wynne et al., 2018; [4] Paquin and Provost, 2010; [5] Allford et al., 2008; [6] Pospisil, 1992; [7] Hahn, 2005; [8] Camacho, 1992.

gaskets near the bottom and near the lid of each trap. A hose is attached to the top of the trap and the surface, so that all parameters can be sampled at each trap (Hahn, 2005). Larger macrofauna, including decapods, amphipods, and isopods can also be collected using baited traps (e.g., Fenolio et al., 2017; Hutchins and Orndorff, 2009; Purvis and Opsahl, 2005). Lastly, direct collection of stygofauna usually occurs in situ through the use of plankton nets which can differ in mesh size depending on the environment and size of the target fauna (Fig. 2b.4) (Camacho, 1992). An additional but compatible method for inferring population densities, habitat use, and investigating morphological traits, involves visual observations in the field (e.g., Behrmann-Godel et al., 2017; Bichuette and Trajano, 2015).

The use of one technique over another depends on many factors including historic preference, cost, collectors' experience, and habitat features such as the depth of the water table, type of overlay strata and availability of existing bore holes. These aspects, once linked to the conventional trade-off between effort and accuracy of each method, have also triggered further fragmentation in a research area that is already strongly influenced by regional practices. In fact, the various methods do not necessarily generate comparable results when sampling the same location (e.g., Allford et al., 2008), and most of them perform poorly for rare taxa or highly mobile species which can avoid some trap types (Hancock et al., 2005 and references therein). Overall, this has had an impact on the generation of comparable and reliable regional and global datasets for environmental modelling, a compelling task under the current climate change scenario (Hendrich et al., 2015; Waldvogel et al., 2020).

Whilst traditional techniques will always be important for accessing and describing subterranean fauna, we consider that new innovations in DNA analysis have the potential to add value rather than replace these techniques. We suggest that eDNA has the potential to overcome many of the drawbacks of traditional sampling methods and could advance the field by providing a complimentary, standardised and affordable biomonitoring tool. Thus, we provide in the next sections a comprehensive assessment - the first for caves and groundwaters - on the characteristics, technical aspects, caveats, and perspectives, of the use of eDNA in subterranean ecosystems.

3. eDNA: A ground(water)-breaking tool

3.1. Using DNA for biomonitoring ecosystems

The conventional workflow for eDNA analysis involves four steps: collection of samples (e.g., water and/or substrate), extraction of DNA, amplification of DNA barcodes (and NGS), and final sequencing and interpretation of DNA barcodes (e.g., Thomsen and Willerslev, 2015; Wang et al., 2019) (Fig. 3). Methods for collecting eDNA samples vary according to the characteristics of the environment being targeted and the most common techniques employed within respective disciplines (e.g., aquatic environments, Rees et al., 2014a and references therein; terrestrial habitats, Ruppert et al., 2019 and references therein). The same considerations apply to sample filtration methods (when needed), preservation of samples, use of contamination controls in the field and/or in the laboratory, as well as DNA extraction procedures, among many other aspects (Garlapati et al., 2019 and references therein).

Amplification of DNA can be accomplished via barcoding or metabarcoding. While barcoding is most commonly carried out through Sanger sequencing and targets a single source sample, eDNA metabarcoding usually refers to the method of targeting and sequencing many DNA fragments that are contained in a mixed biological sample (i.e., groundwater or sediment that may contain DNA from micro- to macro-organisms). When metabarcoding reads (i.e., millions of short DNA sequence fragments) are generated and filtered, a threshold of genetic variation is imposed to infer biodiversity from sequence data (e.g., Operational Taxonomic Units, OTUs; see Blaxter et al., 2005). In theory, each unique OTU reflects a haplotype or 'species' variant and can be represented by many thousands of reads, depending on the ubiquity of the original organism in the sampled environment. The use of eDNA to estimate biodiversity can be limited (reviewed in Rees et al., 2014a) if accurate sequence reference databases do not exist for the target species in question (Bissett et al., 2016; Coissac et al., 2016; Creer et al., 2016; Dormontt et al., 2018). In order to infer taxonomic nomenclature, each OTU should be queried against a reference library of known sequences (i.e., Barcode

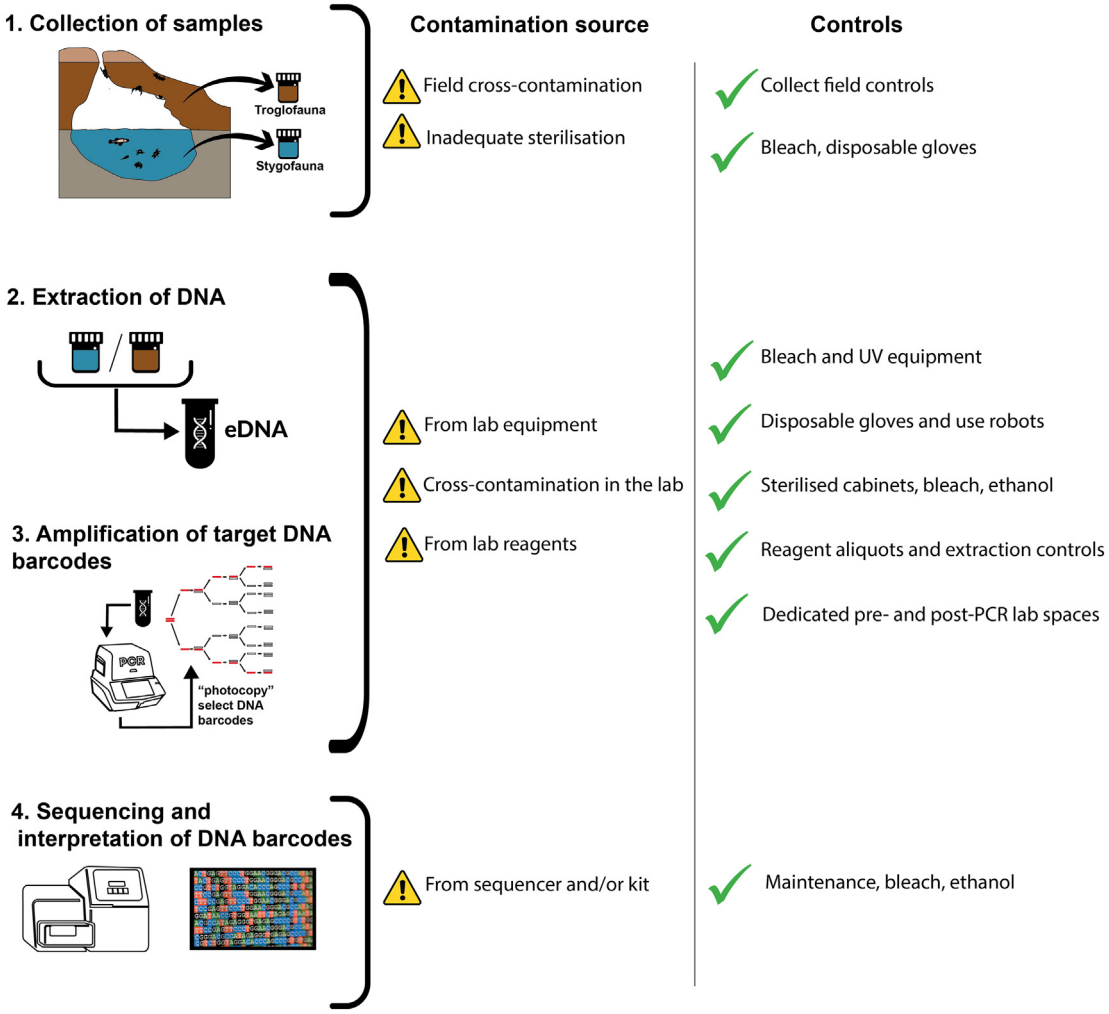

Fig. 3. Example of a conventional eDNA workflow applied to subterranean studies and main contamination/technical issues involved together with control procedures to prevent/eliminate the contamination.

Reference Library - BRL). To reflect accurate levels of genetic variation within a species and also within and between populations across a species' distribution, the BRL should contain homologous sequences of multiple individuals and associated taxonomic metadata. GenBank and the Barcode of Life Data System (BOLD) are frequently used to BLAST search metabarcoding data (Meiklejohn et al., 2019), but it is widely accepted that a custom BRL for a community under investigation is the most desirable means by which estimates of biodiversity can be gained from metabarcoding reads (Kress et al., 2015). Ideally, BRLs should be supported by associated voucher specimens and images that can be used subsequently for identification verification, integrated descriptive taxonomy and/or sequencing for phylogenetic studies (Meiklejohn et al., 2019). Whilst potentially an expensive exercise, development of a custom subterranean BRL has the important advantage of accurate identification of OTUs as named taxa as well as the additive synthesis of a dynamic database that will grow over time facilitating both the nomenclature and identification of species from subterranean habitats.

3.2. Needle vs the haystack

Two approaches for DNA metabarcoding and HTS include sequencing either the 'haystack' (complete diversity) or the 'needle' (specific taxon) of DNA sequences in an environmental sample (Fig. 4). Sequencing the 'haystack' can be broadly described as targeted sequencing of broad biological diversity (i.e., at the level of ecological community), or the DNA of multiple taxonomic groups contained in an environmental sample (i.e., water, soil, scat, etc.). Such technique can inform on the presence or absence of prokaryotic and eukaryotic organisms at a location relative to the quality of eDNA and sampled substrate (van der Heyde et al., 2020a). This approach involves selection of genetic markers (i.e., eDNA primers) that are universal in nature; in other words, the primers of choice can target and amplify a particular metabarcode sequence across numerous species or groups as long as the DNA is present and not too degraded in the environmental sample. Such "universal" primer sets (i.e., assays) are designed to bind to a conserved homologous region of a gene shared by numerous species or groups of taxa using PCR (i.e., Tanaka, 1993). The assays amplify across a variable region that is later used for molecular taxonomic identification (Mousavi-Derazmahalleh et al., 2020 and references therein), OTUs (Edgar, 2016) or Amplicon Sequence Variants (ASV; Callahan et al., 2017) to describe the genetic diversity as characterised within the eDNA sample of choice (van der Heyde et al., 2020a). Choice of assays is influenced by the suitability (or presence) of a *locus* to target and the availability of reference sequences (i.e., cytochrome *c* oxidase subunit I (*COI*) and mitochondrial 16S rRNA genes are generally favoured for use in metazoans). Currently, there is no single 'silver bullet' universal metabarcode assay that will capture all the prokaryotic or all eukaryotic diversity contained in an environmental sample (Alexander et al., 2019). This is because assay and primer design requires a-priori knowledge of sequence data from all the taxa being examined. In addition, the evolution of genes containing homologous regions targeted for universal primer-binding and PCR-amplification can result in mutations (i.e., genetic differences; Kocher et al., 1989) that can affect primer-binding efficiency depending

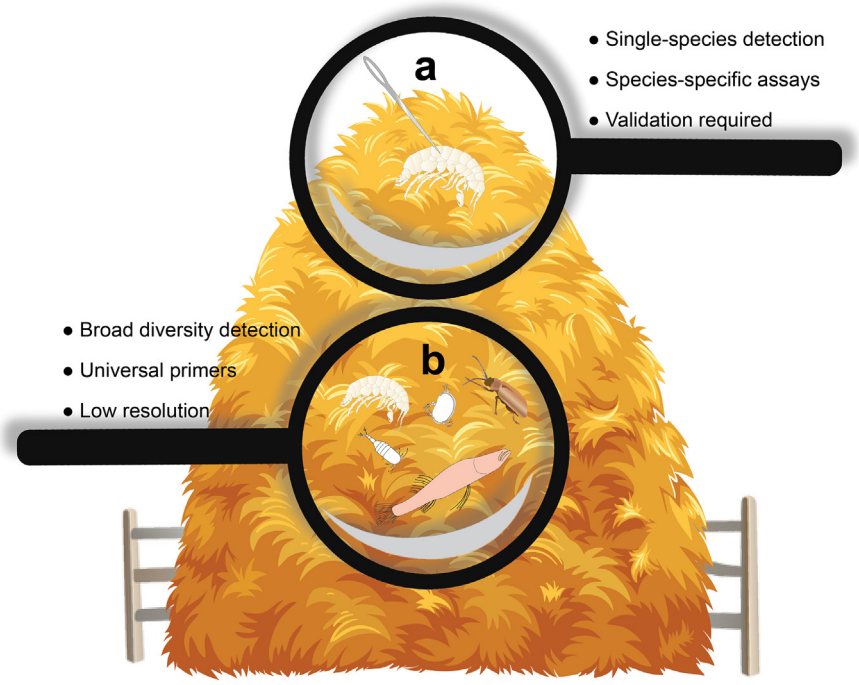

Fig. 4. Diagram depicting the "needle vs the haystack" design and the main characteristics of each approach. (a) Singular species approach ('needle') and (b) ecological community based study design ('haystack').

on the taxonomic group in question. Future endeavours might aim for the development of a 'silver bullet' eDNA assay, one that will allow for the effective detection of all fauna for measuring and monitoring community composition (Giebner et al., 2020), if it is even possible. More likely, the use of multiple assays will be necessary to encompass the broadest organismal diversity spectrum in a given sample.

The second approach for DNA metabarcoding involves design, execution, and sequencing of species-specific assays to detect the 'needle' (specific taxon) contained in the 'haystack' (complete diversity) of mixed DNA in eDNA samples. Targeted sequencing of the 'needle' is most commonly referred to as a single-species detection assay (e.g., rare or cryptic or invasive species, i.e., Harper et al., 2018). Considerations when designing and validating metabarcoding assays, both for the 'needle' and the 'haystack' approach, should include: 1) in silico validation – design of primers specific to the species of interest, 2) in vitro validation – possess high PCR sensitivity to allow detection even at low densities and low DNA concentrations and 3) in situ validation – consideration of assays when applied to environmental samples across spatiotemporal scales (Thalinger et al., 2020; Website: https://edna-validation.com/). Whilst single-species probe assays (e.g., Taqman; Taylor et al., 2002) are not a metabarcoding or HTS approach, they are frequently applied to eDNA samples to determine the presence or absence of species via quantitative PCR using fluorescently labelled DNA oligonucleotides, and may be an economical choice for some studies. Species-specific metabarcoding assays, despite being more expensive, offer an advantage over species-specific probe assays in that intra-species genetic variation and phylogeographic structure can be assessed with HTS (e.g., Deiner et al., 2017; Marshall and Stepien, 2019). To prove the cost-effectiveness and maximise HTS of species-specific assays, they can be conducted in multiplex PCR reactions (Tsuji et al., 2018). The shift from traditional barcoding via Sanger sequencing, where one biological sample produces one DNA barcode sequence, to metabarcoding (i.e., the 'haystack') via HTS, (i.e., one eDNA sample to produce thousands of DNA barcode sequences), has allowed researchers to genetically explore many different types of mixed biological substrates (Giebner et al., 2020). Unlike standard barcode regions (~600–700 bp in length (Hebert et al., 2003)), metabarcoding usually involves targeting the same barcode regions but at a shorter length due to the limits of the sequencing capacity of the HTS technology of choice. Benchtop high-throughput DNA sequencers have evolved over the last 10+ years. Historically, the 454 GS Junior, released in 2010, generated the longest DNA reads (~400–500 bp) but had the lowest throughput (i.e., number of DNA reads produced); the Ion Torrent Personal Genome Machine, released in 2011, produced intermediate throughput but with the shortest reads (~200 bp), and the Illumina MiSeq, released late 2011, has the highest throughput with up to 600-cycles of sequencing-by-synthesis chemistry (~600 bp) (Loman et al., 2012). To date, Illumina has developed eight benchtop HTS platforms, for both DNA and RNA, that provide exceptional data quality and accuracy at massive scales, and are most widely employed for metabarcoding studies (https://sapac.illumina.com/systems/sequencing-platforms.html).

3.3. Current applications for subterranean fauna

Subterranean and groundwater ecosystem management strategies in many locations around the world rely on surveys and ongoing biomonitoring to assess and monitor potential changes in biological community composition (Gibson et al., 2019; Mammola et al., 2019a; Steube et al., 2009). Traditional sampling approaches such as haul net surveys and litter traps include sampling the habitat followed by morphological identification and confirmation of species present with DNA sequence data (i.e., Sanger sequences). However, as with any sampling methodologies, new techniques can add value and improve the quality of surveys. Current gaps in subterranean survey data include: time delays, inconsistent identifications and nomenclature between studies, and difficulty in capturing rare and elusive taxa. eDNA-based methods have real potential to dramatically add value to existing methods and expand knowledge of subterranean ecosystems.

Examples of subterranean habitats targeted through eDNA methods include springs (e.g., Niemiller et al., 2018a), caves (e.g., West et al., 2020), and aquifers accessed through wells (e.g., Mouser et al., 2021) or bores (e.g., White et al., 2020). Sediment samples are usually collected through sterile vials (e.g., West et al. (2020) used 50-ml Falcon tubes), while groundwater samples are often collected at variable volumes through

sterile plastic bottles (i.e., West et al., 2020) or Falcon tubes (e.g., Vörös et al., 2017), or alternatively filtered directly in the field (e.g., Mouser et al., 2021). An alternative approach to the conventional water filtering, but still in its infancy for subterranean systems, is provided by passive eDNA collection by directly submerging filter membranes in the water column (Bessey et al., 2021). Given the hard accessibility and high heterogeneity that subterranean environments often display, passive eDNA filtering provides a method with great potential, but further research on the accuracy of this method is required to fully validate its applicability underground. Fixation of subterranean eDNA is also variedly reported in the literature, and include freezing the filtered sample (i.e., White et al., 2020), mixing it with mixed solutions of sodium acetate and absolute ethanol (i.e., Vörös et al., 2017), or using lysis solutions such as the Longmire's buffer (i.e., Mouser et al., 2021). Other important aspects involved in the eDNA collection are the filter sizes (most commonly, 0.22 μm or 0.45 μm pore sized are used depending on the turbidity of the water, White et al., 2020) and material (i.e., cellulose, polythersulfone, nylon, etc., see Moldovan et al., 2020 for further details), and number/types of controls both in the field and in the laboratory (as per other research fields, a combination between positive and negative controls is recommended, Mächler et al., 2018). Regarding the extraction of eDNA, Qiagen kits are the most frequently employed for subterranean studies (e.g., Boyd et al., 2020; Mouser et al., 2021; White et al., 2020), sometimes coupled with inhibitor removals (Niemiller et al., 2018a).

Numerous studies of subterranean fauna advocate for eDNA metabarcoding as a solution to the problems associated with traditional sampling and sequencing technologies (e.g., Gladstone et al., 2021; Zagmajster et al., 2018). To date, both the 'needle' and the 'haystack' approaches have been employed for groundwater ecosystems. For stygofauna, studies that employ the 'needle' approach include: the cave amphibian *Proteus anguinus* Laurenti, 1768 (Dinaric Alps, Bosnia and Herzegovina) (Vörös et al., 2017), the Sweet Home Alabama cave crayfish *Cambarus speleocoopi* (Alabama, USA) (Boyd et al., 2020), the Hay's Spring amphipod *Stygobromus hayi* Hubricht & Mackin, 1940 (Rock Creek, Columbia, USA) (Niemiller et al., 2018a) and the Pilbara blind cave eel *Ophisternon candidum* Mees, 1962 (north-western Australia) (White et al., 2020). Studies targeting single species via Taqman probe assays comprise: the cave amphibian *Proteus anguinus* Laurenti, 1768 (Dinaric Alps, Bosnia and Herzegovina) (Gorički et al., 2017), the Caney Mountain cave crayfish *Orconectes stygocaneyi* Hobbs III, 2001 (Missouri, USA) (DiStefano et al., 2020), two species of cave fishes (Ozark cavefish *Troglichthys rosae* Eigenmann, 1898 and Eigenmann's cavefish *Typhlichthys eigenmanni* Girard, 1859) and five species of cave crayfishes (Benton cave crayfish *Cambarus aculabrum* Hobbs & Brown, 1987, bristly cave crayfish *C. setosus* Faxon, 1889, Delaware county cave crayfish *C. subterraneus* Hobbs, 1993, Oklahoma cave crayfish *C. tartarus* Hobbs & Cooper, 1972, and Caney Mountain cave crayfish *Orconectes stygocaneyi* Hobbs, 2001) in the Ozark Highlands (Missouri, USA) (Mouser et al., 2021).

For the 'haystack' approach, expansion into community level eDNA metabarcoding has been investigated in Iran for twenty springs (Hashemzadeh Segherloo et al., 2021), and in Australia for: a calcrete aquifer in Western Australia (Saccò et al., 2020b), alluvial aquifers in eastern Australia (Korbel et al., 2017), Christmas Island karst systems (West et al., 2020), and the Beetaloo sub-basin environment in the Northern Territory (Oberprieler et al., 2021). Results from stygofaunal studies have demonstrated that using the 'haystack approach' for eDNA analyses is especially useful for meio-fauna, a group that is notoriously difficult to identify using microscopy. However, it is known that universal assays do not necessarily detect all taxa (Korbel et al., 2017; West et al., 2020). In contrast, the 'needle' approach with design and validation (in silico, in vitro and in situ) of single-species detection assays have demonstrated the need for sample replication when targeting subterranean fauna with low detection probabilities (Mouser et al., 2021). In combination, both approaches, 'haystack' and 'needle', have the potential to significantly contribute to the understanding of subterranean fauna, at both the community and single-species levels, in these notoriously difficult to sample environments.

While many opportunities for studying stygofaunal communities using groundwater eDNA metabarcoding exist, there is a clear gap in research for troglofaunal detection and community composition. To our knowledge, the only published work on detection of troglofauna (both in water and sediment samples from underwater) is the one carried out by West et al. (2020). In that study, DNA metabarcoding techniques implemented a combination of mitochondrial 16S rRNA and nuclear 18S genes. Interestingly, this study was designed to target stygofauna, and the detection of troglofaunal DNA in aquatic environments was a secondary outcome, indicating that specimens can fall into the water column from the terrestrial habitat. Therefore, further research to assess the best protocols to survey the diversity of cave-dwelling groups is urgently needed. Advancements in other terrestrial research areas can indicate the way forward, given that metabarcoding of soils to detect arthropods is a well-established area of research, where many troglobitic relatives exist (Kirse et al., 2021; Oliverio et al., 2018; Rota et al., 2020).

4. eDNA goes subterranean: Limitations, caveats, and how to overcome them

Dire warnings have now been issued for subterranean fauna based on the burgeoning impact of over-exploitation of groundwater, pollution, and impending global climate change (Culver and Pipan, 2019; Mammola et al., 2019b). Regionally, mining and quarrying, impoundments, groundwater extraction, commercialization of caves, and amateur and scientific collections are immediate threats across the various groundwater dependent ecosystem types. Major shortfalls in invertebrate conservation have been identified (Cardoso et al., 2011), in addition to other impediments that apply to the conservation of subterranean biodiversity and ecosystems (Mammola et al., 2021; Niemiller et al., 2018b), including: 1) most species are undescribed (the Linnean shortfall), 2) the distributions of most species are unknown (the Wallacean shortfall), 3) the abundances of most species and how they change spatially and temporally are unknown (the Prestonian shortfall) and 4) the ecology of species and sensitivity to habitat changes are largely unknown (the Hutchinsonian shortfall). These knowledge gaps are the consequence of limited research on invertebrates, especially related to their taxonomy and life history (Cardoso et al., 2011, 2020). eDNA can help fill in the gaps of traditional sampling methods to: 1) identify undescribed species irrespective of informal or ad hoc nomenclature, 2) broaden knowledge of species boundaries and geographic ranges, 3) improve detection of rare and elusive (e.g., active dispersers) subterranean species, 4) be applied to a wide range of habitats, 5) detect fauna at all organismal life-history stages irrespective of gender and life history stage (i.e., identification usually relies on morphological characters of a specific sex and adult life stages) and 6) facilitate non-destructive detection of species that are often threatened or endangered. In order to progress eDNA analysis and its broad uptake for subterranean fauna, there are seven key interconnected issues that need to be addressed: marker development, species delineation, reference databases, repeatability, DNA degradation, temporal variation, and abundance.

4.1. Marker development

To date, eDNA assays for accurate detection of subterranean communities have comprised single-species assays of well-known stygofauna (see above) and also studies at the ecosystem level. The latter studies targeted microbes (i.e., Saccò et al., 2020b), protists, fungi and more broadly metazoans (i.e., Herrmann et al., 2020), including stygofauna (i.e., Oberprieler et al., 2021) or both microbes and stygofauna (Korbel et al., 2017). Excluding the custom designed assays for species-specific detection, the most commonly used assays in subterranean studies are bacterial 16S rRNA (microbes), and a combination between 16S mitochondrial ribosomal RNA, *COI* and eukaryotic 18S rRNA for protists, fungi and stygofauna. This is in line with other research areas, such as terrestrial (i.e., Thomsen and Sigsgaard, 2019), freshwater (i.e., Ficetola et al., 2021) and marine studies (i.e., Jeunen et al., 2019), as the use of these universal and well

conserved genes allows assessment of the broad biodiversity spectra characterising the ecosystems. However, as discussed earlier, a combination of assays (both species-specific ('needle') and broad ('haystack') eDNA metabarcoding) are required to target the full variety of taxa in subterranean communities. Within stygofaunal and troglofaunal groups, many unknown (i.e., dark) species exist (e.g., estimated at >80% for the western half of Australia; Guzik et al., 2011). In order to characterize these dark taxa, 'universal stygofauna and troglofauna' assays are required at the family level to avoid narrowing in too early in the metabarcoding process. However, even at high taxonomic levels inefficiencies in existing 'universal' primers can cause gaps in community level metabarcoding data (i.e., crustaceans are notoriously difficult to detect using standard *COI* barcoding primers; Asmyhr and Cooper, 2012; Korbel et al., 2017). Therefore, genus and family level assays will be extremely important in the future because they will allow for targeting specific taxonomic groups as well as the many dark species.

4.2. Species delineation

It is common practice for OTUs to consist of sequences clustered at a threshold percentage (typically 97%); however, the use of arbitrary clustering thresholds is strongly debated (Callahan et al., 2016). For surface invertebrates, species delineation thresholds are minimally established. Frequently invoked thresholds for the *COI* gene in invertebrates include 16% for crustaceans (Lefébure et al., 2006a), 2% for insects (White et al., 2014), and 1.4–2.6% for molluscs (Barco et al., 2013), but it is well known that a single threshold certainly cannot be applied to all taxa (Krishnamurthy and Francis, 2012). Such differences are exacerbated for subterranean fauna which often have divergent taxon groups (Abrams et al., 2012; Juan et al., 2010; Page et al., 2018) that have experienced extreme selection pressures and population isolation within heterogeneous habitats over long periods of time (Borko et al., 2021; Fišer et al., 2012). In these cases, evolutionary rates are minimally understood compared to surface counterparts (Juan and Emerson, 2010; Lefébure et al., 2006b). Since most subterranean fauna are invertebrates, the appropriate similarity threshold for clustering is unknown. Therefore, it may be more suitable to use denoising algorithms, such as DADA2 (Callahan et al., 2016) or UNOISE2 (Edgar, 2016), to control sequencing error and produce a dataset of unique sequence variants, rather than pooling similar sequences together. Using unique sequence variants also makes it possible to compare between datasets, whereas clustered OTUs may have different 'representative sequences' even if they belong to the same species. The next step then is, of utmost importance for assigning taxonomy, a reference database.

4.3. Reference databases

To make meaningful inferences of community composition and ecology with metabarcoding data, taxonomic rank assignment, ideally to species level, is required. As discussed earlier, currently GenBank and the BOLD are the most widely used reference databases for assigning taxonomy to reads. Some DNA sequences from subterranean species currently exist in the literature and on GenBank from targeted Sanger sequencing of samples from phylogenetic and phylogeographic studies (e.g., Fišer et al., 2009; Matthews et al., 2020). However, given that much of subterranean diversity is dark or unknown, closely related sequences will often not exist in current databases, and even if they do, they won't be available for all known barcoding *loci*. In the absence of a unified subterranean sequence database (BRL – see above), BLAST analysis against public DNA sequence databases is an appropriate option for providing identity to OTUs. Limitations of these public databases include: sequence coverage often varies within and between barcoding *loci*, variable success of *loci* for taxon groups (e.g., *COI* is not suitable to identify fungi), incorrect taxonomy, lack of taxonomy, and undescribed subterranean species generally being poorly characterised. Further, the identity of species in databases such as GenBank should always be treated with caution as a proportion of them are incorrect and there are no voucher specimens to validate them. A custom BRL for a community under investigation is the most desirable means by which species can be assigned to metabarcoding reads (Kress et al., 2015). A BRL has the ability to maximise the accurate and efficient identification of the taxa present using the reference sequences and translating them into meaningful estimates of biodiversity.

For subterranean and groundwater fauna, the number of dark and cryptic species versus known species is innumerable (Guzik et al., 2011; Trontelj et al., 2009; Trontelj and Fišer, 2009). To identify the number of dark species, subterranean biologists frequently use genetic lineages of sequenced individuals (e.g., Guzik et al., 2019; Matthews et al., 2020) alongside a commonly invoked integrated taxonomic approach to species delineation such as that proposed by Fišer et al. (2018) and Matthews et al. (2020), comprising phylogeography, geographical boundaries, population connectivity and/or morphology. Any BRL should be combined where possible with voucher specimens for morphological analyses and formal taxonomic description to provide names for delimited species; this last step being essential to successful biologically meaningful BRL (Delić et al., 2017; Fišer et al., 2009) and eDNA metabarcoding studies (Cristescu, 2014). In the future, the use of whole genome approaches to produce BRLs will open up a range of possibilities beyond a molecular identification system (see Nevill et al. (2020) for a general discussion).

4.4. Repeatability

Ecological surveys provide critical information on species presence, distribution, and abundance. In order to obtain confidence that the proportion of specimens gained from surveys is representative of the existing community, consistent and repeatable sampling strategies that optimise the detection of subterranean species (stygofauna and troglofauna) are required. One of the few studies that has assessed subterranean faunal community composition of groundwater using eDNA metabarcoding methods (Korbel et al., 2017) identified a number of challenges, in particular, that different components of groundwater can provide varying results. For example, groundwater wells reflect an artificial environment and that, prior to purging, present an incomplete picture of an aquifer community. In addition, groundwater can move in many directions both horizontally and vertically, and this aspect can make the source of DNA unclear. Because collection methods and substrates strongly impact species detection, research in freshwater (Buxton et al., 2017) and marine (Koziol et al., 2019) environments have recommended both repeated sampling (Eberhard et al., 2009; Gibson et al., 2019; Zagmajster et al., 2018) and sampling of various substrates (Koziol et al., 2019). However, we ultimately acknowledge that aquifers present certain difficulties for sampling eDNA in a balanced way, since the reliance on using groundwater bores/wells that are sparsely distributed and usually drilled for other purposes, can impact the outcomes of ecological and/or molecular ecological studies.

Adopting eDNA analysis as a complementary tool to existing survey methods has the advantage of increasing the efficiency of these processes without compromising accuracy and comprehensiveness. Here we make recommendations for eDNA water sampling design, processing, and lab testing, and advocate for consideration of methodologies described by Korbel et al. (2017). Samples can comprise water, soil collection and sediment. Soil and sediment are known to bind DNA for longer (resulting in slower decay rates) than free DNA in the water column (Sakata et al., 2020). For replicate numbers, current marine methodologies include ~5 (usually 1 L volume per each replicate) replicates per sample location to account for variation within and between locations, which is consistent with the recommendations for subterranean sampling by Allford et al. (2008). Nonetheless, eDNA studies underground must consider flexible protocols depending on the characteristics of the habitat and the sampling method employed. Indeed, the development of best practice that accounts for the variable heterogeneity and accessibility of subterranean habitats will provide timely guidance able to significantly improve representativity and repeatability of sampling processes. Further, when dealing with high volumes of low-quality DNA, control and contamination mitigation methods are essential for the generation of accurate and reliable sequencing outputs

(Sepulveda et al., 2020). This is particularly true for studies on subterranean environments, where sample collection often involves two completely different frameworks: subterranean (the ecosystem sampled) and surficial (the system where samples reside once samples are collected from the subsurface) environments. As a result, there is a real need to maintain control samples in order to track contamination, i.e., its source in the field, how to control it, and how to track it (Fig. 3). Essential protocols include decontamination of equipment between samples and sites for at least 10 min with a 10–50% bleach solution after each site, followed by rinsing with site water or Reverse Osmosis (RO). It is also imperative to keep samples out of UV light and as cold as possible before and after filtering, with filter membranes remaining frozen at all times, unless preservation buffers are employed (e.g., Mouser et al., 2021).

4.5. DNA degradation

DNA is an unstable molecule that follows an exponential decay pattern with a half-life governed by a number of external factors such as temperature and pH (Allentoft et al., 2012; Lindahl, 1993). The longevity of eDNA within an environment varies greatly depending on the substrate and environmental conditions (Barnes et al., 2014; Pedersen et al., 2015; Seymour et al., 2018; Shogren et al., 2018; Strickler et al., 2015), with eDNA in aquatic systems lasting as long as a month (Dejean et al., 2011) to less than a day (Barnes et al., 2014). eDNA persistence in the environment can substantially impact species detection and quantification (Goldberg et al., 2018) and can affect the reliability of results (Barnes et al., 2014). Degradation of eDNA varies across species, habitats, and environmental conditions (Collins et al., 2018; Shogren et al., 2018; Williams et al., 2018), as does DNA dispersion (Goldberg et al., 2018). In a model epigean freshwater crustacean species (white clawed crayfish), degradation rates of eDNA were low and target eDNA was still detectable 14–21 days after the removal of crayfish (Troth et al., 2021). However, groundwater can have unusual properties affecting longevity of eDNA. For instance, they can be extremely cold or extremely warm habitats (e.g., groundwater in Western Australia's Pilbara region can be up to 35 °C (Humphreys, 2001; Eberhard et al., 2009; Mokany et al., 2019), while calcrete aquifers in central western Australia can vary between 18 °C and 28 °C (Jones et al., 2021)). DNA degradation is also accelerated under conditions of higher temperature and increased UV (Goldberg et al., 2018), microbial community composition (Barnes et al., 2014), pH (Seymour et al., 2018) and conductivity (Barnes et al., 2014). Under standard conditions (i.e., neutral pH), Strickler et al. (2015) showed that most degradation in aquatic environments occurred within 10 days, but some eDNA fragments persisted up to 58 days. However, aquatic habitats that are colder, more protected from solar radiation, and more alkaline are likely to hold detectable amounts of eDNA for longer than those that are warmer, sunnier, and neutral or acidic. Overall, the relatively short lifespan of eDNA within aqueous environments is therefore potentially useful in providing accurate, temporal biodiversity measures for selected areas (Balasingham et al., 2017). For solutions to DNA degradation in aquatic environments, Goldberg et al. (2018) made a number of recommendations, in particular, to increase the number of sampling locations in large and highly degraded (acidic or other) habitats and to increase filter pore size in high-particulate systems. Considerations such as these are likely to be informative for subterranean systems given the variability in physical and chemical composition of these environments.

4.6. Temporal variation

Temporal variation can exist within subterranean environments and potentially it has an impact on the ability to detect species using eDNA. For example, studies from Europe suggest that at the surface and in caves there is usually a decrease in arthropod abundance during the winter season (Moldovan et al., 2018; Reddy and Venkataiah, 1990). In shallow subterranean ecosystems known as Mesovoid Shallow Substratum, this seasonal decrease has also been reported in several localities in Romania and Slovakia (Nitzu et al., 2011, 2014; Rendoš et al., 2012), but in similar habitats in Portugal the opposite has been observed (Eusébio et al., 2021). These results are dependent on the arthropod groups present, their relative life-history strategies/stages, and seasonal cues (Eusébio et al., 2021). In Australia, Hyde et al. (2017) and Saccò et al. (2020a, 2020b, 2021a) identified aquifer and groundwater recharge as a major factor in seasonal variation in species' abundance, energy flows, food web interactions and life-history stages. Since other studies of arthropods demonstrate that detection probabilities of technical replicates vary substantially between seasons (Troth et al., 2021), seasonality should definitely be considered in any eDNA surveys.

4.7. Abundance

Abundance is especially difficult to assess with eDNA metabarcoding. The abundance of certain DNA fragments in the environment does not necessarily reflect the abundance of the taxa because of the effect of biomass (Elbrecht and Leese, 2015) and other traits (e.g., keratinized skin, Adams et al., 2019) on DNA deposition and/or degradation rates. Additionally, there are biases in the DNA metabarcoding process that can cause DNA from certain taxa to be preferentially PCR-amplified or PCR-inhibited (Elbrecht and Leese, 2015; Schenekar et al., 2020). As a result, most eDNA studies are limited to presence/absence data or rely on other proxies, such as the number of replicates with positive detection rather than sequence abundance. There are, however, ways of quantifying the amount of DNA from certain taxa; for example, digital droplet PCR (ddPCR) has been shown to quantify the number of DNA sequences, even at very low concentration (Mauvisseau et al., 2019). This method can be used to estimate fish population abundances (Capo et al., 2021) with taxon-specific assays. Knowledge of stygofauna abundance is generally very poor. Korbel et al. (2017) suggested that differences in stygofaunal community structure between wells and aquifers and differences in the data between traditional sampling and eDNA metabarcoding, question the suitability of using eDNA as a 'stand-alone' method for stygofauna monitoring.

5. Bringing light to the underworld: Innovations on the horizon and future research directions

The inclusion and further development of eDNA technology can greatly increase understanding of subterranean ecosystems and, thus, enable progress in subterranean ecology studies beyond what is possible using current sampling methods. The use of eDNA for monitoring in subterranean ecosystems has proven successful across a variety of topics, including understanding range extensions in important indicator taxa such as the cave-dwelling amphibian *Proteus anguinus* (Gorički et al., 2017), an endangered endemic cave crayfish (Boyd et al., 2020) and blind cave eel (White et al., 2020). However, eDNA methods are currently at a nascent stage in their application, and below we highlight some of the innovations on the eDNA horizon that may transform subterranean monitoring and assessment.

5.1. Bio-banking of samples

Curated, well-supported infrastructure that includes time-stamped sample archives containing tissue or DNA extract vouchers and associated databases of biodiversity information, will future-proof collections and enable the identification of current biodiversity baselines and allow future predictions of ecosystem health in the face of environmental change (Jarman et al., 2018). Bio-banking will also enable temporal resolution and the possibility of re-analysing past samples with future technologies. Biobanks have already been established for human health (Sachs et al., 2018), native plant seed (Svalbard Global Seed Vault), livestock (Blackburn, 2018) and bulk biodiversity samples (National Museum of Natural History, Washington D. C. Biorepository), with the latter believed to be the largest museum-based natural history biorepository in existence exceeding 4.2 million standard 2-ml cryovials overall. Given the high risk of extinction of subterranean fauna (Camacho and Valdecasas, 2003; Niemiller et al., 2013;

Niemiller et al., 2018b) and the need for comparative data (morphological and genetic) to enable identification of putative new dark or cryptic species, biobanking assets housed within publicly accessible natural history institutions are essential (Paton et al., 2020). Whilst numerous major repositories for subterranean fauna specimens already exist (e.g., Western Australian Museum (Perina et al., 2019) and the Museo Nacional de Ciencias Naturales (MNCN) Madrid (Camacho et al., 2017)), not all specimens are stored in conditions suitable for future DNA extraction, or with enough information (Ponder et al., 2001), emphasising the need for frozen tissue and DNA collections. For completeness, specific eDNA extract vouchers and membranes should also be stored in public institutions to avoid technological redundancy (Jarman et al., 2018) and enhance future reference collections and reproducibility of results.

5.2. Removing barriers to eDNA data

For the purposes of monitoring and assessing biodiversity of subterranean fauna, an increasing number of commercial service providers, the development of relatively user-friendly lab workflows, together with bioinformatics interfaces/pipelines (e.g., Mousavi-Derazmahalleh et al., 2020; Zafeiropoulos et al., 2020), have substantially reduced the usual barriers (i.e., molecular biology and bioinformatics expertise) to the uptake of eDNA metabarcoding by both researchers and environmental consulting companies. Further streamlining in the future will make eDNA datasets easier to produce and more accessible (Ruppert et al., 2019). These innovations, combined with a continual decrease in the cost of NGS, and improvements in sequencing technologies, will lead to ever greater volumes of more accurate DNA sequence data, that can be achieved in shorter timeframes and generally faster workflows. However, the large number of dark taxa in subterranean systems means that assignment of names to DNA sequences will be a continuing problem. For this reason, a future focus on data-sharing will also be crucial. Shared databases for improved information transfer among stakeholders and subterranean taxon-specific databases for molecular species identification will be important (Berry et al., 2021).

5.3. Sequencing in the field

For subterranean fauna, field-based collections are typically extremely labour intensive (i.e., remote area fieldwork, deep underground sampling, specialist equipment required, few laboratory facilities nearby, etc.). Recent advances in DNA sequencing technology and the increased availability of portable laboratory equipment provide the opportunity to conduct real-time, eDNA-based biodiversity assessments of aquatic ecosystems including groundwater. For example, an Oxford Nanopore Technologies MinION field based eDNA metabarcoding workflow for jellyfish detection has been designed and tested, and will be applied to biodiversity surveys of endemic and introduced species (Ames et al., 2021). Recent improvements in this technology have greatly reduced error rates, making nanopore platforms such as the MinION (Logsdon et al., 2020; Lu et al., 2016) much more feasible for eDNA-based biodiversity assessments (Truelove et al., 2019). Such equipment has huge potential as it requires little more than a power outlet and a table to set up, avoiding the need to take samples back to laboratories or when refrigeration is difficult to access.

5.4. Future research avenues

As discussed above, the health, viability, and functioning of subterranean communities and ecosystems are increasingly under threat from pollution, climate change, and water extraction that impact the groundwater profile (Mammola et al., 2019a; Mammola et al., 2019c). Therefore, there is an urgent need for research on subterranean communities and the elements within them. In their roadmap guide to future research in subterranean ecosystems, Mammola et al. (2020) identified fifty priority research questions across six subject areas in subterranean biology. Here we use eight relevant questions from their study to highlight the areas of research to which eDNA data can contribute. In the area of community ecology of subterranean ecosystems, eDNA analysis, in conjunction with additional technologies such as isotope analyses (Saccò et al., 2020b, 2020c, 2021a), can help to unravel the key food-web processes influencing subterranean community dynamics (Q23) and also how stochastic events (e.g., rainfall) interact with long-term trends in subterranean ecosystems (Q24). In the area of macroecology and biogeography, eDNA analyses can provide new insights on patterns of subterranean biodiversity (Q27) and also help determine species richness patterns of subterranean organisms at local and global scales (Q28), particularly facilitating detection of species in areas that are difficult to access or are hydrologically intermittent (Gorički et al., 2017; Niemiller, Taylor & Bichuette, 2018b). Relevant to conservation biology, eDNA approaches provide an opportunity for standardising sampling effort, particularly when surveying groundwater (e.g., by sampling specific quantities of water to produce a quantitative assessment of the diversity present), allowing comparisons of relative species richness patterns to be unbiased and comparable over time (Q31). The utility of this approach has been demonstrated in natural freshwater systems such as ponds for the detection of amphibians (Thomsen et al., 2012), and for quantifying relative fish abundance (Jerde et al., 2013; Lacoursière-Roussel et al., 2016). The advantages of eDNA methods for monitoring subterranean ecosystems in a standardised way, allows for long-term studies on changes in these environments over time, and inferences of how climate change (Q33), pollution (e.g., herbicides or high levels of nutrients) or other above-ground (e.g., mining, agriculture, tourism, vegetation clearance) or below-ground disturbances (e.g., fracking) may be affecting subterranean ecosystems (Q34). However, it is important that base-line data are generated, considering the natural changes that occur across seasons, prior to any human-induced changes to subterranean ecosystems. In addition, eDNA metabarcoding analyses can provide valuable information on microbial diversity and how microbes are influenced by different energy sources and quantity (Q47), as well as natural and human-induced changes above- and below-ground (Saccò et al., 2020b). These attributes can then be linked with changes in the relative abundance of other organisms in the ecosystem.

6. Conclusions

Given the wide use of eDNA in other freshwater (e.g., Leese et al., 2018; Minamoto et al., 2012; Pilliod et al., 2013), marine (e.g., Lobo et al., 2017; Stat et al., 2017) and soil environments (e.g., van der Heyde et al., 2020b; Yan et al., 2018), there is great potential for the application of eDNA analysis to improve understanding of subterranean ecosystems. However, all monitoring methods have both advantages and disadvantages. One of the challenges now facing the implementation of eDNA analysis as a monitoring tool for subterranean ecosystems is identifying how it can best be integrated with existing survey approaches. Here we have identified key eDNA methodologies and areas of subterranean fauna research that will benefit from future advances in eDNA technologies.

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Mattia Saccò: Conceptualization, Methodology, Visualization, Writing – original draft. **Michelle T. Guzik:** Validation, Methodology, Writing – original draft. **Mieke van der Heyde:** Writing – original draft, Writing – review & editing. **Paul Nevill:** Writing – original draft, Writing – review & editing. **Steven J.B. Cooper:** Writing – review & editing. **Andrew D. Austin:** Writing – review & editing. **Peterson J. Coates:** Writing – review & editing. **Morten E. Allentoft:** Writing – review & editing. **Nicole E. White:** Writing – original draft, Writing – review & editing.

Declaration of competing interest

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.

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